Universidade de Lisboa
Faculdade de Farmácia

ROLE OF REDOX - ACTIVE SOD MIMICS ON THE MIGRATION AND INVASION CAPABILITIES OF HUMAN BREAST CANCER CELLS

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Orientadores: Prof. Doutora Ana Sofia Gregório Fernandes
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Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia (especialidade Toxicologia)

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Preface

Cancer disease is one of the most severe human pathologies due to its high morbidity and mortality, preceded only by cardiovascular diseases. Despite therapeutic advances, cancer represents a concerning health and economic problem on a global scale.

The molecular mechanisms driving breast cancer cells migration/invasion and metastasis are not totally understood. Although reactive oxygen species seem to be relevant for cancer invasiveness and metastasis, there are only few studies in breast cancer focusing on the redox regulation of these processes. Many studies suggest the potential therapeutic usefulness of superoxide dismutase mimics in breast cancer, so it was important to investigate the impact of these redox-active compounds alone or in combination with chemotherapeutic agents in cellular processes related to metastasis, since they have not yet been duly studied in this context.

The work developed in this thesis resulted from a strong collaboration between the Chemical Biology and Toxicology (CBT) Group (iMed.ULisboa) and the Laboratory of Pharmacology and Therapeutics (CBIOS-Research Center for Biosciences & Health Technologies) and also with the Radiation Oncology Department of Duke University.

In structural terms this thesis is divided into five Chapters. Chapter 1 (General Introduction), addresses knowledge on breast cancer, its characteristics and subtypes and reviews current information on migration/invasion mechanisms, oxidative stress, redox regulation and the role playing by some classes of superoxide dismutase mimics in this context, as well as on breast cancer therapeutics and anticancer agents. Chapter 2 (Aims) states the reasons and objectives, that led to this investigation. The results obtained are reported in Chapters 3 and 4 and discussed in the light of the current knowledge on this subject. In order to not extend the Chapter 4 too much, it was decided to describe some more theoretical aspects, complementary information and optimizations concerning the methods used in the Annex, at the end of the thesis. Lastly, in Chapter 5 (Final Discussion, Conclusions and Future Prospects) a global and integrated discussion is presented, as well as the main conclusions regarding the study. Some suggestions for future research are also presented.

The results obtained in this thesis contribute to a better understanding of superoxide dismutase mimics role in migration/invasion and metastasis, representing a further step towards a potential future clinical use of these promising redox-active compounds in breast cancer therapy.

Acknowlegments

I would like to express my heartfelt thanks to many people who have contributed, in one way or another, to the elaboration of this thesis, making this task easier and more enjoyable.

My deepest gratitude to my supervisor Professor Ana Sofia Fernandes. Thank you for accepting to be my supervisor and for being my teacher, for the constant guidance and support from the beginning and for all the knowledge that I`ve collected from you. I was fortunate in having benefited from your superb and invaluable input in every phases of the work, which contribute to my scientific development. I also thank your patience, total disponibility and comprehension relative to my overlap between the work of this thesis and my teaching activity during all these years. No words are enough to express my admiration, thankfulness and friendship. You are a brilliant person in so many ways and a model of a teacher and researcher.

I am especially grateful to Professor Nuno Guerreiro de Oliveira for accepting to be my co-supervisor and for his support, teaching, guidance and precious advices. It was a privilege to be your doctoral student. Thank you for helping me to grow and mature scientifically. Professor, you will always be “The Master”! I also thank you for helping me to improve my skills in scientific writing, which was very important for this thesis. I want to thank your friendship and concern related to my particular situation, since I couldn`t work in full time for this thesis.

I want to express my deepest gratitude to Professor Matilde Castro for receiving me so warmly in her research group and for accepting to be my co-supervisor. I also want to thank you for all support, guidance, precious advices and friendship. Professor Matilde, it was a privilege working with your research group. I also would like to express my great admiration and to say that I consider you as an example and inspiration to me, in every possible ways.

I am very grateful to Professor Nuno Saraiva from CBIOS (Research Center for Biosciences & Health Technologies, Universidade Lusófona) for all his support, guidance, teaching and friendship. I`ve learned so much from you, since from several experimental methodologies including equipment handling, as well as regarding the analysis, treatment and interpretation of results. Your help and work was precious, providing me with the expertise I needed to develop this study. I am deeply grateful for that.

I would like to thank all the other researchers that were involved directly or indirectly in this work. I thank Professor Judite Costa and Professor M. Fátima Cabral from the Chemical Biology and Toxicology (CBT) Research Group (iMed.UL, Universidade de Lisboa) for the previous work related to the SOD mimic Cu[15]pyN_5 synthesis and characterization, which permitted its application in this work. I also thank you for your sympathy and friendship.

My acknowledgment to Professor Ines Batinic-Haberle from Radiation Oncology Department of Duke University for the possibility of studying the SODm MnTnHex-2-PyP^{5+} in the work developed in this thesis.

I would like to thank Professor Maddy Parsons from King`s College (London) for the precious help regarding data analysis from random cell migration assay. I also would like to thank to Guia Carrara and Professor Geoffrey Smith from University of Cambridge for helping with the focal adhesions evaluation.

I am also grateful to Professor Joana Miranda for her support and friendship during the period that I have worked in the Lab of CBT Research Group (iMed.UL).

A special thank to Professor Lídia Pinheiro, my dear friend, for introducing me to Professor Matilde Castro, which led to my admission in CBT Research Group.

I want to thank my colleagues from CBT Research Group, especially Patrícia Guerreiro, Susana Proença, Madalena Cipriano, Sérgio Camões and Pedro Pinheiro for all their support, constant help and friendship. It was a pleasure to work near you and thanks for providing me some memorable and enjoyable moments, including our trips and participation in scientific meetings in Coimbra and Porto.

I also want to thank my colleagues at CBIOS, specially João Costa and Sara Cerqueira for all their support, help and friendship. A special word for João, it was a pleasure working by your side in CBIOS Lab, during our thesis experimental work. Thank you for being my partner in the Training Camps in Spetses, Greece (2014) and in Alicante, Spain (2015). I have very good memories from these times!

I want to acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT, Portugal), through funding UID/DTP/04567/2016 to CBIOS and funding UID/DTP/04138/2013, to iMed.ULisboa. I also thank the European Cooperation in Science and Technology (COST, European Union) action BM1203 EUROS for funding this work and also for funding my Training Schools in Spetses, Greece (2014) and in Alicante, Spain (2015).

I am especially grateful to my dear and closest friends for the constant support, motivation, encouragement and unconditionally friendship. Thank you for understanding the demands of this work, my absences and the time invested in the realization of this thesis.

Last but certainly not the least, I express my deepest gratitude to my dear family for the constant support, encouragement and unconditionally love. A very special word for my Mother. The person I am today owes much to you and to your example! No words are enough to express my gratitude, in particular during these intense and laborious years of my doctoral work, simultaneously with my teaching activity. I couldn`t do it without your precious help, including the financial support. Thank you for everything!

I want to mention two special persons in my life, who helped me to grow up with care and deep affection. This thesis is dedicated to the memory of my Grandparents.

Abstract

Breast cancer metastases are determinant for cancer mortality and multiple mechanisms related to migration/invasion undergo redox regulation. Superoxide dismutase enzymes catalyze $O_2^{\bullet -}$ dismutation, and have many roles in health and disease, including cancer. Additionally, SOD mimics (SODm) have been proposed in different clinical contexts. Manganese(III) porphyrins (MnPs) are promising SODm with several applications, including in cancer, either alone or in combination with chemo/radiotherapy. Moreover, copper(II) complexes have been designed as SODm with potential therapeutic applications in cancer, being Cu[15]pyN_5 a redox-active complex previously studied as a chemosensitizer in mammary cells.

In the present work, the impact of Cu[15]pyN_5 and MnP MnTnHex-2-PyP^{5+}, alone or in combination with doxorubicin (dox) was assessed in metastasis-related processes in two human breast cancer cells, MCF-7 (low aggressiveness) and MDA-MB-231 (highly aggressive). Both SODm were not cytotoxic and did not alter dox cytotoxicity pattern. Cu[15]pyN_5 and dox significantly increased intracellular ROS, partially due to H_2O_2 accumulation. Co-treatment with MnTnHex-2-PyP^{5+} and dox altered intracellular ROS, increasing H_2O_2. Cu[15]pyN_5 decreased MCF-7 directed cell migration. While MnTnHex-2-PyP^{5+} did not modify cell migration, co-exposure decreased collective cell migration and chemotaxis. Additionally, MnTnHex-2-PyP^{5+} reduced dox-induced increase in random migration of MDA-MB-231 cells. Co-treatment of dox with Cu[15]pyN_5 reduced proteolytic invasion of MDA-MB-231 cells and MnTnHex-2-PyP^{5+} or dox decreased proteolytic invasion, being the effect more pronounced upon co-treatment. To further explore the cellular mechanisms, focal adhesions (FA) number and NF-kB activation were studied. In both cells, dox increased FA number, being significant for MCF-7 cells. This increase was more evident in co-treatment, especially for MDA-MB-231 cells. In MCF-7 cells, dox and MnTnHex-2-PyP^{5+} alone increased NF-κB activation (2-fold), while co-treatment led to a reduction to levels similar to non-treated cells. In MDA-MB-231 cells, dox and MnTnHex-2-PyP^{5+}, *per se*, did not change NF-κB activation. Conversely, co-treatment led to a significant increase.

Overall, although differential effects were observed, the alterations induced by Cu[15]pyN_5 or MnTnHex-2-PyP^{5+} in dox-treated cells may be beneficial in breast cancer treatment and deserve to be further explored.

Keywords: Macrocyclic copper(II) complex, manganese(III) porphyrins, doxorubicin, cell migration/invasion in breast cancer, superoxide dismutase mimics/SOD mimics

Resumo

A formação de metástases a partir de um tumor primário é um fator determinante para a mortalidade por cancro e, nesse sentido, têm sido feitos inúmeros esforços com vista a um melhor entendimento dos processos de migração/invasão e, consequentemente, da mestastização. As espécies reativas (ER) têm sido implicadas na cancerigénese, a vários níveis, incluindo na migração e invasão celulares. Atualmente sabe-se que muitos destes processos estão sujeitos a regulação redox.

As enzimas superóxido dismutase (SOD) catalisam a reação de dismutação do anião superóxido ($O_2^{\bullet -}$). Estudos pré-clínicos e clínicos têm demonstrado benefícios em várias patologias humanas, incluindo no cancro. No entanto, visto que a aplicação clínica das SOD nativas apresenta diversas limitações, têm sido sintetizados e desenvolvidos compostos químicos que mimetizam a sua ação, sendo designados genericamente por compostos miméticos da superóxido dismutase (SODm). Os SODm promovem a reação de dismutação do $O_2^{\bullet -}$, contribuindo, deste modo, para o aumento dos níveis de peróxido de hidrogénio (H_2O_2). Estas alterações no balanço redox celular podem ser benéficas do ponto de vista da potenciação dos efeitos terapêuticos de agentes antitumorais convencionais, como a antraciclina doxorrubicina (dox).

Dada a importância fisiológica do cobre e devido à sua atividade redox em particular, tem havido interesse no desenvolvimento de complexos de Cu(II) como SODm, com vista à sua potencial aplicação terapêutica em várias patologias, incluindo no cancro. Neste contexto, o Cu[15]pyN_5 é um complexo macrocíclico de Cu(II) com atividade redox, tendo sido previamente sintetizado, caracterizado e estudado por este grupo de investigação como sensibilizador da quimioterapia (oxaliplatina) em células mamárias.

As porfirinas de manganês(III) (MnP) são SODm antioxidantes polifuncionais, que interagem com diferentes ER e reagem com unidades redox de proteínas envolvidas na transcrição. As MnP são SODm potentes com efeitos benéficos no tratamento do cancro em combinação com regimes de quimioterapia/radioterapia. Apesar dos ensaios clínicos em curso, pouco se sabe sobre os efeitos das MnP no processo de metastização. A MnP estudada neste trabalho, MnTnHex-2-PyP^{5+}, é considerada uma ferramenta útil para estudos de mecanística redox e apresenta-se como um potencial fármaco para o tratamento de várias patologias, incluindo o cancro.

No presente trabalho, foi avaliado o papel dos SODm Cu[15]pyN_5 e MnTnHex-2-PyP^{5+}, administrados isoladamente ou em regime de co-tratamento com a dox, em processos relacionados com a metastização, usando duas linhas celulares humanas representativas de

cancro de mama: as MCF-7 (adenocarcinoma), células com baixa agressividade e as MDA-MB-231 (cancro de mama avançado), que são células altamente agressivas.

Os SODm Cu[15]pyN_5 e MnTnHex-2-PyP^{5+} não apresentaram citotoxicidade nas duas linhas celulares utilizadas, assim como não alteraram o efeito citotóxico da dox. Os níveis intracelulares de ER foram avaliados através de microscopia de fluorescência, tendo sido utilizadas duas sondas diferentes: a dihidroetídio (DHE) e a dihidrorodamina 123 (DHR). A fluorescência resultante da oxidação da sonda DHE diminuiu quando ambos os modelos celulares foram expostos à MnTnHex-2-PyP^{5+}. No tratamento com a dox, verificou-se o aumento significativo da fluorescência, assim como nas células em co-tratamento, mas em menor extensão. No entanto, no co-tratamento o nível de fluorescência obtido foi superior ao obtido nas células expostas apenas à MnTnHex-2-PyP^{5+}. Nos ensaios com a sonda DHR, observou-se um aumento nos níveis de ER em ambas as células expostas à MnTnHex-2-PyP^{5+}, ao Cu[15]pyN_5 ou à dox. Verificou-se, também, que este aumento foi mais pronunciado após o co-tratamento, tendo sido parcialmente revertido pela catalase. Estes resultados sugerem que a acumulação de H_2O_2 possa ser, em parte, responsável pelos efeitos observados nos diferentes ensaios realizados.

A migração celular foi avaliada, em primeiro lugar, através do ensaio de ferida. Enquanto que o tratamento com a MnTnHex-2-PyP^{5+} ou a dox isoladamente não alterou de um modo apreciável a migração celular coletiva, o respetivo co-tratamento conduziu a uma diminuição significativa da motilidade das células MCF-7 e, numa menor extensão, das células MDA-MB-231. Em relação às células MCF-7, a diminuição da sua motilidade no co-tratamento foi parcialmente revertida pela catalase o que sugere, uma vez mais, o envolvimento do H_2O_2. Estes dados estão de acordo com o que se conhece acerca do papel fundamental do H_2O_2 nos efeitos biológicos das MnP. Em relação ao Cu[15]pyN_5 verificou-se que reduziu, significativamente, a motilidade das células MCF-7. No co-tratamento com a dox esta redução também se verificou, embora sem significado estatístico. Nas células MDA-MB-231, apenas foi observada um ligeiro decréscimo na sua motilidade celular, após o tratamento com os SODm isoladamente ou em co-tratamento com a dox.

Em relação à quimiotaxia, avaliada pelo ensaio de inserção de “transwells”, o Cu[15]pyN_5 levou a uma diminuição significativa da migração celular direcionada das células MCF-7. No entanto, não se verificou o mesmo efeito nas células MDA-MB-231. No regime de co-tratamento com a dox, observou-se uma pequena redução da migração quimiotática de ambas as linhas celulares mas sem significado estatístico. Em relação à MnTnHex-2-PyP^{5+}, esta não alterou a migração quimiotática das células MCF-7. No entanto, quando estas

células foram expostas simultaneamente à MnTnHex-2-PyP^{5+} e à dox, observou-se uma diminuição significativa da quimiotaxia. Para as células MDA-MB-231 observou-se uma tendência semelhante. Além disso, a MnTnHex-2-PyP^{5+} reduziu o aumento induzido pela dox, na migração aleatória das células MDA-MB-231.

Neste trabalho, foi também analisado o efeito destes SODm, isoladamente ou em co-tratamento com a dox, na quimioinvasão das células MDA-MB-231, a qual foi avaliada pelo ensaio de inserção de " transwells", cuja membrana estava coberta com uma camada de Matrigel. Visto que as células MCF-7 não têm um fenótipo claramente invasivo, este ensaio foi realizado somente com as células MDA-MB-231. De facto, foi testado e as células MCF-7 não apresentaram quimioinvasão nas condições do ensaio (dados não apresentados). Em relação aos estudos em que foi realizado o co-tratamento com a dox, quer com a MnTnHex-2-PyP^{5+} quer com o Cu[15]pyN_5, verificou-se que ambos originaram uma diminuição significativa da invasão proteolítica das células MDA-MB-231.

Posteriormente, foram também avaliadas as atividades das metaloproteinases de matriz MMP-2 e MMP-9, por zimografia, em meio de cultura condicionado das células MDA-MB-231. Não foram observados efeitos significativos nas células expostas à MnTnHex-2-PyP^{5+} ou à dox sozinhas, nem em co-tratamento, o que sugere que outros mecanismos podem ser responsáveis pelos efeitos observados na invasão celular no ensaio de quimioinvasão. Na sequência destes resultados das zimografias e com vista a um melhor entendimento dos resultados obtidos no ensaio da quimioinvasão, foi realizado um estudo de degradação de gelatina fluorescente com as células MDA-MB-231 expostas à MnTnHex-2-PyP^{5+}, à dox isoladamente, ou a ambos. Foi observado que o tratamento com os dois compostos isoladamente diminuiu a invasão proteolítica das células MDA-MB-231, embora este efeito tenha sido ainda mais pronunciado e significativo após o co-tratamento.

Posteriormente, para esclarecer melhor os mecanismos celulares subjacentes aos efeitos observados, foi avaliado o número de adesões focais que correspondem a estruturas celulares importantes nos mecanismos de adesão celular, bem como a ativação do fator de transcrição NF-κB. Este fator poderá aumentar indiretamente a expressão de outros genes envolvidos na migração, invasão e metastização de células tumorais, podendo também ser regulado por mecanismos redox.

O número de adesões focais foi avaliado por microscopia de fluorescência confocal, enquanto que a ativação do NF-κB foi avaliada através de um ensaio dual com o gene repórter luciferase. Estes ensaios foram realizados em ambas as linhas celulares expostas à MnTnHex-2-PyP^{5+} e à dox isoladamente ou, em regime de co-tratamento.

Em relação ao número de adesões focais por célula, foi observado um aumento induzido pela dox, em ambas as linhas celulares. Nas células MDA-MB-231 expostas ao co-tratamento foi observado um aumento significativo das adesões focais por célula. Este resultado está de acordo com o aumento da área celular observado nestas células com o co-tratamento (dados não apresentados). Além disso, as alterações observadas em relação ao H_2O_2 podem contribuir para as alterações e resultados mencionados neste trabalho, enfatizando o papel do H_2O_2 na dinâmica da adesão celular. Relativamente à ativação do fator de transcrição NF-κB, os resultados diferenciais observados nas duas linhas celulares podem ser atribuídos às diferenças inerentes nos níveis de H_2O_2 e nas enzimas antioxidantes das células MCF-7 e MDA-MB-231, visto que a ativação de NF-κB tem uma dependência dupla e oposta dos eventos oxidativos e do estado redox da célula.

Globalmente, embora tenham sido observados resultados diferenciais de acordo com os parâmetros analisados e dependendo da linha celular e do tipo de migração, no geral o estudo desenvolvido nesta tese aponta para dois potenciais fármacos, que podem ter impacto na progressão do cancro de mama através de mecanismos diferentes e complementares, dado que poderão auxiliar a reduzir a migração e invasão de células cancerosas. Além disso, as alterações induzidas quer pelo Cu[15]pyN_5, quer pela MnTnHex-2-PyP^{5+} em células tumorais de cancro de mama tratadas com dox foram, consistentemente, no sentido de um efeito terapeuticamente favorável. Deste modo, os resultados obtidos no decurso desta tese contribuem para demonstrar a utilidade e segurança dos tratamentos baseados em SODm como potencial estratégia terapêutica no cancro de mama.

Palavras-chave: Complexos macrocíclicos de cobre (II), porfirinas de manganês(III), doxorrubicina, migração/invasão celular no cancro de mama, compostos miméticos da superóxido dismutase

List of publications and communications

From the results presented in this thesis, the following publications were published in international refereed journals:

Full papers

1. Flórido A, Saraiva N, Cerqueira S, Almeida N, Parsons M, Batinic-Haberle I, Miranda JP, Costa JG, Garrara G, Castro M, Oliveira NG, Fernandes AS (2019) The manganese(III) porphyrin MnTnHex-2-PyP^{5+} modulates intracellular ROS and breast cancer cell migration: Impact in doxorubicin-treated cells. *Redox Biology* **20**: 367-378

2. Fernandes AS, **Flórido A**, Saraiva N, Cerqueira S, Ramalhete S, Cipriano M, Cabral MF, Miranda J, Castro M, Costa J, Oliveira NG (2015) Role of the copper(II) complex Cu[15]pyN5 in intracellular ROS and breast cancer cell motility and invasion. *Chem Biol Drug Des* **86**: 578-588

Abstracts

1. Saraiva N, **Flórido A**, Costa J, Parsons M, Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS (2018) Beneficial impact of a manganese(III) porphyrin in cancer cell migration. *Free Radic Biol Med* **120(1)**: S117-S118

2. Saraiva N, **Flórido A**, Costa J, Parsons M, Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS (2018) Impact of a Superoxide dismutase mimic in cancer cell migration. *Biomed Biopharm Res* **15** (2): 258

3. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Proença S, Miranda J, Castro M, Oliveira NG, Fernandes AS. (2015) Impact of the SOD mimic MnTnHex-2-PyP on the adhesion and migration of doxorubicin-treated MDA-MB-231 cells. *Toxicol Lett.* **238(S)**: S241-42

4. Fernandes AS, **Flórido A**, Saraiva N, Cerqueira S, Cipriano M, Cabral MF, Miranda J, Castro M, Costa J, Oliveira NG. (2014) Effect of Cu[15]pyN5 on MDA-MB-231 cell migration and invasion. *Toxicol Lett.* **229(S)**: S152

5. Fernandes AS, **Flórido A**, Cipriano M, Batinic-Haberle I, Miranda J, Saraiva N, Guerreiro PS, Castro M, Oliveira NG. (2013) Combined effect of the SOD mimic MnTnHex-2-PyP5+ and doxorubicin on the migration and invasiveness of breast cancer cells. *Tox Lett*. **221(S):** S70-1

Whitin the scope of this thesis, the following oral communications were presented in scientific meetings:

1. Saraiva N, **Flórido A**, Costa J, Batinic-Haberle I, Castro M, Oliveira NG, Fernandes AS. Redox regulation of breast cancer progression: insights from SOD mimics. Physioma. 10-11 October 2019, Lisbon, Portugal

2. Costa J, Saraiva N, **Flórido A**, Almeida N, Vidovic B, Keser V, Jackson C, Parsons M, Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS. Modulation of cancer cell migration by redox-active compounds, Mechanobiology of migrating Cells: From Basic Science to the Clinic, 1st – 2nd April 2019, Coimbra, Portugal

3. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS. Impact of SOD mimic MnTnHex-2-PyP on adhesion, migration and invasion of doxorubicin-treated breast cancer cells. 8th Postgraduate iMed.UL Students Meeting, 14 e 15 de Julho de 2016, Faculdade de Farmácia da Universidade de Lisboa, Lisboa, Portugal

4. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS. Role of MnTnHex-2-PyP on the adhesion, migration and invasion of doxorubicin-treated breast cancer cells. XLVI Reunião da Sociedade Portuguesa de Farmacologia, XXXIV Reunião de Farmacologia Clínica, XV Reunião de Toxicologia, 4 a 6 de Fevereiro de 2016, Centro de Investigação Médica Faculdade de Medicina da Universidade do Porto, Porto, Portugal

5. Flórido A. Effect of superoxide dismutase mimics (SODm) in migration and invasiveness of breast cancer cells. Conference (Advanced Training Plan), 12th November 2015, FFUL, Lisbon, Portugal

6. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Proença S, Miranda J, Castro M, Oliveira NG, Fernandes AS. Effect of MnTnHex-2-PyP on the adhesion and migration of doxorubicin-treated MDA-MB-231 cells. “Redox Biology in Health and Disease”, 2-8 October 2015, Alicante, Spain

7. Flórido A, Saraiva N, Cerqueira S, Cipriano M, Miranda J, Costa J, Cabral MF, Castro M, Oliveira NG, Fernandes AS. Role of SOD mimetics in breast cancer metastization. CBIOS Scientific Sessions, 15th April 2015, Lisbon, Portugal

8. Cerqueira S, **Flórido A**, Oliveira NG, Fernandes AS, Saraiva N. *In vitro* techniques to assess the effect of SOD mimetics in breast cancer cell spread and adhesion. I Jornadas CBIOS, 9-10 October 2014, Lisbon, Portugal

9. Flórido A, Saraiva N, Cerqueira S, Cipriano M, Miranda J, Costa J, Cabral MF, Castro M, Oliveira NG, Fernandes AS. Effect of the redox-active complex Cu[15]pyN5 in doxorubicin-treated breast cancer cells. "Biochemical Basis of Healthing Ageing", 22-28 September 2014, Spetses, Greece

The results obtained in this thesis were also presented in scientific meetings in the following poster communications:

1. Saraiva N, **Flórido A**, Costa J, Parsons M,Batinic-Haberle I, Miranda JP, Castro M, Oliveira NG, Fernandes AS. Impact of a Superoxide dismutase mimic in cancer cell migration. III Jornadas do CBIOS, 4 de Outubro 2018, Lisboa, Portugal

2. Saraiva N, **Flórido A**, Costa J, Parsons M, Batinic-Haberle I, Miranda JP, Castro M, Nuno Oliveira NG, Ana Sofia Fernandes, P-240 - Beneficial impact of a manganese(III) porphyrin in cancer cell migration, SFRRI international meeting, June 2018, Lisbon, Portugal

3. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Proença S, Miranda J, Castro M, Oliveira NG, Fernandes AS. (2015) Impact of the SOD mimic MnTnHex-2-PyP on the adhesion and migration of doxorubicin-treated MDA-MB-231 cells. EuroTox 2015, 13-16 September, Porto, Portugal

4. Flórido A, Saraiva N, Cerqueira S, Batinic-Haberle I, Proença S, Miranda J, Castro M, Oliveira NG, Fernandes AS. Impact of MnTnHex-2-PyP on the adhesion and migration of doxorubicin-treated MDA-MB-231 cells. 7th Postgraduate iMed.UL Students Meeting, Faculdade de Farmácia da Universidade de Lisboa, 15-16 July 2015

5. Saraiva N, **Flórido A**, Cerqueira S, Batinic-Haberle I, Miranda J, Castro M, Oliveira NG, Fernandes AS. Effect of the SOD mimic MnTnHex-2-PyP on the adhesion and migration of doxorubicin-treated breast cancer cells. EU-ROS COST (Action BM12013) Meeting, 21-24 April 2015, Munique, Germany

6. Flórido A, Saraiva N, Cerqueira S, Ramalhete S, Cipriano M, Miranda J, Costa J, Cabral M, Castro M, Oliveira N, Fernandes A. Effect of the redoxactive complex Cu[15]pyN5 in doxorubicin-treated breast cancer cells. XVIII Congress of the Portuguese Biochemical Society. December 17-20 2014, Coimbra, Portugal

7. Fernandes AS, **Flórido A**, Saraiva N, Cerqueira S, Cipriano M, Cabral MF, Miranda J, Castro M, Costa J, Oliveira NG. Effect of Cu[15]pyN5 on MDAMB- 231 cell migration and invasion. Eurotox 2014, 7-10 September 2014, Edinburgh, Scotland

8. Flórido A, Saraiva N, Cerqueira S, Cipriano M, Miranda J, Costa J, Cabral MF, Castro M, Oliveira NG, Fernandes AS. Cu[15]pyN5, a redox-active complex, modulates doxorubicin effects in human breast cancer cells**.** 6th Postgraduate iMed.UL Students Meeting, Faculdade de Farmácia da Universidade de Lisboa, 2 July 2014

9. Fernandes AS, **Flórido A**, Saraiva N, Cipriano M, Cerqueira S, Costa J, Cabral MF, Miranda J, Castro M, Oliveira NG. Role of the redox-active complex Cu[15]pyN5 in doxorubicin-treated breast cancer cells. EU-ROS COST (Action BM12013) Meeting, 5-8 March 2014, Istanbul, Turkey

10. Fernandes AS, **Flórido A**, Cipriano M, Batinic-Haberle I, Miranda J, Saraiva N, Guerreiro PS, Castro M, Oliveira NG. (2013) Combined effect of the SOD mimic MnTnHex-2-PyP5+ and doxorubicin on the migration and invasiveness of breast cancer cells. Eurotox 2013, 01-04 September 2013, Interlaken, Switzerland

11. Flórido A, Cipriano M, Batinic-Haberle I, Miranda J, Saraiva N, Guerreiro P, Castro M, Oliveira NG e Fernandes AS. Co-treatment with MnTnHex-2-PyP and doxorubicin impairs the migratory and invasive capacities of breast cancer cells. 5th Postgraduate iMed.UL Students Meeting, Faculdade de Farmácia da Universidade de Lisboa, 18 July 2013

12. Flórido A, Cipriano M, Costa J, Cabral MF, Guerreiro P, Miranda J, Castro M, Oliveira NG e Fernandes AS. Role of the redox-active complex Cu[15]pyN_5 in doxorubicin-treated breast cancer cells. 4th Postgraduate iMed.UL Students Meeting, Faculdade de Farmácia da Universidade de Lisboa, 20 December 2012

Alongside with the work developed under this thesis, Ana Flórido was also involved in other studies that resulted in the following outputs:

Publication on international refereed journal

1. Oliveira CA, D'Almeida Peres D, Graziola F, Chacra NAB, Araújo GLB, **Flórido A**, Mota J, Rosado C, Velasco MVR, Rodrigues LM, Fernandes AS, Baby AR. (2016) Cutaneous biocompatible rutin-loaded gelatin-based nanoparticles increase the SPF of the association of UVA and UVB filters. *Eur J Pharmacol Sci* **81:** 1-9 (full paper)

Poster communications

1.Oliveira CA, Silva ALF, Peres DD, **Flórido A**, Chacra NAB, Saraiva N, Mota J, Rosado C, Velasco MVR, Rodrigues LM, Fernandes AS, Baby AR. Development of cationic and anionic rutin-loaded gelatin nanoparticles: a platform for innovative sunscreens. Abstract submission: 28th IFSCC 2014, 27 – 30 October 2014, Paris, France

2.Oliveira CA, Silva ALF, Peres DD, **Flórido A,** Chacra NAB, Saraiva N, Mota J, Rosado C, Velasco MVR, Rodrigues LM, Fernandes AS, Baby AR. Rutin-loaded protein based nanotechnology: a platform for innovative sunscreens. 27th Brazilian Congress of Cosmetology, 12-14 May 2014, S. Paulo, Brazil – 2nd Award NurnbergMesse Brasil, awarded at the 27th Brazilian Congress of Cosmetology (S. Paulo, 2014)

List of abbreviations

AP-1	Activator protein-1
APS	Ammonium persulfate
ATCC	American Type Culture Collection
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CAT	Catalase
CoA	Coenzyme A
CSC	Cancer stem cells
Cu[15]pyN_5	Copper(II) complex of 3,6,9,12,18-pentaazabicyclo [12.3.1] octadeca-1(18),14,16-triene
Cu, Zn-SOD	Copper, zinc – superoxide dismutase (SOD1)
CV	Crystal violet
2D	Two dimensional
3D	Three dimensional
DAPI	4`,6-diamino-2-phenylindole
DHE	Dihydroethidium
DHR	Dihydrorhodamine 1 2 3
DMEM	Dulbecco`s Modified Eagle`s Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dox	Doxorubicin
ECM	Extracellular matrix
EC-SOD	Extracellular superoxide dismutase (SOD3)
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMT	Epithelial-mesenchymal transition
ER	Endoplasmatic reticulum
ERK 1/2	Extracellular signal-regulated kinase 1/2
FA	Focal Adhesions
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FCS	Foetal Calf Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green fluorescent protein

GPx	Glutathione peroxidase
GR	Glutathione reductase
GRX	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HIF-1α	Hypoxia-inducible factor 1α
H9c2	Rat cardiomyoblasts cell line
IB	Immunoblotting
IDC	Invasive ductal carcinoma
IL-8	Interleukin-8
JNK	c-jun N-terminal kinases
Keap1/Nrf2	Kelch ECH associating protein 1/nuclear factor erythroid 2-related factor 2
$\log K_{CuL}$	Stability constant of Cu(II) complex with ligand L
MAPK	Mitogen activated protein kinases
MBC	Metastatic breast cancer
MCF-7	Adenocarcinoma breast cancer cells
MCF-10A	Epithelial breast normal cells
MDA-MB-231	Metastatic breast cancer cells
Mito-ETC	Mitochondrial electron transport chain
MMPs	Matrix metalloproteinases
MnSOD	Manganese-superoxide dismutase (SOD2)
MnPs	Manganese (III) porphyrins
$MnTBAP^{3-}$	Mn(III) meso-tetrakis (4-carboxyphenyl) porphyrin
$MnTE\text{-}2\text{-}PyP^{5+}$	Mn(III) 5,10,15,20-meso-tetrakis (*N*-ethylpyridinium-2-yl) porphyrin
$MnTM\text{-}4\text{-}PyP^{5+}$	Mn(III) 5,10,15,20-meso-tetrakis (*N*-methylpyridinium-4-yl) porphyrin
$MnTnHex\text{-}2\text{-}PyP^{5+}$	Mn(III) 5,10,15,20-*meso*-tetrakis (*N*-*n*-Hexylpyridinium-2-yl) porphyrin
$MnTnBuOE\text{-}2\text{-}PyP^{5+}$	Mn(III) 5,10,15,20-*meso* tetrakis(*N*-*n*-butoxyethylpyridinium-2-yl) porphyrin
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NOX	NADPH oxidase

PBS	Phosphate buffered saline
pCU	- log [Cu^{2+}]
PFA	Paraformaldehyde
PI	Propidium Iodide
PI3K/Akt	Phosphatidylinositol 3-kinase/protein kinase B
PML	Promyelocytic Leukemia Protein
RT-PCR	Real-time reverse-transcription polymerase chain reaction
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide dismutase
SODm	Superoxide dismutase mimics
TBHP	*Tert*-butylhydroperoxide
TEM	Transendothelial migration
TF	Transcription factor
TNF-α	Tumor Necrosis Factor-α
TRX	Thioredoxin
V79	Chinese hamster lung fibroblasts

(charges are omitted throughout the document for clarity)

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Chapter 1

GENERAL INTRODUCTION

Incidence and mortality due to cancer have been increasing over the past 50 years and it is likely that the number of new cases rises about 70% in the next two decades [1]. Cancer disease is a leading cause of morbidity and mortality and there were more than 14 million estimated cancer cases in 2012, in the world and also 8.2 million cancer related deaths. In 2018, 1,735,350 new cancer cases diagnosed in the United States (US) were estimated, which is the equivalent of more than 4,700 new cancer diagnoses each day and 609,640 cancer deaths, corresponding to nearly 1,700 deaths per day [1]. Indeed, regarding these statistics there is no doubt that cancer represents a concerning health and economic problem in US and also on a global scale.

With regard to breast cancer, the most common cancer in women worldwide, its incidence is also increasing [1,2]. In US in 2018, nearly 64,000 new cases of *in situ* breast lesions were expected to be diagnosed in women. Regarding invasive breast cancer, more than 266,000 new cases were expected to be diagnosed in women. In fact, according to Siegel *et al.* [1], there is an estimative that about one in eight women in US during her lifetime will develop invasive breast cancer. In addition to the statistics related to women, in US in 2018, approximately 2,500 new cases of invasive breast cancer were expected to be diagnosed in men [1].

Despite all of the treatment advances, increased people awareness, and the possibility for an earlier detection through appropriate screening, breast cancer nowadays still remains, globally, a leading cause of death in women with cancer. Indeed, for US women, breast cancer death rates are higher than those of any other type of cancer, except for lung cancer [1]. Contributing to these higher rates, metastases of primary breast cancer to distant sites constitute the principal cause of cancer fatalities [3,4].

1.1. Development, progression and types of breast cancer

The development of the human mammary gland begins early, during the embryonic life period [5]. Human mammary glands are constituted by bilayered ductal structures of outer myoepithelial cells, inner luminal cells and mammary stem cells that reside between these two populations. These structures contain also extracellular matrix (ECM) and ECM components include, among others, collagens, fibronectin and elastin. Several other cell types exist in the mammary glands, namely, endothelial cells, fibroblasts, immune and inflammatory cells and adipose cells. The basement membrane of the referred structures is a special type of ECM which separates epithelial, endothelial cells and adipocytes from the surrounding microenvironment (stroma) [6].

The biology of tumors can no longer be understood simply by looking to the cancer cells characteristics only, but instead it must be considered during carcinogenesis, the participation of the “tumor microenvironment”. In the ECM of breast stroma, which is a mixture of cells and connective tissue that can be highly heterogeneous in density and cellular composition, there are numerous stromal cell types, as aforementioned. In addition to these cell types, cancer-affected microenvironment contains malignant cells termed as cancer-associated fibroblasts (CAFs), which are the most numerous cell type and also infiltrating macrophages, named by tumor associated macrophages (TAMs) [2,7]. All the mentioned cells are under regulation and modification throughout development and tumor progression [2,8]. Actually, it is known that there is a reciprocate communication between cancer and normal cells within the tumor-associated stroma, since cancer cells may send signals to stimulate normal cells, which in turn provide cancer cells with several growth factors, cytokines, chemotactic factors and ECM degrading proteases. Altogether these factors acting by different mechanisms can promote breast tumor onset and progression [9,10].

Breast carcinomas, *i.e.,* malignant cancers of epithelial origin, are complex tumors that result mainly from the accumulation of both genetic and epigenetic alterations occurring in epithelial cells of the mammary gland, and also from the interaction between tumor cells and its stroma. A large number of breast carcinomas originate from dedifferentiated luminal epithelial cells, being classified as ductal or lobular lesions, depending on their original location [11]. Specifically, interactions between transformed epithelial cells and their surrounding stroma may be determinant for the fate of evolving cancers, since signals from the microenvironment profoundly influence the survival and migration of cancer cells. As an example of the influence abovementioned are exosomes, which are generated from cancer cells and also from the stroma, that contribute to the invasive growth in the primary tumor by local discharge. Some of these exosomes move by diffusion to distant organs to prepare as mentioned by some authors the so-called “metastatic niche”, important for the settlement and development cancer cells and consequent subsequent metastization at distant organs [12, 13].

In addition, it was signalized the existence of a subclass of neoplastic cells within tumors, termed cancer stem cells (CSCs). In fact, an increasing body of evidence supports the notion that cancers are propagated by a small population of cells present in the malignant tissue, that possess the ability to form a hierarchy similar to the one present in normal tissues, the CSCs, which are able to proliferate originating more stem-like cells, to exhibit resistance to current therapies and to remain quiescent during long periods of time. However, it is still not clear whether the CSCs originates from the normal stem cells of the tissue that deregulate

their self-renewal ability or, from normal mature cells or progenitor cells that acquired stem cell characteristics. Breast cancer stem cells (BCSCs) constitute a subpopulation of tumor cells that have high capacity for tumor generation *in vivo* taking into account that BCSCs have both self-renew capabilities and potential to differentiate generating, therefore, cells with a variety of phenotypes within breast tumors [14]. Importantly, attempts have been made in order to find a universal phenotype for the BCSCs; however being breast cancer a disease with high heterogeneity, it is not expected that a single BCSC phenotype would apply to all breast cancers [14].

Breast cancer is still a challenge from the scientific and clinical point of view due to its remarkably biological heterogeneity, since it encloses a heterogeneous group of disease subtypes that exhibit distinct biological and clinical characteristics [15]. Breast cancer tumor heterogeneity is one of the hallmarks of malignancy, which includes intertumor heterogeneity that is observed in breast cancers from different patients, as well as, intratumor heterogeneity as a consequence of the presence of heterogeneous cell populations within an individual tumor [7]. However until nowadays, the origins of both inter- and intratumoral heterogeneity are not completely understood [16]. If we can understand how certain genes collectively function in this heterogeneous biological setting, in order to promote progression and also to modulate the response to therapy, it would be possible to achieve a better disease management [17]. In fact, like breast cancer, many other human tumors are histopathologically diverse, containing regions with various degrees of differentiation, proliferation, vascularity, inflammation and/or invasiveness [9]. It has been observed that the composition of cancer cells, and also stroma, stromal fibroblasts, and immune cells is highly variable even in nearby regions of the tumors [18].

Breast cancer consists of a group of malignancies that arise from different areas of the mammary glands, namely the ducts, the lobules, or the tissue in between. Based on which type of cell is involved in the origin of breast cancer, they can be divided into two groups [19]:

1- *carcinomas*: breast cancers originated from the epithelial constituent of the breast, and containing the cells that line both the lobules and terminal ducts;

2- *sarcomas*: a rare form of breast cancer (<1% of primary breast cancer) arising from the stromal components of the breast, including myofibroblasts and blood vessel cells.

Based on the degree of invasiveness comparing to the primary tumor, many different types of human breast cancer have been identified so far. Accurately being able to

distinguishing between the various subtypes is vital, since each subtype has different prognoses and treatment implications. Based on the criteria of pathological features and invasiveness potential, common breast cancers can also be divided into three major groups, which are non-invasive (or *in situ*) **(1)**, invasive **(2)** and metastatic breast cancers (MBC) **(3)** [2,19]:

1 – Non-invasive (or *in situ*):

- Ductal carcinoma *in situ* (DCIS): one of the most common types of human breast cancer. It is a pre-invasive breast cancer which develops inside of pre-existing normal ducts. Although it is not invasive *per se,* has potential to become invasive, therefore the early detection and adequate treatment will be determinat to prevent the development of an invasive form of cancer;

DCIS is a group of non-invasive carcinomas with inherent heterogeneity, characterized by the proliferation of malignant cells inside the breast ducts without invasion through the basement membrane.

2 – Invasive breast cancer (IBC):

Considering the normal breast lobules and ducts, cancer cells invade and spread outside of these structures, growing into the surrounding breast stromal tissue. About two-thirds of women with an IBC have the age of 55 or older at diagnosis. Based on the tissue and cell types involved, IBC can be further divided into two subtypes:

- Invasive Ductal Carcinoma (IDC): is the most common type of breast cancer, corresponding to about 80% of all breast cancers; affects women in middle age and includes several subtypes: tubular carcinoma, mucinous carcinoma, papillary carcinoma, and cribriform carcinoma;

- Invasive Lobular Carcinoma (ILC): is the second most common type of breast cancer and accounts for approximately 10-15% of all breast cancers; although it can affect women at any age, it is more common in older women, in the early 60s;

Considering together all the cases of breast cancer disease, 90-95% correspond to invasive subcategories. Invasive carcinomas that have the potential to spread to other sites

of the body, such as the lymph nodes or distant organs and to develop metastases are classified as metastatic breast cancers (MBC).

3 – Metastatic breast cancer (MBC):

Also referred as advanced breast cancers, are late stage breast cancers (stage IV) that have spread to other organs in the body. In MBC, malignant cells originated from breast primary tumors are able to invade other tissues and organs, resulting in the development of a systemic disease. Metastases from breast cancers can be detected in lymph nodes in the armpit, and/or in distant sites such as the lung, liver, bone and brain. From clinical point of view, patients may be initially diagnosed with metastatic disease (or *de novo* metastatic breast cancers), or in alternative they may develop metastases months or even years after receiving the initial treatment. After the removal of the primary tumor, if microscopic tumor cells or micro-metastases remain in the body, it is possible that the cancer may return and, then, disseminate. Regarding breast cancer, the risk of returning and metastasizing is not clearly understood or predictable, since it varies from patient to patient, largely depending on the unique molecular biology of the tumor itself and the stage at the time of the original diagnosis. Taking into account all the women being diagnosed with an early-stage breast cancer, it is estimated that nearly 30% of them will develop a metastatic form of the disease.

The actual accepted sequential progression model of breast tumorigenesis consists of a gradual transition from epithelial hyperproliferation to DCIS and then to IDC [2]. This model is well supported with information provided by both clinical data and data obtained through epidemiological studies, as well as by data from molecular clonality studies. However, regarding breast cancer progression, the evolution from DCIS to IDC is a key event that, even nowadays, is still not well understood [20].

Due to gene expression profiling it was possible to introduce individual molecular markers in the breast cancer diagnosis. Breast cancer is determined mostly by expression of estrogen receptor/progesterone receptor (ERα/PgR) and human epidermal growth factor receptor 2 (HER2), according to microarray-based expression profiling studies. Estrogen receptors (ERs) are membrane and nuclear estrogen receptors (ERα, ERβ), while human epidermal growth factor receptors (EGFRs, or HERs) 1 to 4 constitute a family of tyrosine kinase receptors that are expressed both in normal tissues and in many types of cancer [19,20].

As aforementioned, breast cancer is a complex and heterogeneous disease with varied morphological manifestations, molecular features, behaviors, and responses to therapy. Based on the gene expression pattern of each cancer cell specimen, breast cancers can be

classified into several subtypes [19,20]. This classification is in constant evolution, as advances in DNA and RNA microarrays, as well as in immunohistochemical (IHC) staining permit researchers to precisely define the molecular heterogeneity of breast cancer different subtypes, which allows the selection of the most appropriate treatment [20]. The advances in gene expression studies enabled to demonstrate that ERα+ and ERα- breast cancers are, in fact, distinct diseases in molecular terms. ERα mediates estrogen action, is expressed in about 70% of breast cancers, being required for estrogen-dependent growth of breast cancers. In breast cancer progression, tumor cells acquire growth autonomy, no longer need estrogen and become resistant to antiestrogens, such as tamoxifen. These breast cancers are antiestrogen-resistant, being generally invasive and metastatic and responding poorly to chemotherapy and radiation treatment [19,20,21]. The biology of breast cancer as well as its therapeutics does not depend only on the influence of the full-size ERα, but also many of its isoforms [19]. In fact, high expression of a truncated isoform of ERα, ERα 36 was found to be correlated with metastatic phenotype and poor prognosis in breast cancer patients [22]. It was observed that the most commonly used ERα antagonist, tamoxifen, had an adverse effect on ERα36 exacerbating stem cell qualities, thus promoting the progression of the disease instead of suppressing it. This useful discovery could be related to the fact that only about 70% of ERα patients respond to hormonal therapy, potentially increasing the precision of personalized treatment [22].

Regarding HER2 signaling amplification, it results in HER2 protein overexpression which is linked to tumor cell proliferation and cancer progression. Moreover, breast cancer cells that express HER2 are more likely to develop metastases [19,20].

With the advance of gene expression profiling techniques, the list of intrinsic genes that differentiate breast cancer subtypes is now constituted of several clusters of genes related to ER expression (the luminal cluster), HER2 expression, proliferation, and a cluster of genes called the basal cluster [19]. Considering a molecular point of view, breast cancer whole-genome profiling demonstrated that breast tumors fall into five general categories:

- ***Luminal A breast cancer*:** ERα and/or PgR +, HER2 - ; low levels of protein Ki-67 (low grade); correspond to about 40% of all breast cancers; its growing is slow and generally have the best prognosis; treatment normally involves hormonal therapy;

- **Normal-like breast cancer:** ERα and/or PgR +, HER2 - ; low levels of protein Ki-67 (low grade); is similar to luminal A disease since has a good prognosis which is still slightly worse than luminal A cancer's prognosis;

• ***Luminal B breast cancer*****:** ERα and/or PgR +, either HER2 + or HER2 -, high levels of Ki-67 (high grade); less than 20% of all breast cancers; grow slightly faster than luminal A breast cancers, being the prognosis slightly worse;

• ***HER2-enriched breast cancer*****:** correspond to 10%-15% of breast cancers; absence of ER and PgR expression, high expression of HER2 and proliferation gene clusters, and low expression of luminal and basal clusters; develop faster than luminal cancers being the prognosis, generally, worse; can be treated successfully with targeted therapies related to HER2 protein, such as Herceptin (or trastuzumab) and Tykerb (or lapatinib);

It should be explained that the HER2-enriched subtype is not equal to clinically HER2+ breast cancer. Indeed, while about 50% of clinical HER2+ breast cancers are HER2-enriched, the other half can include any molecular subtype but are mostly HER2+ luminal subtypes. However, nearly 30% of HER2-enriched tumors are clinically classified as HER2 - [19].

•**Triple-negative/basal-like breast cancer (TNBC):** is ERα -, PR- and HER2 - ; corresponds to nearly 20% of all cases of breast cancer; women with BRCA1 gene mutations, African-American women and women younger than 40 years of age are the most often diagnosed; TNBC has higher aggressiveness comparing with the other types of breast cancer being a high grade breast cancer; inexistence of efficient targeted therapies.

The evolving paradigm of the molecular sub-classification of breast cancer suggests new directions to investigate the interactions between primary tumor and systemic environment during the metastatic cascade [23]. *In situ* and invasive breast carcinomas of the same histological subtype, surprisingly share the same genetic and epigenetic alterations and expression patterns. In an opposite way, mRNA profiles of breast tumors belonging to different subtypes (luminal, HER2+, and basal-like) are significantly different. Therefore, in order to compare DCIS and IDC, the mutation *status* and expression of several tumor suppressors and oncogenes have been analyzed. For example, *p53* mutations are more frequent in basal-like and HER2+ subtypes compared with luminal tumors. Basal-like breast carcinomas have generated considerable interest as a distinct subset thought to be originated from normal basal/myoepithelial breast cells, which are defined as those lacking both ERα and HER2 expression. As aforementioned, these carcinomas have a more aggressive clinical behavior, distinctive metastatic pattern and, therefore poor prognosis.

Advanced tumor grade (grade III), mutant *p53 status*, ERα-, and PgR- are characteristics that represent a basal-like phenotype [24].

Both ERα+ and HER2+ tumors have luminal epithelial characteristics, whereas a large fraction of TNBC have stem cell/basal-like properties In fact, TNBC clinical phenotype usually consists of the basal-like molecular subtype, although TNBC and basal-like breast cancers are not synonymous [25]. While TNBC is clearly defined by the absence of three marker expressions, being notorious for its aggressive nature, there is no universally accepted profile of basal-like breast cancer. The nature of TNBC biological specificity that is similar to basal-like cancers, which are tumors arising in BRCA1 mutation patients and claudin-low breast cancers, is actually being explored in order to find targets for novel biological and chemotherapeutic agents. As aforementioned, approximately 15–20% of all breast cancers fall into the TNBC group, which have been attracting much attention, due to the poor prognosis associated and the lack of an effective treatment target [24].

Regarding TNBC, the most frequently observed histology is infiltrating ductal carcinoma, although a rare histological subtype, the medullary carcinoma is also generally triple negative. Recent evidence supports that epigenetic regulation and noncoding RNAs may play important roles in breast cancer development, contributing to the heterogeneity and metastatic aspects of breast cancer, particularly that of TNBC. Although the molecular background of TNBC is complex and remains to be fully understood, recent data obtained from molecular profiling analysis of TNBCs enabled the identification of four stable subtypes: luminal androgen receptor, mesenchymal, basal-like immunosuppressed, and basal-like immune-activated [19]. For example, in TNBC subtype enriched for androgen receptor (AR) signaling, it is observed by immunohistochemical (IHC) assessment that AR is present in 13%–37% of TNBC. However, the prognostic value of AR expression in TNBC is not clear yet, and is difficult by lacking of standard testing methodology and also due to heterogeneity within the patient population [26].

TNBC have a considerable clinical relevance as they primarily affect women at young age and have an especially poor prognosis. These tumors are often resistant to conventional chemotherapy treatments and has a significantly worse clinical outcome than other breast cancer types. While most breast cancer cases are related to increasing age, TNBC occurs preferentially in younger/pre-menopausal women. Comparing to patients with luminal breast cancers, TNBC patients have higher probability to suffer an early relapse. Unlike other breast cancer subtypes which have several targeted regimens such as ER antagonists and HER2 monoclonal antibodies, TNBC's non-surgical treatment has been limited to conventional chemotherapy, since these tumors do not respond to most effective and least toxic therapies, including hormonal therapy (tamoxifen) or Herceptin [27]. However, recently the poly ADP

ribose polymerase (PARP) inhibitor Olaparib was approved for BRCA1 and BRCA2 mutation carriers, who are more likely to develop TNBC [19].

Claudin-low molecular subtype shows low expression for genes encoding tight and adherent junction proteins. This subtype is enriched for TNBC which have histologic subtypes with a prominent spindle cell component, including metaplastic and medullary carcinomas. While distinct from basal-like cancers, claudin-low tumors are triple-negative being considered another subtype of triple-negative disease. It lacks epithelial cell junction proteins such as E-cadherin, being marked by intense immune cell infiltrate and stem cell-like features. Regarding metaplastic carcinomas which have, like others, mesenchymal phenotype, it is observed that are typically negative for ERα, PgR and HER2 expression. In addition, metaplastic carcinomas which are frequently classified as basal-like or claudin-low, often carry *p53* mutations and generally respond less to chemotherapy than other TNBCs [28].

In conclusion, in order to be able to improve clinical outcomes it is essential to have a better understanding of both complexity and biology of TNBC disease. Therefore, it is very important to continue the research work to further elucidate which specific molecular pathways in TNBC allow tumor progression, being possible future targets to novel therapies.

While IDC and ILC account for approximately 90-95% of all breast cancer cases, as aforementioned, some rare types of breast cancers can be observed in clinical setting, such as inflammatory breast cancer (IBC) [2,19]. IBC is a rare, aggressive, locally advanced breast cancer that generally has a poorer prognosis. IBC tends to occur in younger women, being more frequent in African-American women, as well as in women with overweight or obese. Indeed, in Western countries no more than 5% of all patients have IBC however in African countries this % may be higher than 10%. IBC is considered the most lethal and the least understood form of advanced breast cancer, whose hallmark is the lymphovascular invasion [2]. Typical IBC include several symptoms, such as, inflammation-like breast swelling, purple or red color of the skin, and pitting or thickening of breast skin, all of which are probably caused by the blockage of skin lymph vessels induced by the cancer cells. IBC lethality is a consequence of its capability to invade the lymphatic system and the absence of a palpable tumor mass, not being usually detected by mammograms or ultrasounds. As a consequence, by its ability to metastasize rapidly, most IBC tumors are characterized as stage IIIB at the time of detection [29], which means that when the first diagnosis is done, they are already metastasized to distant sites of the body, making it more difficult to treat successfully. In this way, IBC requires immediate aggressive treatment with chemotherapy prior to surgery, being treated differently than the more common types of cancer [30].

Different from other MBC cells, IBC cells invade by forming tumor spheroids that retain E-cadherin based cell–cell adhesions and a strong uniform E-cadherin expression in primary

tumors [18,31]. The majority of IBC tumors are ERα −, PgR− and HER2+ [32]. In view of their ERα and PgR *status*, the presence of a high E-cadherin level despite its highly aggressive nature is intriguing and contrasts with the observations in most other breast cancers [18].

Metastatic breast cancer (MBC) which is seen in 30–40% of all patients with breast cancer, is one of the most aggressive human malignancies, as aforementioned, presenting an extremely low 5-year overall survival [19,24]. MBC is characterized by a rapid disease progression without specific symptoms, usually preventing an early diagnosis and curative treatment. Indeed, MBC remains the chief cause of cancer-related death among women in the Western world. In most cases, lethality in cancer patients is caused by metastases rather than the primary tumor. To be able to improve cancer survival it is essential to understand the nature of the genes involved in the metastatic spread. Therefore, for providing potential therapeutic targets is still crucial the identification of novel alterations at molecular level related to breast tumorigenesis and also breast cancer mechanisms underlying metastasis.

1.2. Hallmarks of cancer disease – breast cancer migration, invasion and metastasis

Carcinogenesis is a complex multistep process in which cells must acquire a succession of capabilities – the Hallmarks of Cancer. These hallmarkers are depicted in Figure 1.1 and were proposed and described by Hanahan and Weinberg [9].

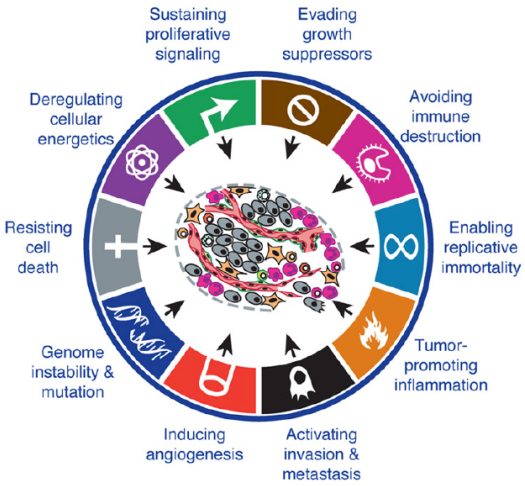


Figure 1.1 – The Hallmarks of Cancer (reproduced from [9]).

The capabilities aforementioned which are acquired during the multistep development of human tumors, will allow the tumor cells to progress and evolve to the stage of malignancy. Underlying the capacities referred in Figure 1.1 are genome instability and inflammation.

Later, two new hallmarks have been proposed: reprogramming energy metabolism and evading immune destruction; the second one meaning the active evasion of cancer cells from attack and elimination by immune cells, which reveals the duality of the immune system that both antagonizes and promotes tumor development [9].

If a certain capability is really significant for the biology of the tumors, then its inhibition should impair tumor growth and progression. Each one of the core hallmarks of cancer is regulated by signaling pathways with some degree of redundancy. Consequently, a targeted therapeutic agent inhibiting one key pathway in a tumor may not completely shut off a hallmark capability, allowing some cancer cells to survive with residual function until they or their progeny eventually adapt to the selective pressure that is being exerted by the therapy. In fact, in certain preclinical models, where potent angiogenesis inhibitors succeed in suppressing this hallmark capability, tumors can adapt and change from a position of dependence on angiogenesis, enhancing the activity of another hallmark capability instead – invasiveness and metastasis [9]. In the context of the present thesis, it is of great relevance this hallmark of cancer related to activating migration/invasion and metastasis in breast cancer.

Regarding breast cancer, localized disease is treatable, and the 5-year survival rate is 98% for early stage cases. However, this rate decreases dramatically for patients with distant metastasis, being only ~ 25% [23] .As aforementioned, breast cancer metastasis accounts for the majority of deaths from breast cancer. Considering all breast cancer patients, 10%-15% will eventually develop distant metastases within a time frame of three years after the initial detection of the primary tumor [19]. This shows that invasion and metastasis directly affect breast cancer mortality, being a very important subject which is studied in this thesis. Metastasis which is the most life-threatening feature of human cancer is responsible for nearly 90% of cancer patient mortality, being one of the hallmarks of malignancy. As aforementioned, metastases development is the cause for the majority of breast cancer deaths, rather than the primary tumors [33,34]. However metastases formation remains the less understood event of cancer pathogenesis.

Analysis of circulating tumor cells (CTCs) with emerging techniques has shown promising results in predicting and identifying the early stages of breast cancer metastasis in patients. It is suggested that CTCs in the blood may play an active role in metastases development, and the presence of CTCs can be correlated with poor prognosis and with an increase in metastatic sites in patient data [19]. CTCs contribute for the development of metastases in the microenvironment of organs such as bone, brain, liver and lung which are particularly suitable for CTCs settlement and also for the creation of secondary lesions [34]. Nowadays it

is known that breast cancers have a pattern of dissemination that is specific to certain types of organs. At least 80% of patients with MBC will develop bone metastases during the course of their disease, being the frequency of bone metastases (occurrence rate at 47%-60%) much higher than that of liver (occurrence rate at 19%-20%), lung (occurrence rate at 16%-34%) or brain (occurrence rate at 10%-16%) [19,35,36].

Despite several decades of intensive investigation, the exact pathophysiologic mechanism(s) of metastasis are not well understood, existing even some controversy about this issue.

Until 2000 the mechanisms underlying invasion and metastasis were practically unknown. At that time, it was clear that carcinomas arising from epithelial tissues would progress to higher levels of pathological malignancy, and the associated cancer cells typically developed alterations in their morphology, as well as in their attachment to other cells and to ECM. In addition, besides the gain and loss of interactions between cells and matrix attachment proteins (*e.g.* E-cadherin), the principal regulators of invasion and metastasis were barely unknown [9]. Afterwards, it has become clear that some specific components of ECM and also the cancer microenvironment are major factors promoting metastasis initiation and outgrowth. This cancer progression seems to be driven by both genetic and microenvironmental factors. These genetic alterations leading to changes in cell-cell interactions contribute, therefore, to the dissociation of cells from the primary tumor mass. These events are followed by local invasion and migration through proteolytically modified ECM. Breast cancer invasion emerge from diverse cell interactions, falling into three major categories: soluble factor signaling, cell–cell adhesion, and ECM remodeling [10].

The concept of epithelial–to-mesenchymal transition (EMT) has evolved to highlight the importance of “tissue architecture plasticity”, as well as the cross-talk between tumor cells, different cells in the stroma and specific molecules in ECM. Although EMT is an essential physiological process occurring in embryonic development, wound healing, and tissue remodeling, it has been implicated in tumor progression and metastasis. Indeed, early stage tumors progression into more invasive ones has been associated with EMT activation. During EMT, it is observed that malignant epithelial cells lose differentiation characteristics (*e.g.* cell-cell adhesion, apical-basal polarity and immobility), acquiring instead mesenchymal features (*e.g.* increased motility, invasiveness and resistance to apoptosis) [19,28,37]. As aforementioned, EMT plays a central role in the beginning of breast cancer invasion, being regarded as the possible first step in the complex process of metastasis. Indeed, EMT is a required step to metastatic tumor progression during detachment of tumor cells from the primary tumor site and attachment to metastatic sites. To reinforce this notion, EMT has

been characterized as a hallmark for cancer invasiveness and metastasis in many types of cancer including breast cancer [10,38,39].

One protein prominently associated with EMT is the epithelial cell adhesion E-cadherin, a key cell–to-cell adhesion molecule in adherent junctions. E-cadherin down-regulation during metastasis is considered a hallmark of EMT [39]. Moreover, other event also associated with EMT is the loss of claudins in tight junctions of cells. It is known that EMT usually involves the disruption of tight and adherent junctions, which contribute to the separation into individual cells. During EMT, epithelial cadherins that are expressed normally in the epithelial cells are down-regulated, while cadherins expressed in the mesenchyme (*eg.* N-cadherin) are up-regulated, as well as mesenchymal proteins such as vimentin and fibronectin. In fact, it was observed that E-cadherin reduction contributed to increased cell mobility and also promoted tumor cell invasion [40,41]. Consistent with this notion, IDC exhibit a decrease in E-cadherin expression and an increase in N-cadherin expression. Other molecular markers of EMT include up-regulation of matrix-degrading proteases, and up-regulation and/or nuclear translocation of transcription factors related to the specific gene program of EMT, such as β-catenin and members of the Snail1 family [42]. In addition, in EMT reorganization of the cytoskeleton occurs to acquire a more spindle-like morphology and increased motility that involves dynamic actin microfilament networks.

Although the precise role of EMT in tumor invasion and metastasis is a matter of interest, the molecular mechanism related to EMT regulation has not been fully understood [41]. Moreover, EMT confers tumor cells with CSC–like characteristics, through transcriptional and translational mechanisms, with consequent therapeutic resistance and tumor recurrence [39]. CSCs that are resistant to treatment, not only have the capacity to give rise to differentiated tumor cells but they can also lead to tumor progression, recurrence and metastasis [43].

The mechanisms that maintain EMT`s transcriptional and translational repression and also cellular invasion are poorly understood. In fact, recent studies on pancreatic carcinoma models refuted EMT as a prerequisite for metastasis, suggesting instead that EMT may be more relevant to resistance to therapy [44] Thus, it remains to be determined whether EMT is required and necessary for breast cancer metastasis.

EMT is considered a transient/dynamic process that brings high plasticity to cancer cells. The term “transition” in EMT, emphasizes the transient and reversible nature of the process, so that under specific conditions within the tissue, mesenchymal cells can also undergo the inverse plastic change that generates epithelial tissue, so called, mesenchymal–epithelial transition (MET), a process that contributes also to formation of tissues and organs during development. Although MET can be correlate with the establishment of secondary tumors following metastasis, it is a process less characterized and studied than EMT [6].

Tumor metastases formation is not only a consequence of abnormalities in cancer cells, being also influenced by surrounding cells and tissues [45]. As aforementioned, microenvironment plays a central role in tumor cell proliferation, survival and migration. As invasive cancer cells breach the basement membrane, they acquire a migratory phenotype and colonize distant organs during the process of metastasis, they face a new group of environmental challenges. “Phenotypic switching” during EMT may be related to cell surface receptors remodeling and thus altered responses to signals from the tumor microenvironment. Also, the circulatory system and secondary tumor microenvironment may not have the most favorable conditions to cell growth and survival [28].

The formation of metastases is a complex and multistep process which requires a sequence of biological phenomena such as cell proliferation at the primary tumor sites, loss of cellular adhesion and eventually EMT, increased motility and invasiveness, entry and translocation through the lymph and blood circulation, exit to a new tissue and, finally, the adaptation of these cells to the distant tissue microenvironments, that will result in the successful colonization of the distant site, including survival and adaptation to the microenvironment of these tissues, to finally form a macroscopic secondary tumor [19,33,46,47].

The multistep process of invasion and metastasis has been schematized as a sequence of several discrete steps, the invasion-metastasis cascade (Figure 1.2) [33].

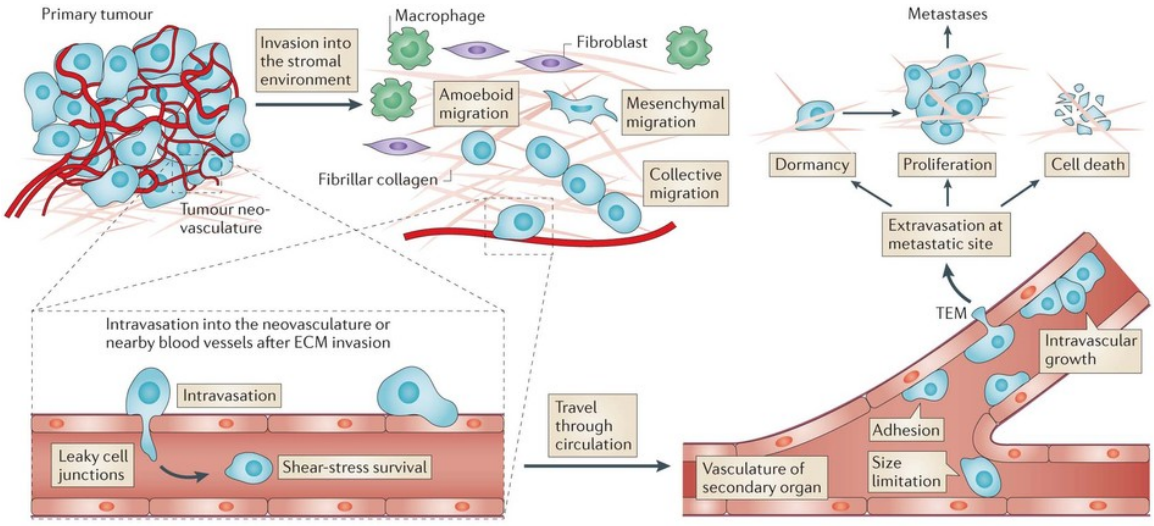


Figure 1.2 – Cancer cell metastatic dissemination (reproduced from [33]).

In more detail, during metastatic dissemination, cancer cells from a primary tumor could acquire migratory and invasive properties, which allow them to invade the surrounding tissues [33]. After that, few of these cells will migrate towards periferic circulation and this invading cancer cells could enter the blood or lymphatic circulation by intravasation. Through

the bloodstream or the lymphatic circulation, especially in breast cancer, these cells spread throughout the body. If cancer cells could survive to the shear stress and the protective immune cells then they will leave the circulation at potential secondary tumor site by extravasation [9,33]. At this point three scenarios are possible: cancer cells can enter in a state of dormancy or they can proliferate in the new microenvironment and, if it is the case, it will occur, in the stroma, the formation of small nodules (micrometastases), that will continuing to proliferate until the growth of macroscopic tumors could be clinically detected. This process is referred as “colonization” [9] and implies recruitment of new blood vessels by induction of angiogenesis [42]. However, most of the cancer cells that undergo extravasation will not be able to colonize in the new microenvironment, since they will undergo cell death [33].

Preclinical findings also indicate that cells from within a heterogeneous primary tumor intravasate into the vasculature, survive in the circulation and extravasate into the parenchyma of a distant tissue. Into the foreign environment, it is thought that disseminated tumor cells (DTCs) exist in a state of dormancy for unknown and varying periods of time. Some micrometastatic *foci* will ultimately form overt tumors, apparently through their ability to recruit the necessary stroma and vasculature, while avoiding to be detected and eliminated by the immune system of the host [23].

Cancer cells extravasation from circulation into a tissue microenvironment where they can be able to survive and proliferate is a limiting step in the metastatic process. This extravasation process involves a cross-talk between cancer and endothelial cells (ECs), and leukocytes, platelets and proteins of the coagulation cascade play supportive roles [28]. Therefore, to be able to extravasate firstly the cancer cell needs to adhere to ECs in the vasculature (margination) and after that, it has to disrupt the tight interactions between ECs to pass through the blood vessel [41]. Interestingly, once metastatic tumor cells enter blood vessels during intravasation, they normally come together forming aggregates sometimes with the aid of platelets which protect them from the hostile environment in the blood vessels. However, during the process of extravasation, the tumor *emboli* has to somehow adhere to the ECs in much the same as neutrophils do when they extravasate from the blood vessels to the site of tissue injury [48].

It seems that EMT can both support tumor cells survival in the blood stream and also to promote extravasation at the distant metastatic site [6]. Since mesenchymal carcinoma cells are thought to proliferate at reduced rates and since many carcinoma metastases display the same degree of differentiation as their primary tumors, it is thought that mesenchymal invasive cancer cells undergo a MET after extravasation in distant organs to form overt

(macro)metastases [28]. In fact, MET which is the opposite of EMT as aforementioned, has been proposed as a mechanism for establishment of the metastatic neoplasm. The extravasated tumor cells may remain indolent as DTCs or micrometastasis or form macrometastasis by MET, which is considered to contribute substantially to the colonization of DTCs into metastatic tumors at the secondary site.

Since local invasion is a necessary initial step in metastatic dissemination to distant organs, the potential of cells to undergo EMT also defines the metastatic potential of the tumor. However, as aforementioned, there is the possibility that EMT is dispensable for intravasation, and cancer cells rather intravasate by collective migration of epithelial cell clusters or even by “passive dropping” into the circulation, promoted by a disorganized and leaky vasculature existent in the primary tumor [6,28].

In some types of cancer such as breast cancer and melanoma, macroscopic metastases may be clinically detected decades after a primary tumor has been surgically removed or pharmacologically destroyed; this metastatic tumor growth reflect dormant micrometastases that, at some point, switch to a proliferative state and therefore, evolve to macroscopic metastases [9]. Metastatic dissemination is considered to be the late onset of breast cancer progression. However, an epidemiological study based on an analysis of more than 12,000 breast cancer cases demonstrated that metastasis initiation may occur 5.8 years before the diagnosis of the primary tumor [49]. The referred study also suggests that some types of precancerous cells may have capability to invade and spread to distal tissues prior to the formation of large tumors. The candidates of such cells that have early metastatic potential may include CTCs found in the peripheral bloodstream or DTCs located in the bone marrow. In fact, DTCs detection occurs in about 31% of stage I to III breast cancer patients with micrometastases and poor prognosis [50].

A very important aspect in breast cancer metastasis is that the identification of the mechanisms underlying cancer cell extravasation, can contribute for the development of new therapies which can lead to reduce metastases formation [33]. Research related to the understanding of the capability for invasion and metastasis and the subjacent molecular mechanisms has increased significantly over the past two decades and nowadays there has been an intense research work searching for novel drugs that inhibit cell migration/invasion [51].

Reactive oxygen species (ROS) may regulate several signaling pathways related to cellular migration and invasion in cancer and many mechanisms related to these events which are fundamental to metastization undergo direct redox regulation [52-55]. This is a fundamental issue and aim of this thesis (Chapter 2), which will be developed in the next section.

1.3. Reactive oxygen species, redox regulation and cancer

1.3.1. Redox homeostasis

The term reactive species (RS) includes oxygen, nitrogen, chlorine, bromine and sulphur transient species with high chemical reactivity [56]. The ones with oxygen are referred as reactive oxygen species (ROS) and there are two types of ROS: free radicals, which contain one or more unpaired electron(s) in their outer molecular orbitals and non-radical ROS, which do not have unpaired electron(s) but are chemically reactive and can be converted to radical ROS. Examples of ROS usually seen in biological systems are superoxide radical anion ($O_2^{\bullet -}$), nitric oxide (NO) and hydroxyl radical ($HO^{\bullet}$), which are extremely unstable and reactive species. Figure 1.3 shows the generation pathways of ROS. For example, $O_2^{\bullet -}$ is formed in biological systems by the partial reduction of molecular oxygen (O_2). Non-radical ROS include, *e.g.*, hydrogen peroxide (H_2O_2), which can be formed by reduction of $O_2^{\bullet -}$, being relatively stable with half-life in the range of seconds [57,58]. In the presence of reduced transition metals, such as Cu(I) and Fe(II), H_2O_2 can be reduced and originate $HO^{\bullet}$ [59].

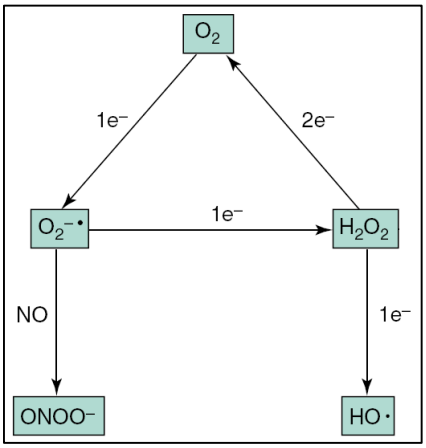


Figure 1.3 – Generation of the most relevant ROS (adapted from [60]).

ROS can be generated owing to exposure to several components present in environment, *e.g.*, pollutants and tobacco smoke, or it can be continuously generated inside aerobic cells, mainly in mitochondria [57,58]. In fact, $O_2^{\bullet -}$ formation is a consequence of mitochondrial electron transport chain uncoupling during oxidative phosphorylation [60]. Moreover, the catalytic action of a variety of intracellular oxidases like NADPH oxidases (NOX) gives rise to $O_2^{\bullet -}$ [56]. Beyond internal stimuli, other factors could also affect intracellular redox

homeostasis, *e.g.,* an imbalance of nutrients and a hypoxic environment [55]. The ROS H_2O_2, for example, exists in the cell due to product release from normal metabolic processes, as well as a result from the exposition to environmental causes including cigarette smoke, radiation, and UV light. A great level of ROS can be formed in consequence of increased expression of enzymatic sources of ROS, increased production by non-enzymatic sources, *e.g.*, abnormal mitochondrial respiration, impaired antioxidant activity or more plausibly, a combination of these factors [61].

Presently it is perfectly established that ROS are essential for biological functions [53,57]. The maintenance of redox homeostasis is pivotal for normal cell physiology, and ROS are known to regulate several cellular events, including cell growth, differentiation, apoptosis, metabolism, regulation of enzyme's activity and transduction pathways, as well as in the mediation of inflammation by stimulating cytokine production [57,61]. Therefore, redox regulation is an essential process in order to maintain both cellular homeostasis and cell survival. Physiologically, cells control ROS levels by a balance between ROS generation and their elimination through ROS-scavenging systems (Figure 1.4), such as the antioxidant enzymes superoxide dismutases (SOD1, SOD2 and SOD3), catalase (CAT) and glutathione peroxidases (GPx) [57], as well as non-enzymatic compounds comprising a number of low molecular weight molecules with the ability to scavenge ROS (*e.g*. glutathione) [58].

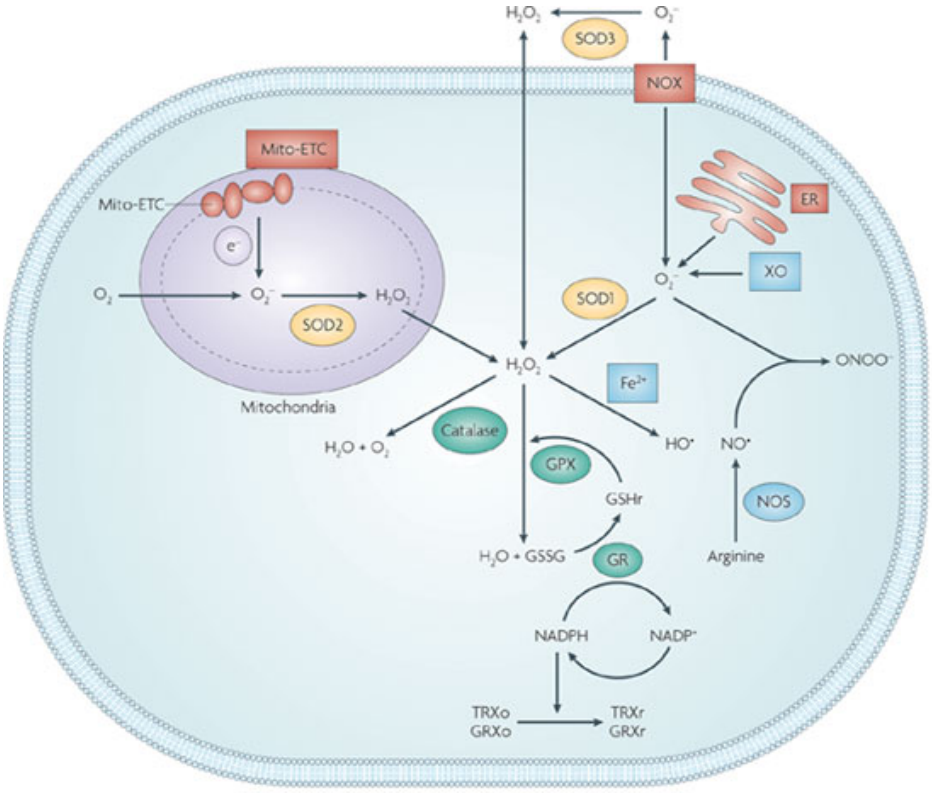


Figure 1.4 – Cellular redox regulation (reproduced from [57]).

Superoxide dismutases (SODs), which enzymatic activity was discovered in 1969 by McCord and Fridovich [62], are metalloproteins with oxido-reductase capacity which protect cells from the toxicity of ROS, acting as the "first line of defense" and being key components of signaling pathways that regulate cell physiology [60]. They efficiently catalyze the dismutation of $O_2^{\bullet -}$ into O_2 and H_2O_2 [58,60,63], accordingly with the chemical equations represented as follows:

$$O_2^{\cdot -} + M^{n+1} \longrightarrow O_2 + M^{n+}$$

$$O_2^{\cdot -} + M^{n+} + 2H^+ \longrightarrow H_2O_2 + M^{n+1}$$

The oxidized form of the metalloenzyme SOD (M^{n+1}) reacts with one $O_2^{\bullet -}$ to form O_2 and the reduced form of the enzyme (M^{n+}). In the second step of the dismutation reaction, the reduced enzyme reacts with another $O_2^{\bullet -}$ and two protons to form H_2O_2, regenerating the oxidized form of the SOD enzyme. The H_2O_2 formed is then removed by CAT and peroxidases (*e.g.* GPx).

Three different SOD isoform enzymes have been characterized in mammals, which can be distinguished by their cellular localization and the metal which is in the active center: copper, zinc SOD (Cu, Zn-SOD or SOD1) mainly located in the "cytosol"; manganese SOD

(MnSOD or SOD2) located in mitochondria matrix and extracellular SOD (EC-SOD or SOD 3) [58,64]. They constitute the only group of mammalian enzymes dismutating $O_2^{\bullet -}$ into H_2O_2 [64].

The involvement of the three SOD isoforms in several pathological conditions has been unraveled by modulating the expression of the SOD enzymes using knockout and trangenic models [59]. In fact, SOD1 down-regulation has been associated with reduced fertility, neuronal death and increased susceptibility to paraquat toxicity [65,66]. Regarding SOD2 activity, their loss or reduction has been associated with neurodegeneration, heart failure, and dilated cardiomyopathy [65,66]. The importance of SOD2 is also highlighted by the fact that, in contrast to SOD1 and SOD3, the SOD2 knockout mice do not survive past three weeks of age [66].

CAT enzyme is present in the peroxisomes of most aerobes and catalyzes the direct decomposition of H_2O_2 to O_2 and H_2O [56]. The catalytic activity of CAT is among the highest rates of enzyme activity known, being proportional to the H_2O_2 concentration [58]. Human CAT is a tetrameric haemin-enzyme consisting of four identical subunits of 60 kDa, each containing an heme group with Fe(III) at its active site [56].

In addition to the catalytic action of CAT, peroxides can be detoxificated by GPxs, which are selenium-containing peroxidases that degrade a variety of peroxides, namely H_2O_2, by coupling their reduction with the oxidation of reduced glutathione (GSH) into GSSG [56, 58]. GPx enzymes are widely distributed in animal tissues and, in humans, at least four isoenzymes have been identified, being the level of each isoform dependent of the tissue type [56].

Regarding cell proliferation, ROS have been shown to influence this process. A moderate increase in ROS can promote cell proliferation and differentiation [55,57]. In fact, at low to moderate levels, ROS can act as secondary signaling molecules contributing to sustain cellular proliferation and differentiation, as well as to activate stress-responsive survival pathways. For example, H_2O_2 can serve as signal for proliferation, differentiation and migration [55]. On the other hand, since ROS are highly reactive with biological molecules, such as lipids, proteins and DNA, excessive ROS levels can cause oxidative damage to those biomolecules, which contribute to alter their functions and also several signaling pathways [45,55,57,58]. In fact, high levels of ROS may induce cell death if the cellular tolerability threshold is exceeded [57]. Disruption of redox homeostasis due to either an increase in RS generation or to decay in scavenging capacity and the accumulation of the oxidative damage, converge in a situation referred as oxidative stress. The term “oxidative stress” has been classically defined as “an imbalance between oxidants and antioxidants in favor of the oxidants potentially leading to damage” [67]. It is known that oxidative damage is

implicated in several pathological processes, such as tissue injury, inflammatory disorders, cardiovascular, pulmonary and neurodegenerative diseases and cancer [56,60]. Indeed, oxidative stress plays a role in the regulation of cancer cells metastases which involve cell migration, invasion and adhesion [55,57,58,68].

1.3.2. Redox regulation and cancer

A link between ROS and cellular transformation was first identified in 1981, when it was discovered that insulin elevated H_2O_2 levels, which in turn, lead to increase tumor cell proliferation [55]. However, more than three decades later, the role of ROS in cancer indeed remains controversial. In order to delineate whether ROS are oncogenic or tumor suppressive, numerous studies have evaluated ROS levels and production under different conditions [57]. As aforementioned, ROS are known to act as secondary signaling molecules that modulate key signal transduction pathways during cancer development [69]. ROS can act as second-messengers by oxidizing cysteine residues and impact on cell migration-relevant kinases, phosphatases and transcription factors [54]. In fact, ROS can act as signaling molecules to stimulate cell proliferation and to promote cell motility [68]. There are reports sustaining that increased oxidant/antioxidant ratio is directly related to several processes, such as tumor progression, angiogenesis and migration/invasion [70]. Interestingly, dysfunction of mitochondria has been shown to contribute to tumor progression by raising the metastatic potential of tumor cells. However, the mechanism through which ROS can stimulate cell motility is still unclear [68].

Cancer cells typically show high levels of oxidative stress comparing with the correspondent normal cells [61,71], which contributes to carcinogenesis and metastases formation [45,57]. In addition, the levels of ROS-scaveging enzymes such as SOD and GPx have been shown to be significantly altered in malignant cells and in primary cancer tissues [57], suggesting the existence of an anomalous regulation of redox homeostasis and also the capability of tumor cells to stress adaptation. Breast cancer cells also have important differences in both basal redox *status* and antioxidant background [61,72,73].

H_2O_2 has the chemical stability required to establish significant steady-state concentrations *in vivo* and being small and uncharged, these properties will allow it to freely diffuse across plasma membranes, making it an ideal candidate for a signaling molecule. All types of cells produce small quantities of H_2O_2 and several signal transduction pathways in mammalian cells have been reported to be activated by H_2O_2 [61,74].

As aforementioned, ROS can either in a direct or indirect way affect a large number of molecular pathways related to cell proliferation, motility, angiogenesis and invasion [61]. It is known that ROS, including H_2O_2 may modify cell migration rates by directly regulating actin cytoskeleton reorganization [53,54]. Moreover, ROS have a role in several pathways closely related to cellular migration/invasion, namely integrins, small GTPases, mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinase/protein kinase B (PI3-K/Akt), focal adhesion kinase (FAK), nuclear factor kB (NF-kB) and activator protein-1 (AP-1) [52-54].

In what concerns to proteolytic invasion, ROS were shown to regulate the synthesis/activation of matrix metalloproteinases (MMPs) [52,61,64,75] which are major contributors to the degradation of ECM, being essential to cancer invasion and metastasis [64,75,76]. Despite the data demonstrating the redox regulation of pathways related to migration/invasion and metastasis, there are still many gaps in our understanding of the true role of ROS in the invasive processes. In addition, the mechanisms through which tumor cells benefit from redox homeostasis disruptions need to be further clarified [61].

Although human cancer cells have often decreased activities of antioxidant enzymes compared with normal cells, their redox adaptation by activating ROS-scavenging enzymes seems to be a very important mechanism by which cancer cells maintain their redox homeostasis [55,57,77]. Tumor cells have a perturbed redox homeostasis, being associated with a higher production of ROS, and also with aberrant antioxidant systems [61]. Therefore, the suppression of this adaptation mechanism by redox modulation could be an attractive strategy to affect cancer cells in a selective way, meaning that this strategy could have very important therapeutic implications.

Because the increase of ROS in cancer cells may play an important role in initiation and progression of cancer, the intrinsic oxidative stress of cancer cells is often viewed as an adverse event. However, as high levels of ROS can also be toxic to the cells, cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous ROS-generating agents, since these cells would be more dependent on antioxidants to survive [57]. Therefore, manipulating ROS levels by redox modulation can be a way to selectively kill cancer cells, without causing significant toxicity to normal cells.

In consequence of the number of druggable targets involved, the redox therapeutics of cancer is a very attractive topic. However, further novel strategies in this context are definitely mandatory. In addition, ROS modulation may be beneficial in reducing tumor migration/invasion and metastization and also to improve therapeutic effects of conventional anticancer agents [63,78,79]. In this regard, a very important aspect is that redox-active agents could constitute a beneficial therapeutic approach and, in this context, superoxide dismutase mimics (SODm) could be very important and useful in breast cancer therapy.

1.4. Native SOD and Superoxide dismutase mimics (SODm)

SODs provide the first defending line against $O_2^{\bullet -}$, whose beneficial roles have been shown preclinically and clinically in many pathological conditions, including cancer [63,64,78-81]. Many evidences suggest that SOD levels are relevant for breast carcinogenesis. However the role of these enzymes is not yet clearly understood. In fact, there are reports that referred that the three known SOD isoforms can be modified in cancer and these alterations linked to carcinogenesis. For example, the expression level of MnSOD is altered in several tumors, including breast cancer and a considerable number of evidences for an anticancer role of MnSOD have emerged through the years [82]. Nevertheless the role of MnSOD in cancer aggressiveness is also controversial [64,81]. In fact, more recently MnSOD has become as a key enzyme with a dual role in tumorigenic progression [83]. Regarding breast cancer cells, MnSOD expression is frequently up-regulated in advanced breast cancers, being in an opposite way often down-regulated in early cancer stages [43,83].

Although some previous studies suggest that elevating SOD inhibits breast cancer growth/invasiveness [84], there are also studies indicating opposite conclusions [85]. Therefore, this opposite conclusions justifies additional experimental approaches to clarify this issue. Some reports on the SOD effects on MMPs also present conflicting results [64,75]. However, more recent mechanistic studies have shown that SOD inhibits not only oncogenic activity, but also subsequent metabolic shifts during early carcinogenesis [69].

MnSOD (SOD2) is now considered a tumor-suppressor, since it was found to be reduced or even absent in several types of cancers [61]. Some research groups have suggested that SOD2 activity is suppressed in human breast cancers while other groups have found an opposite result [86]. In breast tumor cell lines, SOD2 overexpression induced experimentally was shown to decrease cell proliferation and tumorigenicity, being these observations consistent with a tumor-suppressive role [87]. In fact, it was observed that MnSOD acts as a tumor suppressive protein, inhibiting cell proliferation and triggering apoptosis. MnSOD also affects the activity of particular transcription factors, including NF-kb, AP-1 and p53. It is also observed that MnSOD protects normal tissues from chromosomal instability, which otherwise may ultimately result in developing cancer [81]. In an opposite way, some reports have shown that SOD1 is overexpressed in human breast tumors [61]. Indeed, contradictory reports on the influence of SOD can be found in the literature, as it seems to depend on several variables, especially on the tumor stage. However, since SODs were shown to display a suppressor role in several models of breast cancer, this aspect allowed the opportunity for redox-active therapeutic approaches.

Albeit the beneficial roles of SOD in many disease models including breast cancer, the use of native SOD as a therapeutic agent is limited. In fact, the clinical use of native SOD enzymes has shown several issues including their size, which limits cell permeability, short circulating half-life, antigenicity and high cost [60]. Similarly to other enzymes that are used as drugs, SODs possess a charge density and undergo rapid renal clearance, affecting the pharmacodynamics and pharmacokinetics of SOD enzyme in a negative way [81].

In order to improve their drug-like properties, several attempts to modify SOD enzymes have been performed, namely the development of SOD conjugates, SOD liposome with enhanced delivery properties and also the genetic engineering of the human proteins [56,62,66].

A different strategy that has shown very promising results consists in the development of synthetic compounds with low molecular weight, which have the ability to mimic the functional properties of the native SOD enzyme, being these small compounds designated as SOD mimics (SODm) [60,80,81,83,88]. SODm would cross biological membranes without exhibiting antigenicity [63,78,89]. In addition, considering that the ultimate goal is their pharmaceutical use, SODm could have a number of advantages over the native SOD enzymes. For instance, SODm are more likely to access inter– and intracellular spaces, do not present immunogenicity, have longer half-lives, might be administered orally and have lower costs and easier production in large scale than native SOD enzymes [90].

1.4.1. Superoxide dismutase mimics (SODm)

SODm are catalytical polyfunctional antioxidants with the capability to modulate the cellular redox *status*. They are potent scavengers of ROS which mimic endogenous SOD enzymes activity. Besides removing $O_2^{\bullet -}$, SODm may react with other ROS and also with RNS [80,81]. The first studies with SODm date back to the late 1970`s and early 1980`s. Since that time up to now, a large number of SODm have been developed and this number is still growing [80,81,90].

SODm should exhibit high rates of reaction with $O_2^{\bullet -}$ [60]. In addition, to have a relevant SOD activity *in vivo*, SODm catalytic activity should be comparable or even higher than the one of the native SOD enzyme [90]. SODm containing a redox-active center are able to easily exchange electrons with targets of interest, like RS [78]. Several compounds are true SODm, *i.e*, able to catalytically remove $O_2^{\bullet -}$. In order to react as a true catalyst rather than only as a stoichiometric $O_2^{\bullet -}$ scavenger, the SODm must have a reversible redox behaviour with an appropriate reduction potential. This reversible behaviour means that the SODm is

reduced in the presence of $O_2^{\bullet -}$ and, after that, SODm reduced form should be reoxidized by $O_2^{\bullet -}$ [91].

As aforementioned, SODm are small molecular weight synthetic compounds, mostly having a metal ion in their redox-active center, which catalyze the dismutation reaction through a mechanism similar to that of the active-site metals of native SOD enzymes [56,60]. Only a few metals, namely copper (Cu), manganese (Mn), iron (Fe), and possibly nickel (Ni), have the ability to form complexes with SOD activity [92]. Since many SODm are metal complexes, for a pharmaceutical application, it is essential that they present high stability [92]. In fact, one aspect that is very important is the complex thermodynamic stability frequently evaluated by the determination of its stability constant [92]. Therefore, in order to be use therapeutically, the metals in the active center of SODm must be enclosed in a stable ligand and, in addition, the bond between the metal and the ligand must be tight to avoid ligand exchange and, consequently, loss of activity [93]. Indeed, to avoid dissociation between the metal and the ligand, it is necessary that complexes have high values of stability constants, being therefore the kinetic stability or dissociation rate of the complex other important aspect to consider [92].

Macrocycles are polydentate ligands having a structure consisting of a ring with a minimum of nine atoms and with at least three donor atoms [94]. Compared to non-cyclic ligands, macrocycle ligands usually have enhanced stability [90]. The number and type of donor atoms of the ligand, as well as the proper shape of the complex formed, are very important in order to achieve SODm with fast catalytic rates [90]. In addition, the ligand charges can have a major effect in terms of catalytic rate, since the appropriate placement of positive charges may supply the electrostatic attraction and guidance for the approach of $O_2^{\bullet -}$ to the active site of the SODm [88]. SODm with high catalytic rates are compounds that are effective at very low concentrations, allowing therapeutic regimens with low inherent side effect probability [92].

In view of the pharmaceutical application of SODm, it is important to consider several aspects concerning their stability. Not only the chemical, thermodynamic and kinetics stabilities of the complex itself are important [63,92], but also its pharmacokinetics and the characteristics of the biological environment [90,92,93]. Regarding biological stability, it is essential that the complex is stably formed at physiological pH values. Other important aspect that contributes to the stability of SODm is the redox environment, since most complexes are susceptible to oxidative ligand degradation [92]. In addition, several complexes dissociate due to reduction of the metal center and in the reducing environment of most cells, if the metal center suffers reduction this will contribute to the decomposition of the complex [92]. Other aspect to consider is the presence of endogenous chelators (*e.g.*

albumin, glutathione) in the biological milieu that can compete with the complex for bonding with the metal [90,93]. Regarding all the stability aspects aforementioned, in fact SODm are cell-permeable and thermodynamically stable at physiological pH.

SODm are pharmaceutical candidates in a variety of diseases including cardiovascular, inflammatory, neurodegenerative and oncologic diseases [65,81]. In fact, SODm have shown remarkable effects in experimental models of several diseases, including cancer [80,81,88,95-103]. Regarding cancer, SODm may suppress oxidative injury and biomarkers of cell proliferation and they seem to be effective in cancer prevention, in both animal models and in human studies [69,78,80,81,88,96].

Although the development of SODm started long ago, no SODm has reached clinical use yet. In recent years, due to the explosion of knowledge in cancer biology, the development of optimized SODm [80,104] and the increased interest for translational science, the importance of SODm in oncology has re-emerged, with different SODm entering phase I/II clinical trials, especially in combination with chemotherapy or radioprotection regimens [80,81,83,88,104,105]. More recently, two SODm belonging to different classes entered in phase III clinical trials, one for suppressing oral mucositis in head and neck cancer patients in combination with radioprotection regimens [105] and the second one, in combination, to prevent chemotherapy induced peripheral neuropathy (CIPN) that was induced by oxaliplatin in patients with first-line metastatic colorectal cancer [106].

1.4.1.1. Classes of SODm

Although many compounds with SOD-like activity with distinct chemical structures have been reported, the search for SODm has been mostly focused on complexes which contain a redox-active metal center and rich coordination chemistry [79,80,88]. The metal-containing SODm that have been developed include Mn(III) metalloporphyrins, Mn(III) salen complexes, Mn(II) cyclic polyamines and Mn(II) PLED derivatives [60,80,81,88,96,105]. Cu(II) and Fe(III) complexes have also been developed [81,88,89]. Some nonmetallic compounds, such as nitroxides and water-soluble fullerenes have also been explored as possible SODm [56,80,81,88]. These classes of SODm are briefly described below, with more detail for Mn(III) porphyrins and Cu(II) complexes due to the aims of this thesis (Chapter 2).

a) Mn(III) porphyrins (MnPs)

This class of SODm consist in Mn(III) complexes with a metal ion that is bonded in the cavity of the porphyrin ligand. Porphyrins are macrocyclic compounds that exist in nature in several biological molecules, *e.g.*, in hemoglobin, myoglobin and cytochrome oxidase [88], with a structure of 16-atoms ring containing four nitrogen atoms (N), which is obtained by linking four tetrapyrrolic subunits with four methine bridges. These macrocycle structures have a cavity where they can properly accommodate several metal ions, such as Fe and Mn, forming compounds designated by metalloporphyrins [107]. Considering that metalloporphyrins exist in nature as major prosthetic groups of a variety of biomolecules, few of them already mentioned, metalloporphyrins appeared to be the logical choice for developing SODm. In addition, these compounds have a number of advantageous features, namely, their extreme stability, low molecular weight, they can cross cellular and subcellular membranes, they present no antigenicity, they have chemical accessibility and a porphyrin core structure that can be chemically modified [80,88]. Therefore, metalloporphyrins were the first compounds considered to be developed as SODm [88]. So far, the most stable and effective SODm developed belong to the class of cationic Mn(III) *ortho* (*N*-substituted pyridinium-2-yl) porphyrins (MnPs) [79,80,105]. MnPs of clinical potential, which have Mn in 3+ oxidation state, are extremely stable with regards to the loss of Mn [80,105]. In fact, up to now, very stable complexes based on a porphyrin core have been developed, where Mn is coordinated to a porphyrin ligand [78], which has been modified so that it can be as efficient as a given SOD native enzyme [78-80,88]. MnSOD enzyme and MnP based mimics of MnSOD have nearly identical thermodynamics of the metal site, *i.e.*, both can undergo similar types of reactions, although they are different regarding their kinetic properties. The kinetics of MnSOD is dominated by the large protein structure, which endows it with a high specificity towards $O_2^{\bullet -}$ [80].

Most efficacious MnPs bear pentacationic charge and the lipophilic analogs support long hydrophobic substituents (Figure 1.5).

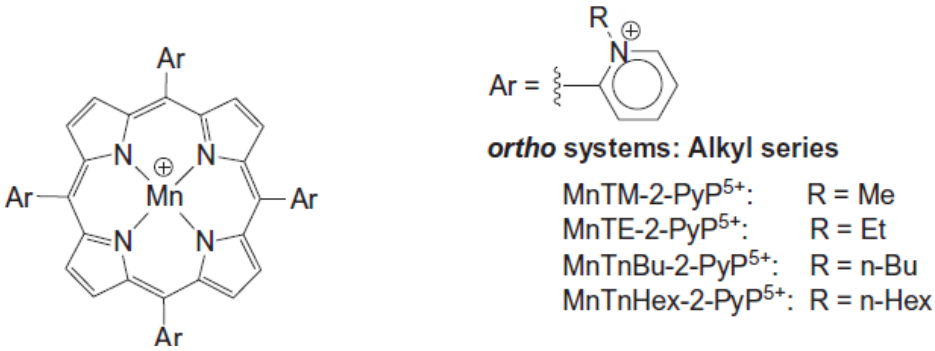


Figure 1.5 - Structures of representative Mn(III) porphyrins (MnPs) [104].

It is observed that the cyclic structure of the porphyrin ligand binds some metals (*e.g.* Mn^{3+}) so tightly, that not even strong acids can cause the loss of the metal [78,88].

As a consequence of the great stability of MnPs, Batinic-Haberle`s group has preferentially studied and explored MnPs, with the main objective to mimic both kinetics and thermodynamics of the $O_2^{\bullet -}$ dismutation by SOD enzymes. In fact, several MnPs have potency similar to SOD enzymes [79]. MnPs have the ability to scavenge several types of ROS, such as $O_2^{\bullet -}$, peroxynitrite ($ONOO^-$) and peroxyl radicals ($ROO^{\bullet}$) [105,108], being the reactivity of MnPs towards RS related to the electron deficiency of the metal site [69,99]. The SOD-like activity of MnPs involves the reversible cycle of reduction and oxidation of the Mn center, that results in changes in valence between Mn(III) and Mn(II) similar to native SOD enzymes [109]:

$$Mn^{III}P^{5+} + O_2^{\cdot -} \leftrightarrows Mn^{II}P^{4+} + O_2$$

$$Mn^{II}P^{4+} + O_2^{\cdot -} + 2\,H^+ \leftrightarrows Mn^{III}P^{5+} + H_2O_2$$

MnPs also have capability to degrade H_2O_2 as a consequence of their extensive conjugated ring system that undergoes reversible one-electron oxidations. However, since this CAT-like activity is less than 1% of that of native CAT enzymes, could be considered negligible [60]. MnPs are among the most efficacious SODm and potent cellular redox modulators within the porphyrin class, being redox-active experimental therapeutics for the treatment of diseases related to a disturbed cellular redox environment, referred as a “state of oxidative stress” [78,79,88]. In fact, MnPs have a remarkable efficacy in treating diseases where physiological redox *status* has been perturbed, such as cancer, diabetes and central nervous system injuries [80,105]. In animal models their efficacy on diseases associated with oxidative stress, is based on a high capability to catalytically remove $O_2^{\bullet -}$ and other RS, such as $ONOO^-$, carbonate anion radical ($CO_3^{\bullet -}$), hypochlorous acid (HClO), NO, $ROO^{\bullet}$ and alkoxyl radicals ($RO^{\bullet}$), thus suppressing the primary oxidative events [78-80,88,96,105].

The newly discovered ability of MnPs to oxidize sulfite (SO_3^{2-}) into sulfite radical ($SO_3^{\bullet -}$) [80] and also hydrogen sulfide (H_2S) into hydrogen disulfide (H_2S_2) [105], points to their possible involvement in sulfur biology [80,105]. It is highly likely given the biologically compatible reduction potential of Mn center, that more reactions of MnPs will be revealed in future. Indeed, bioavailability of Mn with their four oxidation states (Mn^{2+}, Mn^{3+}, Mn^{4+} and Mn^{5+}) allows for ample reactions with biological targets. Given the highly biocompatible redox properties of MnPs and extremely complex cellular redox-biology, numerous as of yet unexplored reactions of MnPs with biomolecules are possible [80,105].Thus far, it is known that the type and the amount of the species which MnPs encounters *in vivo*, the levels of

MnPs and the rate constants of the reactions that ensue are what control the types and the yields of reaction(s) that MnPs undergoes *in vivo* [80,105].

MnPs also affect redox-based cellular transcriptional activity and consequently secondary oxidative stress [88,95,96,102]. Oxidative stress injury`s mitigation exerted by MnPs appears to involve not only the direct scavenging of ROS/RNS, but also the modulation of redox-active transcription factors (TF) as depicted in Figure 1.6. Indeed, there are reports that MnPs can prevent the activation of major TF, such as hypoxia inducible factor-1 (HIF-1α), a major factor in carcinogenesis; NF-kB, a major pro-inflammatory, and AP-1, which controls both cell proliferation and apoptosis [78]. Regarding NF-ķB it has been suggested that MnPs may oxidize cysteines of p50 and p65 NF-kB subunits, which would prevent the DNA binding of NF-kB and its related transcriptional activity. While we have not identified which cysteines are oxidized, data suggest that cys62 of p50 and cys38 of p65 subunit are most likely the culprits [80,105].Therefore, NF-kB oxidation by MnPs will result in suppressing inflammation and/or the survival of cancer cells [78-80]. At present stage, the direct reactions of MnPs with thiols of TF, such as NF-kB or Kelch ECH associating protein 1/nuclear factor erythroid 2-related factor 2 (Keap1/Nrf2), and/or coupled to other species, may present major contributions to the therapeutic efficacy of MnPs [79,80,105]. The dose-dependence and the mechanism behind NF-kB inactivation by some MnPs are under further investigation. In addition, we cannot rule out the indirect impact of MnPs on NF-kB via scavenging RS, which would also signal its activation [79,80,105]. More recently, increasing amount of data supports the notion that MnP/H_2O_2/GSH-driven catalysis of *S*-glutathionylation of proteic cysteine, together with protein function`s modification, is a very important action of MnPs on molecular level [80,105].

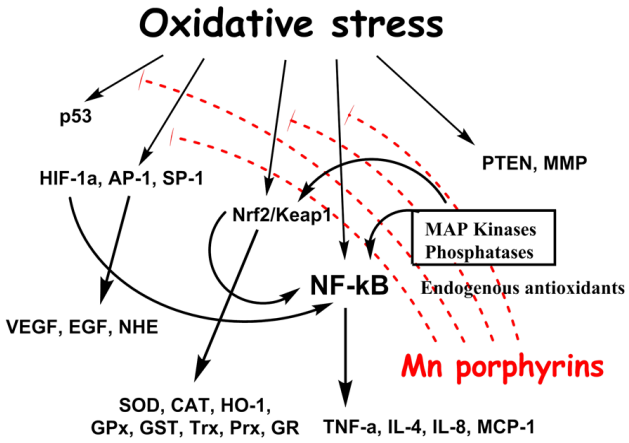


Figure 1.6 - Mn porphyrins (MnPs) affect activities of several transcription factors, such as NF-κB (reproduced from [80]).

The ability to reduce and oxidize $O_2^{\bullet -}$ during the dismutation process suggests that pro-oxidative actions of MnPs may also contribute to their therapeutic effects [79,80,105]. Now it is known that bioavailibility and redox properties control the therapeutic effects of MnPs [80,105]. Bioavailability describes the levels (concentrations) of MnPs at targeted sites (organs and cellular organelles) which is in turn, controlled by lipophilicity, polarity, charge, size, shape and bulkiness of the MnP molecule. Pharmacokinetic studies revealed that MnPs would accumulate in several cellular organelles including mitochondria and the nucleus. Moreover, MnPs could be distributed into several organs in the organism. In fact, MnPs lipophilicity and cationic charge are related with their cellular and organ distribution and, therefore are very important to their bioavailability [79]. Data show that MnPs are highly bioavailable via inhalation route and such knowledge is important for the clinical development of MnPs [80,105]. Moreover, it is recognized that pentacationic charge rather than lipophilicity of pyridyl alkyl chains, controls mitochondrial accumulation of MnPs [79]. In fact, MnPs accumulation in mitochondria and the ability to cross the blood-brain barrier contribute to their remarkable efficacy [78-80,105]. An important aspect is that the reactivity and, in turn, MnPs therapeutic effects are further controlled by the levels or RS in immediate neighborhood of MnPs [79,80,105].

After approximately three decades of research two MnPs, the Mn(III) *meso*-tetrakis (*N*-ethylpyridinium-2-yl) porphyrin, MnTE-2-PyP (BMX-010 or AEOL10113) and also the Mn(III) *meso*-tetrakis (*N*-(2'-n-butoxyethyl) pyridinium-2-yl) porphyrin, MnTnBuOE-2-PyP (BMX-001) have progressed to several clinical trials [80,105].

MnTE-2-PyP was in a clinical trial (NCT02457858) for a non-cancer application, that is the protection of islet cells during transplants, in which it reduces oxidative stress [110]. MnTE-2-PyP is also being tested in clinical trial (NCT03381625) for another non-cancer condition, *i.e.*, atopic dermatitis and plaque psoriasis [80,105].

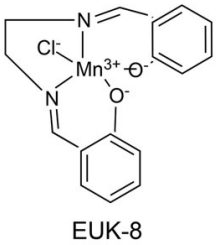
The efficacy of MnTnBuOE-2-PyP for the radioprotection of normal tissue during glioma (NCT02655601), head and neck (NCT02990468) and anal cancer treatment (NCT0338650), was studied in clinical trials in phase I/II at Duke University (USA) [80,81,104,105]. In addition, MnTnBuOE-2-PyP is being studied in other clinical trial (NCT03608020) for radioprotection of normal brain in cancer patients who suffer from brain metastases. It is important to note that MnTnBuOE-2-PyP does not protect cancer cells or cancerous tissue instead it acts as a tumor radio- and chemosensitizer [105].

Additionally, Aeolus Pharmaceuticals conducted phase I trials on di-imidazolyl analog, the Mn(III) *meso*-tetrakis (*N,N'*-diethylimidazolium-2-yl) porphyrin, MnTDE-2-ImP (AEOL10150) as a radioprotector of normal tissue [111].

Considering both the diversity of targets modulated by SODm and also the influence of redox balance in cancer, it is logical to conclude that MnPs might be very useful in cancer therapy including breast cancer therapeutics. This is a very important aspect of this thesis, since one of the most studied and potent lipophilic MnP, the Mn(III) *meso*-tetrakis (*N*-n-hexylpyridinium-2-yl) porphyrin, MnTnHex-2-PyP was investigated in the context of breast cancer invasiveness and metastasis, being this topic addressed in Chapter 4.

b) Mn (III) salen complexes

During the development of synthetic SODm, Mn(III)-salen complexes (salen = N,N`-bis-(salicylideneamino) ethane) were found to exhibit catalytic activity [63,112]. Several relevant SODm of this class were developed by Eukarion, Inc. company, being usually named as EUKs: EUK-8, EUK-113, EUK-134, EUK-189 and EUK-207 [60,92]. As an illustrative example, the chemical structure of EUK-8 is presented in Figure 1.7. The Mn "half" of Mn(III)-salen complexes is coordinated by four axial ligands that can be either N or O atoms [56,60,112]. This is an important aspect since the tetracoordination of Mn allows several possible valence states, thought to be relevant for the scavenging of a wide variety of ROS and thus contributing to the non-selective nature of these antioxidants [60]. Indeed, there are reports referring two key antioxidant properties of Mn(III)-salens, which are scavenging of $O_2^{\bullet -}$ and also H_2O_2 [60,92], which means that Mn(III)-salen complexes exhibit combined catalase-SOD activity [113]. The mechanism that is employed by Mn(III)-salen complexes for $O_2^{\bullet -}$ dismutation is highly similar to the MnSOD mechanism. On the other hand, catalase mimetic activity is exhibited when salen-Mn is oxidised to salen-oxomanganese, using H_2O_2. Moreover, it has been also suggested that these compounds have capacity to react with both $ONOO^-$ and $ROO^{\bullet}$ [56,60].



EUK-8

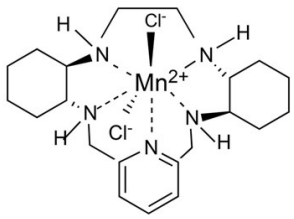
Figure 1.7 - Chemical structure of N,N'-bis(salicylidene) ethylenediamine chloride complex (EUK-8), which forms part of the Mn(III)-salen class of SODm [81].

Compared to other classes of SODm, Mn(III)-salens are considered to have lower catalytic activity. Indeed, they are unstable and exhibit poor SOD-like activity [80]. Therefore, it is possible that the protective effects observed in biological studies are a consequence of

the combination of various antioxidant reactions, instead of a true catalytic SOD activity of Mn(III)-salens [92]. In addition, this group of SODm has other limitations, namely, low water solubility and limited stability [63,92]. For instance, presently the Mn(III) salen, EUK134, is used only in cosmetic skin preparations by Estée Lauder [105].

c) Mn(II) cyclic polyamines

This class of SODm includes the Mn(II)-pentaazamacrocyclic complexes with the macrocycle 1,4,7,10,13-pentazazcyclopentadecane as the template ligand [63,80,81]. In these complexes the Mn(II) is pentacoordinated with atoms of N, being only available for one-electron transfers [56,60], which means that they can transfer one electron to and from $O_2^{\bullet -}$, thus catalyzing its dismutation. However, they do not react with H_2O_2 or $ONOO^-$ [56]. This type of specificity could be desirable for mechanistic studies but considering their therapeutic application, it would be disadvantageous [88]. An important aspect is that although these complexes are specific for $O_2^{\bullet -}$ in test tube reactions, in biological systems due to their higher complexity, that selectivity may not occur [56,60]. During the dismutation of $O_2^{\bullet -}$, the Mn(II) at the active center of the complex undergoes alternate oxidation and reduction, resulting in an interchanging valence state between Mn(II) and Mn(III), as in MnPs [60,88]. Regarding stability, these complexes have excellent kinetic stability although their thermodynamic stability is only reasonable [92]. The most studied SODm of this class is the prototypical complex of the family, M40403 (Figure 1.8) [63,81].



M40403

Figure 1.8 – Chemical structure of M40403 (N,N'-bis{(1R,2R)-[2-(amino)]cyclohexyl}-1,2-diaminoethanetetrahydrochloride) complex forms part of the Mn(II) cyclic polyamines [81].

Metaphore Pharmaceuticals Inc. developed the M40403 compound which has subsequently undergone clinical trials [63,81]. Clinical trials involving M40403 have been either suspended (NCT00033956) or terminated (NCT00101621) [81]. However, a phase II clinical trial led to positive results as the combination of M40403 with morphine have increased the analgesic effects for post-operative pain relief from dental operations [81]. The

cyclic polyamine Mn(II)-pentaaza macrocycle GC4419 (the enantiomer of M40403, previously known as M40419), a potent Mn-based SODm developed by Galera Therapeutics, has completed phase I clinical trials (NCT01921426) considering GC4419 application in chemotherapy and radiation treatment in patients with squamous cell cancer, being presently tested in phase IIb clinical trials. The latter involves the evaluation of the efficacy, safety and tolerability of GC4419 as a treatment to limit the duration of oral mucositis in patients with head and neck cancer which are undergoing chemo-radiation therapy (NCT02508389), the same application as the MnP MnTnBuOE-2-PyP [80,81,105]. For some patients undergoing chemotherapy and radiation treatment it is observed that one of the most usual side effects is oral mucositis which is a painful condition [81]. Therefore, GC4419 has been tested in the clinical trial referred which has demonstrated their efficacy in suppressing oral mucositis in head and neck cancer patients [80,105].

The success of the clinical trials abovementioned has led to the first phase 3 clinical trial (NCT03689712) using a SOD mimic (GC4419) [105].

d) Mn(II) PLED

A more recent class of SODm has been developed by the company PledPharma AB which contains the Mn(II) complexes of dipyridoxyl ethylenediamine diacetate derivatives (PLED-derivatives). Mangafodipir (MnDPDP) (Figure 1.9) is the most studied complex of this class of SODm [114]. Importantly, MnPLED-derivatives (mainly mangafodipir) have been tested in cancer patients and patients suffering from acute myocardial infarction, with promising results [114].

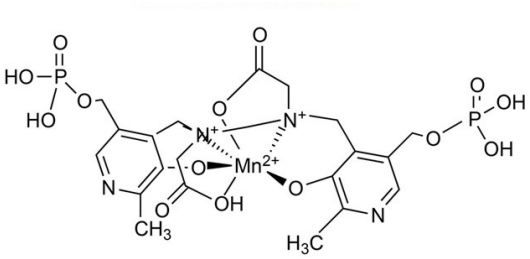


Figure 1.9 – Chemical structure of mangafodipir (MnDPDP), a Mn(II) PLED derivative (adapted from [81]).

Mangafodipir was been clinically approved as a magnetic resonance imaging contrast agent and, in 2010, it has completed a phase II clinical trial. This clinical trial was conducted in patients with colon cancer who had previously undergone surgery. This clinical trial has shown that the pre-treatment with mangafodipir contributed to the reduction of the frequency and severity of adverse effects of FOLFOX (folinate, oxaliplatin, 5-fluorouracil) chemotherapy regimen [114]. Recently, Coriat *et al.* [115] published promising results following

mangafodipir treatment in cancer patients with pre-existing oxaliplatin-associated peripheral sensory neurotoxicity (grade +2), the most problematic toxicity of oxaliplatin. The MnPLED derivative calmangafodipir ($Ca_4Mn(DPDP)_5$) had reached phase II trials in combination with FOLFOX-6 (different doses than FOLFOX) to treat advanced metastatic colorectal cancer patients (NCT01619423) [81]. Recently, in October 2018, it has been initiated a phase III clinical trial where calmangafodipir is used in combination with modified FOLFOX6 (5-FU/FA and oxaliplatin) to prevent chemotherapy induced peripheral neuropathy (CIPN) as a consequence of oxaliplatin action in patients with first-line metastatic colorectal cancer (NCT03654729) [106].

e) Cu(II) complexes

Due to the physiological importance of Cu and its unique redox activity, many Cu complexes with different ligands have been synthetized [116,117] and investigated for their therapeutic and diagnostic potential in human disease, including cancer [89,116,118].

Cu(II) complexes have been designed as chemical models of SOD, since Cu(II) is among the metal ions with ability to catalyse the dismutation of $O_2^{\bullet -}$ [88]. To date several copper(II)-based SODm have been proposed including binuclear or mononuclear complexes with different types of ligands, *e.g.*, derivatives of imidazoles, amines, pyridines and others [56].

Several Cu(II) complexes are efficient SODm *in vitro*, however, *in vivo* they tend to lose activity, mainly those that have acyclic structure. In fact, many of the Cu(II) chelates reported if exposed to *in vivo* conditions, probably will dissociate and release Cu ions [56] and due to the possible production of $HO^{\bullet}$ radical via Fenton reaction, this could raise toxicity concerns [88]. In view of the abovementioned issue and to avoid the dissociation of the metal ion from the complex *in vivo* it is required high stability constants, which can be achieved by using macrocyclic ligands [92]. In addition, if the ligand is a macrocycle, it may have some advantages, namely higher biological stability [119]. Therefore, to be used as potential redox-active drugs, macrocyclic complexes with high kinetic and thermodynamic stabilities towards metal release are thus required [89]. Moreover, for the SODm activity of the complexes, as well as for their stability in the presence of proteins it seems important that the ligand has a macrocyclic structure. Several macrocyclic Cu complexes have been reported to scavenge $O_2^{\bullet -}$ [120,121]. In addition, the use of macrocyclic ligands has shown other advantages, namely in terms of stability in a larger pH range [122].

It must be mentioned that for the development of Cu(II) complexes with SOD-like activity, it is important to find a balance between a certain structural flexibility that allows changing the metal coordination during the course of the catalytic process, as well as a stability high

enough for the application *in vivo* of the Cu(II) complex [122]. Several Cu(II) complexes have been studied so far, however most of them are not thermodynamically stable or do not have high enough activity at the physiological range of pH values [123]. Therefore, the identification of Cu(II) complexes presenting both high stability and activity, together with low toxicity potential is a very important goal to be achieved. In this context, we recently studied a redox active Cu(II) complex with a pyridine-containing aza-macrocycle with SOD-like activity that was previously synthetized and characterized by our group [117]. The assessment of the role of this Cu(II) macrocyclic complex in breast cancer invasiveness and metastasis is an important aim of this thesis, being this topic developed in Chapter 3.

f) Iron(III) complexes

Iron(III) complexes exhibit high kinetic and thermodynamic stability and, therefore, they can be of interest to be studied as SODm. However, Fe ions have a great tendency to react with H_2O_2 via Fenton reaction, thus generating the highly toxic $HO^{\bullet}$ radical [92]. The Fenton reaction may occur either in the presence of Fe complexes or in the presence of free Fe ions, if they are released from the complex during redox cycle [88,92]. Since H_2O_2 is one of the products of the $O_2^{\bullet -}$ dismutation, which is formed due to the complex SOD-like activity, the possibility of the occurrence of Fenton reaction constitute a limitation for the application of Fe complexes as SODm [88,92]. Some Fe(III) porphyrins (FePs) (*e.g.* FeTBAP and FeTMPyP) were able to dismutate $O_2^{\bullet -}$ [56,88]. In addition to the referred SOD-like activity, FePs can also react with $ONOO^-$ [56,88]. Some reports on the protective effects of FePs have been published. However, since FePs can promote $HO^{\bullet}$ radical formation their therapeutic application is somehow limited by the inherent toxicity that may occur [88].

g) Nitroxides

Nitroxides react with free radicals and the resulting products are also free radicals but more stable. Regarding the reaction between nitroxides and $O_2^{\bullet -}$, the reaction rate is low and, therefore, for an adequate activity its necessary a large number of nitroxide molecules to react [124]. In addition, at physiological pH several nitroxides are inefficient as SODm [88]. The SOD-like activity of nitroxides increases when there is a decrease in pH, since the high reactivity is exhibited with protonated superoxide [81]. As a consequence, nowadays these compounds are not considered true SODm by different authors [80].

TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) (Figure 1.10) is a redox-cycling water soluble nitroxide that exhibits superoxide scavenging activity and due to its low molecular weight, its capable of passing through biological membranes. TEMPOL provides cells with protection against damage arising from hypoxanthine/xanthine oxidase or H_2O_2 exposure *in vitro*, with an efficacy that is greater than CAT or SOD administered exogenously [81]. Oral administration of TEMPOL consists in an advantageous alternative to existing therapies, demonstrating significant therapeutic potential in the treatment of multiple sclerosis [81]. In addition, TEMPOL also provides effective treatment in situations of pain conditions. Kim *et al* [125] provide an explanation of how TEMPOL can inhibit both mechanical hyperalgesia and thermal hyperalgesia in models of neuropathic pain induced by CCI and chemotherapy.

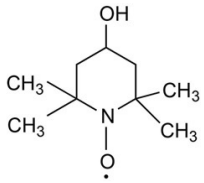


Figure 1.10 - Chemical structure of the nitroxide TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) [81].

Overall, SODm play a role in different cellular redox pathways, allowing the adjustment of deleterious redox imbalances. Regarding the influence of redox balance in cancer and taking into account the variety of targets modulated by SODm these compounds can be very useful in cancer therapy. Despite the amount of encouraging data obtained from both *in vitro* and *in vivo* studies, none of the SODm has already been approved for clinical use in cancer. An aspect that needs further clarification is the timing for the use of SODm as an anticancer strategy [80]. Nowadays it is known that the effect of SOD depends on the stage of the breast cancer but until now, only few studies addressed the impact of SODm in models representative of different cancer stages. More studies are required to fuly define the circumstances in which SODm may be beneficial.

SODm are also useful tools to unravel drugable targets or to characterize the mechanisms of action of novel therapeutics. One important feature of most SODm is their ability to scavenge several RS and impact redox-dependent cellular transcriptional activity. Future work in this field will benefit from a deeper comprehension of the redox basis of breast carcinogenesis. The growing understanding of redox mechanisms, along with the continuous quest for optimized SODm, will impact the progress of those redox-active drugs towards clinic.

1.5. Breast cancer therapeutics and anticancer agents

Cancer therapy is a complex issue that involves the use of different agents to target specific cancer cells molecular and cellular features to cure or, at least hamper the tumor progression. Most of the chemotherapeutic treatments and radiotherapy are based on DNA-damaging drugs which are frequently used in complementary combinations with the purpose of killing the proliferating cancer cells. These drugs can also damage the normal cells DNA, although these cells present a lower replication rate and are usually proficient in all DNA repair pathways, which make non-tumor cells less susceptible to therapeutic drugs.

The major therapeutic approaches used today to treat or control breast cancer are: surgical removal of primary tumors, cancer cells' irradiation to stop their growth and also cytotoxic anticancer drugs, which kill cancer cells or inhibit their proliferation. More recently, treatment options for breast cancer improve greatly and include also immunotherapy and hormonal therapy [126]. As examples of more recent targeted therapeutic strategies for breast cancer subtype HER2+, are the ones related to HER2 protein, such as transtuzumab and pertuzumab [19,126].

Antiproliferative agents, such as doxorubicin (dox; adriamycin, ADR), taxanes (paclitaxel/docetaxel) and cisplatin (cis-diamminedichloridoplatinum, DDP) have long been applied in breast cancer treatment [127]. Various drugs with direct or indirect effects on ROS have been used for breast cancer therapies [55], such as the abovementioned anthracycline drug doxorubicin (dox), which have been used in this thesis as a model of anticancer drug commonly used in metastatic breast cancer treatment.

1.5.1. Doxorubicin (dox)

Since its discovery in 1969, doxorubicin (dox) has been recognized as a potent anticancer agent. With its clinical introduction in the 1970s dox has commonly been utilized for a variety of cancers, including breast cancer and childhood leukemias [128].

Metastatic breast cancer (MBC) is practically incurable with the standard therapy and it turns out that patients with MBC have a median survival of about 2 years after metastases have been detected. Dox is an anthracycline drug widely used in chemotherapy regimen for patients with MBC, being a first line anticancer drug used in MBC chemotherapy, which showed overall response rates of between 35 and 50% in MBC patients who did not receive chemotherapy before [129].

Dox enters the cell via passive diffusion and its major metabolite doxorubicinol, binds to plasma proteins [130]. Dox primary action is to insert into the DNA of replicating cells and to inhibit topoisomerase I and II, which prevents DNA and RNA synthesis resulting in programmed cell death induction [130,131]. These mechanisms seem to play an important role in the antitumoral ability of dox [98]. In fact, it has been shown that dox causes DNA damage with a result of cell cycle block or apoptosis, while other studies support the notion that different doses of dox activate different regulatory mechanisms, inducing either apoptosis or cell death via mitotic catastrophe [132].

Additionally, dox increases ROS levels, in particular $O_2^{\bullet -}$ being a pro-oxidant agent. In fact, dox induces ROS production through two main pathways [131]. The first one is a non-enzymatic Fenton-type reaction by chelating intracellular iron (Fe^{2+}), leading to the conversion of H_2O_2 to the highly reactive $HO^{\bullet}$ radical [131]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Peroxynitrite production via dox, as well as the existence of lipid peroxidation, has also been reported [98,131]. The second pathway by which dox generates ROS is an enzymatic mechanism that involves the mitochondrial respiratory chain (Figure 1.11) [131]. Dox is a quinone that undergoes reduction within the cell, receiving one electron and originating a semi-quinone free radical that in turn donates its unpaired electron to oxygen, forming $O_2^{\bullet -}$. By reducing oxygen to $O_2^{\bullet -}$, dox molecule is regenerated [98,131]. This bioreductive activation may be catalyzed by different oxidoreductases, namely the NADPH cytochrome, the mitochondrial NADH dehydrogenase, P450 reductases of the endoplasmic reticulum and nuclear envelope and the cytosolic xanthine oxidase [56,98,128].

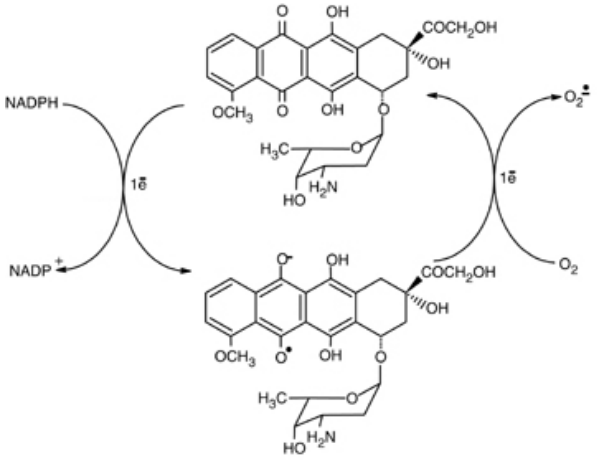


Figure 1.11 – Dox redox – cycling (reproduced from [131]).

Together with the redox cycling process referred, ROS production can also occur as a response to late perturbation of cell metabolism and function resulting from the treatment with dox [98].

The actual strategy in the therapy of breast cancer which includes, as aforementioned, classical chemotherapy, hormone therapy and targeted therapies has been associated with both chemoresistance and serious adverse effects [2]. For example, regarding chemoresistance, exosomes seem to have a significant role. Cancer cells implement exosomes as a drug efflux mechanism potentially involved in drug resistance. Indeed, it was reported that the cytotoxic drugs dox and cisplatin can be eliminated by exosome-secretion [133].

Dox is an effective drug that has been globally applied to treat a variety of solid tumors and hematological malignancies. Despite its excellent anti-tumor activity, dox has a relatively low therapeutic index [132] and its clinical application is limited as a consequence of acute and chronic toxicities associated, such as myelosuppression, immunosuppression and dose-cumulative cardiotoxicity [129]. In fact, the clinical use of this drug is limited since dox generates high levels of ROS, resulting in general toxicity [54]. Dox generates extremely high ROS levels, it penetrates the inner membrane of cardiac mitochondria and competes with coenzyme Q10 in the electron transport chain to induce $O_2^{\bullet -}$ production, which is considered the basis of dox cardiotoxicity [130,134]. As a consequence, this toxic effect becomes dose-limiting. Mitochondria are a primary target involved in dox cardiotoxicity which is mediated by ROS induction through redox cycling of the drug at complex I of the electron transport chain (ETC). This increase in ROS leads to several damaging events that, taken as whole, drastically diminish mitochondrial function [128]. In addition, dox also causes oxidative damage and also induces apoptosis and necrosis to other normal tissues, *e.g.*, kidney, brain and liver [130].

Therefore, decreasing the amount of dox given to patients by using combination therapy with another highly effective non-toxic drug, might suppress vital steps during metastasis which could lead to highly desirable outcomes of breast cancer therapy [135]. In fact, breast cancer treatment is still challenging as drugs are expensive, have serious undesired effects [136] as mentioned for dox and drug resistance is common, mainly in metastatic cases [98,137].

Chemotherapy has a significant role in the management of breast cancer disease. However their efficacy is limited by drug resistance, especially the multiple drug resistance (MDR). Despite the recent improvements, resistance to chemotherapy remains a significant obstacle in breast cancer treatment. After chemotherapy it is possible the survival of a few residual tumor cells. Therefore, if we could have a deeper knowledge of the molecular

mechanisms that allow the survival referred, it could ultimately provide new and more effective chemotherapeutic strategies [138].

Regarding TNBC subtype, current treatment modalities are limited to surgery, radiation, and systemic chemotherapy given the absence of more specific therapeutic targets. Unlike hormone receptor-positive subtypes, nowadays TNBC treatment is limited by the lack of clinically available targeted therapies, however over the past few decades tremendous effort has been expended in the search for molecular targeted therapy for TNBC but with only limited success [26]. Although the reasons for the ineffectiveness of these molecular targeted therapies are unclear, one hypothesis is that breast tumors, in particular TNBCs are highly heterogeneous and, in addition, they use multiple mechanisms, including invasion and metastasis, to enable the aggressive phenotypes. The abovementioned pathways may then become integrated and thus become specific signaling steps which together may contribute to disease progression [139]. In fact, TNBC patients often experience early relapse from distant tumor metastases, although they may initially respond well to the treatments. In addition, because of the lack of targeted therapy ~30%–40% of patients with early-stage TNBC will develop metastatic disease and will die, despite they had received standard multiagent adjuvant chemotherapy [25,26]. Given this situation, the metastatic spread prevention, as well as the treatment of existing metastatic disease in TNBC remains among the top challenges in breast cancer treatment nowadays [25].

There are several reports suggesting that TNBC's resistance to chemotherapy may be explained in part by the EMT hypothesis. Moreover, circulating tumor cells involved in breast cancer metastasis are reported to have mesenchymal features, as well as "gene signatures" of mesenchymal type cells being induce by EMT. In fact, triple-negative type and basal-like type of breast carcinomas have both mesenchymal and stem-cell features, characteristics that can be correlated with resistance to therapy [140]. Thus, the processes of tumor aggressiveness, chemoresistance, metastasis, and invasion appear to be closely inextricably linked to EMT. Indeed, EMT involvement in the development of drug resistance in several cancer types has been reported [25,44]. In addition, there is increasing evidence for a variety of tumor types, which could suggest that cancer cells having CSCs features are more resistant to several conventional chemotherapeutic treatments [9]. BCSCs display increased resistance against both radiation and chemotherapy, indicating the need for new therapeutic regimens that target these cells [14].

Regarding the application of anticancer drugs, the poor oral bioavailability of various drugs due to permeability, solubility or a combination of permeability and solubility issues limits their use. In addition, the pre-systemic metabolism of drugs, drug expulsion via intestinal

drug transporters and variability as a consequence of food effects further restrict application of oral therapies and, therefore, investigations are underway in this direction [126].

The therapeutic redox modulation by SODm may have very promising clinical applications in the chemotherapy field. Most anticancer drugs exert their activity at least partially through the generation of ROS [137]. Also, many adverse effects of chemotherapy are related to oxidative stress [141,142]. SODm may therefore be promising antioxidants for the protection of non-tumoral tissues from chemotherapy and radiotherapy adverse effects. In fact, a promising approach for the future of SODm therapeutics appears to involve combination treatment of the SODm with radiotherapy or chemotherapy [80,81,83,105]. In this context, the combined treatment with dox and a SODm could be beneficial for breast cancer therapy. In this thesis, the combined treatment with dox and a SODm was studied in the context of breast cancer invasiveness and metastasis (Chapter 3 and 4).

1.5.2. Combined treatment with dox and SODm

Regarding anticancer agents development the main goal is to improve both their therapeutic efficiency and selectivity. By exploiting the genetic differences and different basal redox *status* between normal and cancer cells, redox modulation is an emerging concept that may represent important opportunities for cancer therapy [55,57,77,80,83]. Tumor cells have generally higher ROS levels and lower antioxidant defenses than normal ones. Thus, the induction of additional oxidative stress generated by anticancer drugs can lead to the preferential killing of cancer cells. In fact, some chemotherapeutic agent capacity to cause an imbalance in ROS levels can offer a therapeutic opportunity for treating cancer that should be explored.

Concerning breast cancer, redox modulation therapeutics by SODm is a promising approach for clinical applications in association with chemotherapy [83,89,136]. In addition, as abovementioned, many adverse effects of chemo- and radiotherapy are related to oxidative stress [130,134]. In this regard, SODm compounds have already shown to efficiently abrogate chemotherapy and radiotherapy toxic effects in different experimental models [78-81,88], as well as to improve response to therapy [78-80,105,143]. This differential effect of SODm in cancer cells *vs* normal cells is explained in Figure 1.12 [83].

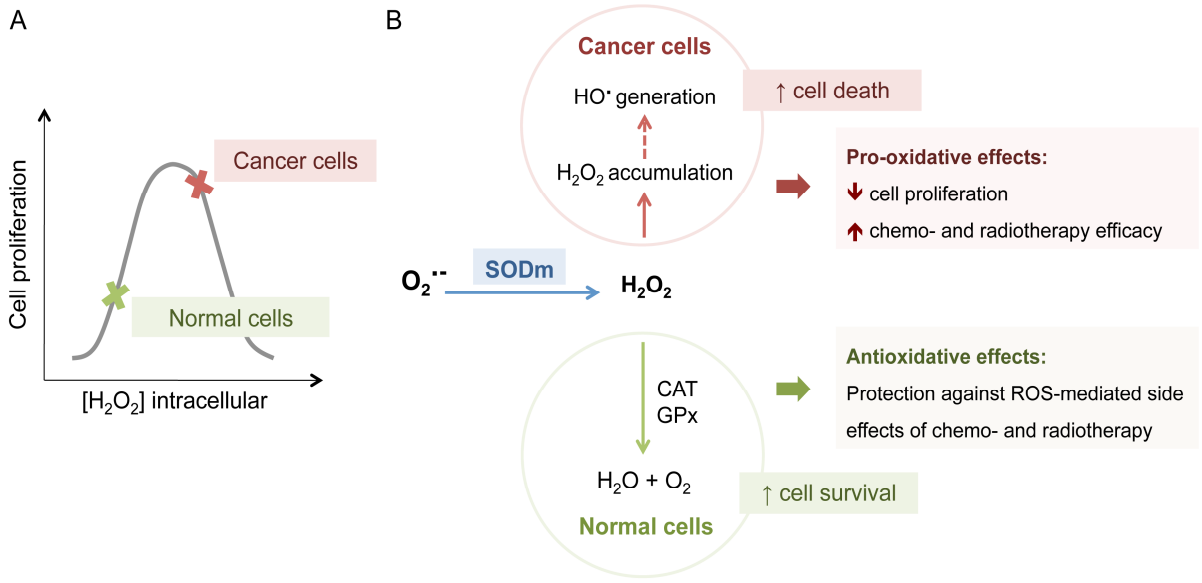


Figure 1.12 – Differential effect of SODm in cancer *vs* normal cells (reproduced from [83]).

As aforementioned, SODm catalize the dismutation of $O_2^{\bullet -}$ into H_2O_2. In normal cells which have the antioxidant defences not compromised, the H_2O_2 formed is efficiently eliminated by CAT and other peroxide-removing mechanisms [136]. Therefore, in normal cells SODm could have an antioxidative effect, since they had the capability to protect normal cells against ROS-mediated side effects of chemo- and radiotherapy [83]. However, cancer cells have often the antioxidant defenses compromised [61,71,136] and, in addition, they have also higher RS levels than normal cells [61,71]. Therefore, as a consequence, in cancer cells the H_2O_2 formed will accumulate. Since this intracellular H_2O_2 level in some tumor cells is close to a cytotoxic threshold, a further increase in H_2O_2 concentration may trigger apoptotic pathways, which are necessary for the therapeutic efficiency of anticancer drugs [136]. Increased levels of ROS induced by anticancer drugs exhausts the remaining antioxidant capacity of tumor cells leading them to death [83,136]. So, in cancer cells SODm could have a pro-oxidative effect, since they have the capability to reduce cell proliferation and therefore tumor growth and, in addition, to improve therapeutic effects of conventional anticancer agents [83,89,144]. Thus, SODm may be useful in chemotherapy, either protecting non-tumor tissues from the oxidative damage induced by conventional anticancer agents like dox, or potentiating its toxic effects in tumor cells, mechanism depending on the cell type and intracellular RS levels [83,89]. Considering the combination with dox, the increase in intracellular H_2O_2 levels seems to be a critical factor [83].

This differential effect of SODm in cancer cells *vs* normal cells is extremely important, being the biochemical basis to design therapeutic strategies in order to selectively eliminate

cancer cells using SODm and ROS-mediated mechanisms [83]. This differential effect was observed in the previous work aforementioned and reported by our group [89], regarding the Cu[15]pyN_5 modulation effect of oxaliplatin cytotoxicity in epithelial breast cells *vs* breast cancer cells.

Concerning the use of the other class of SODm studied in this thesis (Chapter 4), which means the use of MnPs as chemosensitizers, *in vitro* and *in vivo* studies demonstrate that the therapeutic effects produced by MnPs are beneficial for both normal and tumor tissues; in other words, MnPs suppresses tumor growth, while either healing the normal cell/tissue injury or preventing such injury [80,105]. In fact, it has been shown that MnTnHex-2-PyP and MnTnBuOE-2-PyP enhanced temozolide therapeutic efficiency [78]. In a cellular lymphoma model, MnTE-2-PyP accelerated dexamethasone-induced apoptosis. It also potentiated cyclophosphamide in inhibiting lymphoma cell growth and attenuated dox toxicity in H9c2 cardiomyocytes [78,145]. These results highlight the potential of the combination treatment strategy with SODm and conventional anticancer drugs for metastatic breast cancer chemotherapy. Indeed, as mentioned in this introduction SODm are prospective drugs for the treatment of cancer which are being tested in clinical trials in patients treated with radio/chemotherapy [80,81,105,106].

SODm may thus be useful in chemotherapy, either reducing carcinogenesis, protecting non-tumoral tissues from the oxidative damage caused by anticancer agents like dox, or potentiating their toxic effects in tumoral cells. In addition, this potentiation capacity can be an useful approach to overcome drug resistance and some studies have already pointed out a beneficial role of SODm in the sensitization of chemotherapy-resistant tumor cells [146,147].

Overall, the knowledge of the role of SODm, mainly MnTnHex-2-PyP and Cu[15]pyN_5 in breast cancer invasiveness and metastasis should be exploited, thus contributing to develop novel approaches towards breast cancer treatment.

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Chapter 2

AIMS

Reactive oxygen species (ROS) are involved in a number of processes important for metastasis formation, such as adhesion, proliferation, migration and invasion. Despite data demonstrating the redox regulation of pathways related to migration/invasion by ROS, there are still many gaps in the understanding of their role in the invasive processes. While ROS seem to be relevant for cancer invasiveness and metastasis, only few studies are available focusing on the redox regulation of breast cancer progression. Lethal invasion is a major characteristic of metastatic cancer cells. However, the mechanisms of tumor invasion and metastasis are still not well understood. Moreover, despite the modulation of several processes important for the aggressiveness/invasion of cancer cells by SODm, they have not yet been duly studied in this context. Furthermore, while MnSOD seems to play a protective role in early stages, it can be associated with more aggressive phenotypes in advanced breast cancer. As many studies suggest the potential therapeutic usefulness of SODm in breast cancer, it is important to study the impact of these compounds in cellular processes related to the formation of metastases. Such information will also contribute to understand the influence of cellular redox balance in the migratory and invasive capabilities of breast cancer cells.

The global aim of this thesis was to explore the impact of redox-active compounds belonging to distinct classes of SODm, on the migration and invasiveness of human breast cancer cells with different degrees of malignancy. As such, the goal was to evaluate the effect of the manganese porphyrin (MnP) MnTnHex-2-PyP and the Cu(II)-macrocycle complex Cu[15]pyN_5, in some determinant events of metastases formation.

Cu[15]pyN_5 is a macrocyclic copper(II) complex previously synthetized, characterized and studied by our group, as a chemotherapy sensitizer in mammary cells. The MnP MnTnHex–2-PyP is a prospective drug for the treatment of several diseases, including cancer. The possibility of studying this MnP resulted from the collaboration between iMed.ULisboa and CBIOS with the Radiation Oncology Department of Duke University.

This work explores the potential usefulness of Cu[15]pyN_5 **(Chapter 3)** and MnTnHex-2-PyP **(Chapter 4)** in hindering the migration/invasion processes of breast cancer cells with different degrees of invasiveness. This is important since as the basal redox *status* of mammary cells differs with their degree of aggressiveness, SODm may operate differently according to the cell type. In the present study, human cell lines representative of breast adenocarcinoma with low aggressiveness (MCF-7) and metastatic (advanced) breast cancer (MDA-MB-231) were selected as cellular models. MCF-7 cell line is a model of non-invasive estrogen receptor positive breast tumor. In contrast, the human cell line MDA-MB-231 recreates highly aggressive and invasive breast cancer.

The intracellular redox balance may play a crucial role in cells resistance to anticancer drugs. As key redox modulators, SODm can alter the effects of anticancer drugs on cell proliferation, migration and invasion. Thus, this thesis aimed also to study the influence of the redox modulators Cu[15]pyN_5 and MnTnHex–2PyP on the anticancer activity of chemotherapeutics commonly used in metastatic breast cancer, specifically doxorubicin (dox). Dox is a first-line drug used in metastatic breast cancer and one of the most relevant and effective chemotherapeutic agents for breast cancer, which contributes to the generation of $O_2^{\bullet -}$ radicals within cancer cells.

In this regard, the aim of this thesis was to evaluate the role of both SODm, either alone or in combination with dox in the migration/invasion, intracellular ROS levels and other aspects related to the migratory/invasive phenotype in the two representative human breast cancer cells aforementioned.

In order to achieve the referred goals, a first study was developed focused on the SODm **Cu[15]pyN_5 (Chapter 3)**, based on the following specific objectives:

- To characterize the cellular redox *status* upon treatment with Cu[15]pyN_5, in terms of intracellular ROS levels;

- To evaluate the impact of Cu[15]pyN_5 on the cell viability of mammary cells treated with dox;

- To understand the influence of Cu[15]pyN_5, alone or combined with dox, on the migration/invasion capabilities of breast cancer cells with different degrees of invasiveness, by assessing the effect of this SODm in terms of collective and individual cell migration, as well as in chemoinvasion;

- To understand if the effects of Cu[15]pyN_5 were mediated by an increase in H_2O_2 levels, by performing assays in the presence of the H_2O_2-degrading enzyme catalase;

Concerning the study with the reference SODm **MnTnHex-2-PyP** and due to its relevance mentioned in **Chapter 1**, a more in-depth study was developed **(Chapter 4)**. The specific objectives of this work were:

- To assess the effects of MnTnHex-2-PyP on the cellular redox stated, by using probes with differential reactivity towards ROS;

- To evaluate the impact of MnTnHex-2-PyP on the cell viability and cell cycle progression, alone or in combination with dox;

- To study the influence of MnTnHex-2-PyP, alone or combined with dox, in different types of cell migration (collective, chemotaxis, and random);

- To explore the mechanisms underlying the effects of MnTnHex-2-PyP on cell migration by studying the influence of H_2O_2, as well as the impact of the SODm on focal adhesions;

- To investigate the impact of MnTnHex-2-PyP, as single agent or in co-treatment with dox, on cell invasion and to explore the contribution of the proteolytic degradation of extracellular matrix (ECM);

- To explore the molecular pathways modulated by this SODm, by assessing NF-kB activation upon treatment of both cell lines with dox and/or MnTnHex-2-PyP.

Overall, the studies presented in this thesis aim to explore the influence of SODm in breast cancer cells migration and invasion. Such knowledge may contribute to discover potential novel sensitizers for metastatic breast cancer chemotherapy.

Chapter 3

Role of the copper(II) complex Cu[15]pyN_5 in intracellular ROS and breast cancer cell motility and invasion

This Chapter was adapted from:

Fernandes AS, Flórido A, Saraiva N, Cerqueira S, Ramalhete S, Cipriano M, Cabral MF, Miranda J, Castro M, Costa J, Oliveira NG (2015) Role of the copper(II) complex Cu[15]pyN_5 in intracellular ROS and breast cancer cell motility and invasion. *Chem Biol Drug Des* **86**: 578-588

Abstract

Reactive species (RS) have been greatly implicated in cancer at different levels, including in cell migration and invasion, key determinants for the metastatic process which undergo redox regulation. Cu[15]pyN_5 is a redox-active macrocyclic copper(II) complex, previously synthetized, characterized and studied by our group, as a chemotherapy sensitizer in mammary cells. In the work presented in this Chapter, we evaluated the role of Cu[15]pyN_5, either alone or in combination with dox on the migration/invasion and intracellular ROS levels in two representative human breast cancer cells: MCF-7 (adenocarcinoma), with low aggressiveness and MDA-MB-231 (metastatic breast cancer), highly aggressive cells.

Cu[15]pyN_5 was not toxic for these cell lines and did not alter the cytotoxic effect of dox. Cu[15]pyN_5 significantly reduced MCF-7 cells motility, alone and also in co-treatment with dox, although without statistical significance in this case. In MDA-MB-231 cells, only slight decreases in migration were observed in the cells treated with both agents alone or in combination. Cu[15]pyN_5 decreased significantly MCF-7 directed cell migration, but not in MDA-MB-231 cells. The co-treatment with dox and Cu[15]pyN_5 induced a small reduction in chemotatic migration of both cell types.

Chemoinvasion of MDA-MB-231 cells was also evaluated. The co-treatment with dox and Cu[15]pyN_5 decreased significantly the proteolytic invasion of MDA-MB-231.

Intracellular ROS levels were assessed by fluorescence microscopy using the probe dihydrorhodamine 123 (DHR). Cu[15]pyN_5 and dox significantly increased intracellular ROS in both cell lines. The increase was more pronounced upon a co-treatment and was partially reversed by CAT. This increase could be at least partially due to H_2O_2 accumulation. These data suggest that H_2O_2 accumulation could be, at least partially, responsible for the effects observed in the different assays. In summary, the combination of Cu[15]pyN_5 with dox may be beneficial in breast cancer treatment as it could help reduce cancer cell migration and invasion.

Keywords: breast cancer, cell migration and invasion, doxorubicin, macrocyclic copper(II) complex, redox modulation, superoxide dismutase mimics

3.1. Introduction

In accordance with Chapter 1 (General Introduction), breast cancer is the most frequently diagnosed cancer (excluding skin cancers) among women in the United States [1]. It is still one of the leading causes of cancer death in both developed and non-developed countries [1-3]. In fact, the incidence of breast cancer is growing [4].

Heterogeneity in pre-malignant and invasive breast tumors has been extensively reported [2]. As explained in Chapter 1 breast cancer is a heterogeneous group of disease subtypes that exhibit distinct biological and clinical characteristics [3], namely different degrees of proliferation, vascularity, inflammation and/or invasiveness [5], which reflects the aforementioned heterogeneity.

There are also important differences among breast cancer cells in terms of redox *status* and antioxidant background [6-8]. In this regard, differences between malignant and nonmalignant breast cells have also been reported [9].

Redox modulation is an emerging concept that may represent important opportunities for cancer therapy [10, 11]. As stated in Chapter 1, along with cell proliferation, multiple mechanisms related to the migration/invasion of cancer cells could undergo redox regulation. ROS may alter the rates of cell migration by directly regulating actin cytoskeleton reorganization [12, 13]. Moreover, ROS regulate several pathways closely related to cellular migration/invasion, namely integrins, small GTPases, MAPK, PI 3-K, FAK, NF-κB, AP-1, and matrix metalloproteinases (MMPs) [12-14].

In addition, ROS modulation may be beneficial in reducing tumor migration/invasion and metastization and also to improve therapeutic effects of conventional anticancer agents [15-17]. In this regard, a very important aspect is that redox - active agents could constitute a beneficial therapeutic approach. In fact, the redox therapeutics of cancer is a highly attractive topic due to the number of druggable targets involved. However, additional novel strategies are required. In this context and regarding the distinctive redox and catalytic chemistry of copper, several Cu(II) complexes have been reported and suggested for various therapeutic purposes, including cancer [18, 19].

Copper (Cu) is an essential trace transition metal that can exist in oxidised (Cu^{2+}) and reduced (Cu^{+}) states within the human body. This allows it to participate in redox and catalytic chemistry, making it a suitable cofactor for a diverse range of enzymes (*e.g.* SOD) and other molecules involved in many biological processes. Cu distribution and homeostasis are highly regulated, since free Cu ions are potentially harmful to cells. Disruption of Cu

homeostasis is a pathological feature and a potential cause or contribution to many disease states [18, 19].

Cu complexes have been shown to be effective in cancer treatment due to their cytotoxic action on tumor cells [18]. In the case of Cu(II) complexes, an additional mechanism underlying the anticancer effect has been reported. Due to the high levels of H_2O_2 in cancer cells and Cu Fenton chemistry, hydroxyl radical may be generated [20], which can be a further advantage in promoting cancer cells death by this pro-oxidant effect [21].

Cu(II) complexes have been designed as chemical models of SOD, since Cu(II) is among the metal ions with ability to catalyse the dismutation of $O_2^{\bullet -}$ [22]. Copper(II) complexes have been designed as SODm for potential therapeutical application in human pathologies, including cancer. A redox active Cu(II) complex with a pyridine-containing aza-macrocycle (Cu[15]pyN_5; Figure 3.1) with SOD-like activity was previously synthetised and characterized [23]. Aza-macrocyclic ligands are related to biomimetic and catalytic systems, because many coordination compounds with aza-macrocycles are electrocatalytically active in redox reactions [24].

This complex is thermodynamically stable, presenting high values of stability constant (log K_{CuL}) and high pCu value for physiological pH [23]. These features are essential to prevent possible losses of activity and toxic effects in biological milieus. The crystal structure of $[Cu([15]pyN_5)]^{2+}$ determined by X-ray diffraction showed the Cu(II) centre coordinated to all five macrocyclic nitrogen (N) donors, in a distorted square pyramidal geometry (Figure 3.1.B) [23]. The SOD-like activity is known to depend on structural features, namely on the conformation of the active site. Too stable square-planar geometries may not be favourable for the $O_2^{\bullet -}$ dismutation [25], while more distorced arrangements lead to higher SOD activity [26].

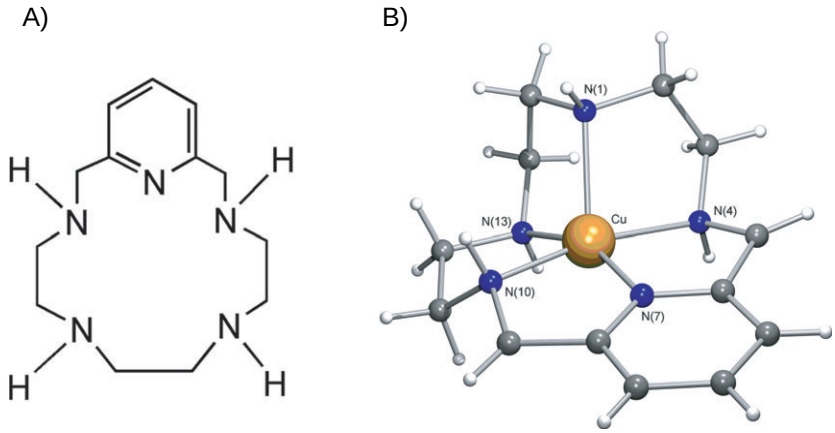


Figure 3.1 – Chemical structure of the macrocyclic ligand [15]pyN5 (A) and molecular structure of its copper(II) complex Cu[15]pyN5 (B) (adapted from [23]).

The complex was also studied as chemotherapy sensitizer in mammary cells and the results showed that Cu[15]pyN_5 was a modulator of the cytotoxicity of oxaliplatin in MCF-10A epithelial breast cells and in MCF-7 breast cancer cells, since it enhanced the therapeutic window of oxaliplatin, by both protecting non-tumor cells and increasing its cytotoxicity in breast carcinoma cells [20]. These results are very important and could be used to design a potential therapeutic strategy to selectively eliminate cancer cells using a SODm.

In this work we studied the effects of Cu[15]pyN_5 in the presence of doxorubicin (dox), an anthracycline drug widely used in breast cancer treatment. Complementary endpoints of cell viability, migration and cell invasion were studied in human cell lines representative of breast adenocarcinoma (MCF-7) and metastatic (advanced) breast cancer (MDA-MB-231). The results described in this Chapter pointed out differential cell line-dependent effects and reinforce the potential interest of the complex Cu[15]pyN_5 in breast cancer.

3.2. Materials and Methods

3.2.1. Chemicals

Dulbecco´s Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin solution, insulin solution from bovine pancreas, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), crystal violet, phosphate-buffered saline, (PBS; 0.01 M, pH 7.4), trypsin, *tert*-butylhydroperoxide (TBHP), doxorubicin (dox), catalase (CAT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Matrigel was purchased from BD Biosciences. Ethanol and acetic acid were purchased from Merck (Darmstadt, Germany). Dox was dissolved in Milli-Q H_2O and stored at -20ºC. Before being used in cell culture experiments, all the aqueous solutions were sterilized by filtration with membrane filters of 0.22 µm pore size. Dimethylsulfoxide (DMSO) was obtained from Merck. Dihydrorhodamine 123 (DHR) and dichlorodihydrofluorescein diacetate (DCFDA) probes were purchased from Molecular probes. A 10 mM stock solution of DHR and DCFDA was prepared in DMSO, aliquoted and stored under nitrogen at -20 ºC. Cu[15]pyN5 was synthesised by our group as previously described [23].

3.2.2. Mammary cell lines

In general, the cell lines mirror both the genomic heterogeneity and the recurrent genome copy number abnormalities found in primary tumors with high fidelity. This is remarkable, considering the fact that many of the cell lines have been carried in culture for many years or decades. This indicates that they have not accumulated substantial new recurrent aberrations during extended culture, supported by analysis showing stable genomic and expression patterns in the cell lines over multiple passages. In addition, important genome aberration ‘‘landmarks’’ like the high-level amplifications associated with poor outcome in primary tumors are well represented [27].

A considerable part of our knowledge on breast carcinomas is based on *in vivo* and *in vitro* studies performed with breast cancer cell (BCC) lines. Therefore, human breast cancer cell lines are useful *in vitro* experimental models for the purpose of this work and two human breast cancer cell lines, with different degrees of aggressiveness, were used in this work: MCF-7 (adenocarcinoma), with low aggressiveness and MDA-MB-231 (metastatic breast cancer), highly aggressive cells [27, 28].

3.2.2.1. MCF-7 and MDA-MB-231 breast cancer cells characteristics

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old caucasian woman breast adenocarcinoma. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers [29].

MCF-7 cells have epithelial cell morphology and regarding cell culture properties they are adherent (Figure 3.2). In fact, MCF-7 cell line is E-cadherin–expressing and anchorage-dependent human breast cancer cell line [30].

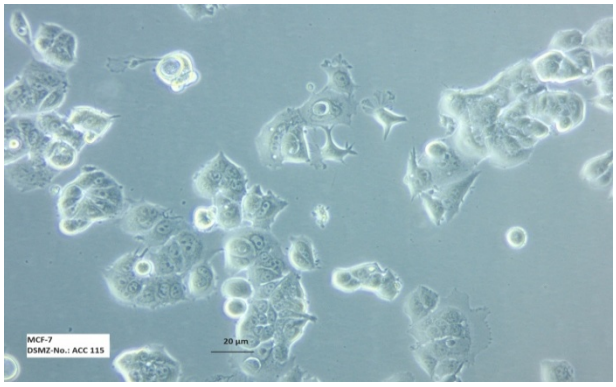


Figure 3.2 – Images from optical phase-contrast microscopy of MCF-7 cells in culture (reproduced from [31]).

As observed in Figure 3.2, these 'luminal epithelial-phenotype' cells grew as interconnected colonies of polygonal cells on plastic. The luminal cell population is characterized by its round or oval shape. Luminal cells appear more differentiated and form tight cell-cell junctions [27]. In fact, MCF-7 cells displayed a cobblestone-like appearance and tight cell–cell junctions [28]. MCF-7 cell line has been used as a model of the mammary epithelium because they maintain a number of characteristics similar to mammary epithelium. Despite being a breast cancer cell line it belongs to a less aggressive and noninvasive/metastatic group [28]. MCF-7 cells represent the non-invasive and oestrogen receptor alpha (ER-α) positive breast cancer, being one of few breast cancers cell lines that express the oestrogen receptor alpha (ER-α) [27, 28].

MDA-MB-231 breast cancer cell line is one of the most commonly used for the *in vitro* experimental study of hormone-independent breast cancer. These cells were isolated by Cailleau and collaborators in 1973, from a pleural effusion (a chest wall recurrence) of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma, in Houston (USA) [32].

MDA-MB-231 cells have epithelial cell morphology and regarding cell culture properties they are adherent (Figure 3.3).

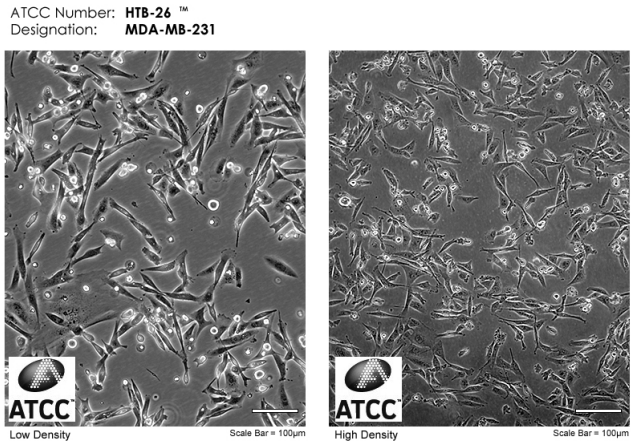


Figure 3.3 – Images from optical phase-contrast microscopy of MDA-MB-231 cells in culture (reproduced from [33]).

As observed in Figure 3.3, MDA-MB-231 cells have spindle-shaped and fibroblastoid phenotype. Initially it was though that these cells were basal-like subtype cells [27] and, in fact, the basal cell population is characterized by its spindle or elongated shape. In addition, basal subtype B cells appear less differentiated and have a more mesenchymal-like appearance [27]. However, it is now recognised as belonging to the claudin-low molecular subtype since it exhibits, for example, down-regulation of claudins, as well as increase in markers of epithelial-mesenchymal transition (EMT) and expression of characteristics associated with mammary cancer stem cells (CSCs) [34].

As aforementioned in Chapter 1, ductal breast carcinomas are highly heterogeneous, and the type most aggressive, even if rare, is triple-negative when tested for oestrogen receptor/progesterone receptor (ER/PgR) and epidermal growth factor receptor-2 (HER2). A correspondence may exist with the invasive MDA-MB-231 cell line since it is a triple negative (ER-α, PgR and HER2 negative) mesenchymal-like cell line [27, 28, 34].

MDA-MB-231 and MCF-7 cells are independently derived lines and, thus, likely differ in many functional and genetic properties [27, 29, 32]. Table 3.1 shows the sources, clinical and pathological features of the tissues used to derive the mammary cell lines used in this work:

Table 3.1 - Sources, clinical and pathological features of the tissues used to derive the mammary cell lines used in this work (adapted from [27, 34]).

Cell line	Gene cluster	ER (α)	PgR	HER2	TP53	Source	Pathology	Age (years)	Ethnicity
MCF-7	Lu	+	+	-	+/- ^{WT}	PE	AC	69	W
MDA-MB-231	Mes	-	-	-	++^{M}	Metast, PE	IDC	51	W
MCF-10A*	BaB	-	-	-	+/- ^{WT}	P.Br	F	36	W

AC, adenocarcinoma; BaB, Basal B; F, fibrocystic disease; IDC, invasive ductal carcinoma; Lu, luminal; Mes, mesenchymal; Metast, metastasis; P.Br, primary breast; PE, pleural effusion; W, white. *Derived from a reduction mammoplasty; MCF-10A are not cancer cells, but immortalised normal breast epithelial cells.

3.2.2.2. MCF-7 and MDA-MB-231 cells culture conditions

MCF-7 cells were obtained from DSMZ and MDA-MB-231 cells were acquired from ATCC. Both cell lines were kept in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. MCF-7 cells medium were also supplemented with 0.01 mg/mL insulin. Cultures were kept at 37 °C, under a humidified atmosphere containing 5% CO_2 in air.

3.2.3. MTT reduction assay

The effect of Cu[15]pyN_5 in cell viability, either given alone or in combination with dox, was studied by the MTT assay [35] in the two human mammary cell lines. In this colorimetric assay, MTT (yellow tetrazole salt) is reduced by mitochondrial enzymes of viable cells into an insoluble purple product, formazan (Figure 3.4).

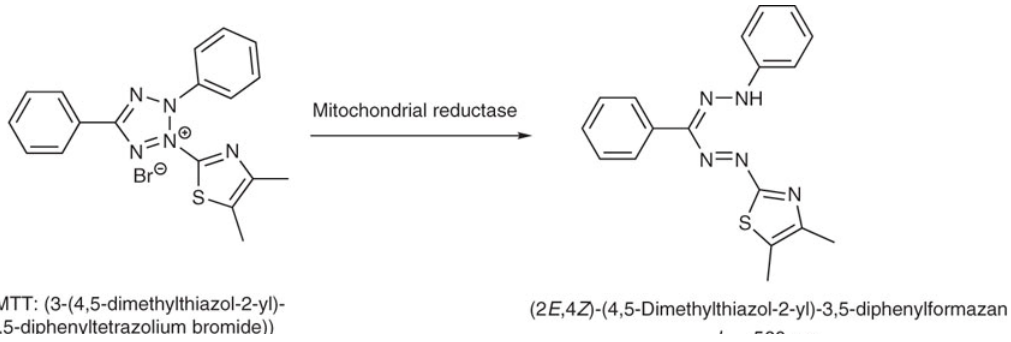


Figure 3.4 – MTT reduction in viable cells by mitochondrial reductase results in the formation of a formazan derivative.

A solubilization solution (usually DMSO) was added to dissolve the insoluble purple product into a colored solution. The absorbance of this colored solution can be quantified at 595 nm. The absorbance is proportional to the number of viable cells and, therefore, is a measure of mitochondrial function [35].

Figure 3.5 shows an image of a 96 well-plate after the procedure of this assay. In this image it is easy to observe the purple colour of the product, as well the different colour intensity as a consequence of different number of viable cells in the wells.

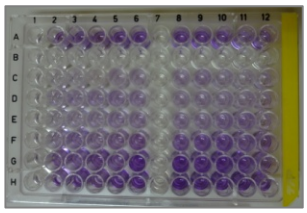


Figure 3.5 – Image of a 96 well-plate at the end of the procedure (own source).

Briefly, approximately 6.5 $\times$ 10^3 cells (for MCF-7) or 5$\times$ 10^3 cells (for MDA-MB-231) were cultured in 200 μL of culture medium per well in 96-well plates. The cells were grown for 48h and then exposed to different concentrations of dox (0.5-20 μM), alone or in combination with the Cu(II) complex (100 μM), for a 24 h-period.

After the treatments, the cells were washed with culture medium and MTT (0.5 mg/mL in culture medium) was added to each well [36]. The cells were grown for a further period of 2.5 h and then carefully washed with PBS. DMSO (200 μL/well) was added to solubilise the formazan crystals and absorbance was measured at 595 nm. Two to ten independent experiments were performed and four replicate cultures were used for each complex concentration in each independent experiment.

3.2.4. Wound Healing Assay

The wound healing technique also known as *in vitro* “scratch” assay is an easy, low-cost, simple but useful and well-developed assay to measure cell migration *in vitro*, that is applicable to a wide variety of adherent cells including mesenchymal and epithelial tumour cells, fibroblasts and endothelial cells [37, 38].

The sequence of basic steps of the wound healing assay consists in: creating a “scratch” in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch and comparing images to quantify the migration rate of the cells (Figure 3.6). In more detail, cells are grown to confluence and then a ‘‘scratch’’ is made,

usually with a plastic pipette tip, in the centre of the monolayer. It is important that the cells are nearly confluent so that the edges of the wound are sharp, and practice is required to obtain reproducible results [37]. It is also important to create scratches of approximately similar size in the assessed cells and control cells to minimize any possible variation caused by the difference in the width of the scratches [38].Time-lapse videomicroscopy or digital image acquisition over time (Figure 3.7) then allows the dynamics of wound closure to be measured under different conditions [37].

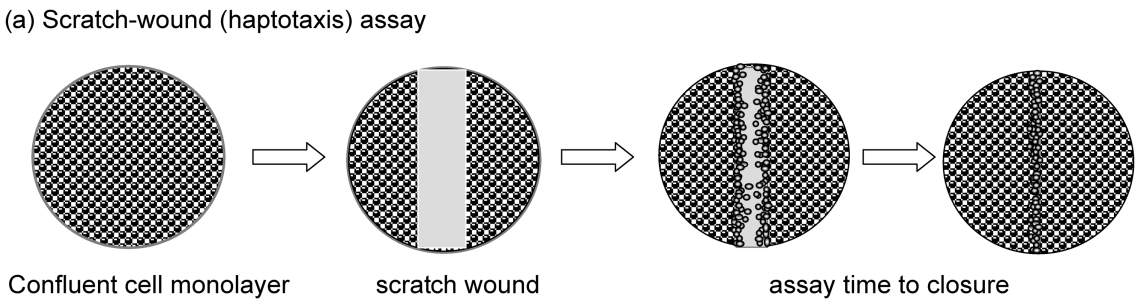


Fig. 3.6- A simple ‘‘scratch-wound’’ assay is illustrated in (a). This is suitable for both tumor cells and measures movement on a solid surface, generally glass or plastic, which may be coated if desired (adapted from [37]).

The wound healing assay has also the advantage of mimic, to some extent, the collective migration of cells *in vivo* [38]. Compared with other methods, this assay can be used for studies on the effects of cell–matrix and cell–cell interactions on cell migration. In addition, with the wound healing assay it is possible to capture images of live cells during migration, in order to monitor intracellular events. This methodology has also been applied to measure migration of individual cells in the leading edge of the “scratch”, instead of monitoring migration of homogenous cell populations [38].

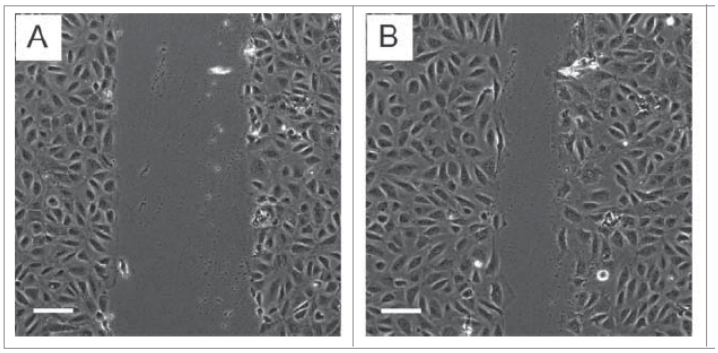


Figure 3.7- Exemplificative phase contrast images from a scratch assay experiment, carried out in endothelial cells, at different time points: A) t = 0 h, B) t > 0 h. Scale bar = 120 mm.

In this work the *in vitro* wound healing assays were optimized according to Liang *et al.* 2007 [38]. MCF-7 cells and MDA-MB-231 cells were seeded in 24-well plate with an inoculum of 2×10^5 cells per well and cultured in complete medium for 24 h. After 24 h, medium was removed and each well was scratched using 200 µL pipette tip, leaving a gap of approximately 0.8 mm in width. The cells were then rinsed twice with PBS to remove the loosen cell debris and assay medium was added, lacking FBS (since FBS is a cellular proliferation promoter and, in addition, serum proteins such as fibronectin and laminin could have the ability to mediate cell migration) and insulin, and containing the test compounds.

The distance between the two scratch edges was evaluated microscopically (Motic AE 2000 inverted microscope) and data images were recorded using Moticam 2500. Scratches were measured using Motic Images Plus V2.0 software at defined time points: 0, 9, and 24 hours after compounds addition.

Zero h was considered as 0% of wound closure. At each time point 3 pictures were taken from the same scratch. Each assay was performed with intern triplicates and at least 4 independent experiments were performed for each condition.

3.2.5. Chemotaxis and Chemoinvasion assays

Directional cellular migration in response to an extracellular gradient of soluble bioactive molecules (chemotaxis) is achieved when a suitable attractant is used [37]. Therefore, chemotaxis is the directional migration of cells toward a chemoattractant, in this case active movement from the upper side of a transwell insert (where the cells are added) to the lower side (where the chemoattractant is placed) of the filter. A suitable attractant could be FBS which is placed in the lower well. FBS has a plethora of chemotactic factors since contains a myriad of chemokines and extracellular matrix adhesion proteins like fibronectin and laminin that are known to attract tumor cells [39]. Serum or culture media containing 5–10% FBS (commonly referred to as complete medium) are routinely used in Boyden chamber motility assays as chemoattractans.

Perhaps the most widely used method for assaying chemotactic migration of cells is based around a modified Boyden chamber. Boyden chambers which are plastic devices consisting of upper and lower chambers separated by a porous filter are used to measure chemotaxis. The original Boyden chamber method has been extensively refined since its initial description. The original chambers were reusable but many types are now disposable.

One of the most popular and used in this work, is the BD Bioscience Transwell™ which can be used for both migration and invasion assays [37].

In this assay, cells are introduced in suspension to the top of a chamber that is separated from a lower chamber by a semipermeable membrane containing pores of a defined diameter (usually 3–14 mm) designed to allow passage to motile cells. The pore size selected should be such that active processes are required and the cells should deform and migrate rather than passively fall through to the underside. This assay is indicative of the motility of individual cells [37]. The polycarbonate filters used for this test produced with different pore sizes must be chosen carefully: the cells under investigation should be able to pass through the pore size by actively ‘‘squeezing’’ through it, but should not passively fall through excessively large pores or get physically blocked by pores that are too small. For this reason, large epithelial and endothelial cells should be tested using filters with pores of 12 mm diameter. Many other types of cells do well with the standard 8 mm size and, in this case, the movement of cells from the upper wells through the 8 µm pores could be considered a directed motility with minimal to negligible random movement [40].

An important aspect is that care should be taken in the preparation of cells for loading. Most epithelial cells will require to be released from plastic flasks prior to assay, and this may be achieved by EDTA and/or trypsin. It is important not to over-trypsinise cells (as key adhesion molecules may be stripped off) but equally, it is necessary to ensure that a single cell suspension is prepared, as clumps will interfere with the assay.

The assay is usually designed so that cells remain attached to the lower surface of the membrane. Non-migrated cells on the upper surface first need to be removed with a cotton bud. Cells which have migrated through the filters can be quantified by a variety of methods, including fixation, staining (*e.g.*, using CV) and manual counting or absorbance measurement.

Chemoinvasion is the migration in the presence of the matrix barrier, which is dependent on invasive capabilities of the cell [41]. A chemoinvasion assay quantifies the invasive potential of most cell types and it can be applied to detect the migratory activity associated with matrix degradation.

The chemotaxis assay can be converted to a chemoinvasion or transmigration assay by adding a barrier of ECM proteins, Matrigel™ or a confluent layer of fibroblasts or endothelial cells to the upper surface of the filter, prior to seeding of tumor cells [37]. A chemoinvasion assay based on the Boyden chamber was originally described by Albini, who used Matrigel™, a natural reconstituted basement membrane product, as an overlay to the filter [37].The ‘chemoinvasion assay’ using Matrigel™, in Boyden blind-well chambers was developed more than 25 years ago as a tool for invasion and metastasis research. Since

then, it was adapted for investigation of how different cells types engage with and penetrate basement membrane, including research in angiogenesis, invasive cell migration, as well as for screening of molecules that inhibit invasion. In fact, this assay is widely used for this application, since is a very rapid, easy, inexpensive, reliable and flexible test that can be used to quantify the invasive potential of most cell types, as well to detect the migratory activity associated with matrix degradation [40, 41].

Matrigel is a solution of tissue basement membrane matrix extracted from the Engelbreth–Holm–Swarm (EHS) mouse tumour. It is rich in extracellular matrix proteins, with laminin, collagen IV and heparin sulphate proteoglycans as major components. Matrigel (from matrix and gel) provide a matrix material with several unique characteristics which became widely known [28]. Matrigel can simply be applied diluted in its cold, liquid form (at 4 ºC) over filters in the Boyden chamber chemotaxis assay, allowing the formation of a polymerized gel when warmed to room temperature or 37 ºC, producing a reconstituted, biologically active matrix ready-to-use. Importantly, it is necessary to avoid bubble formation and to create an uniform coating [40].

Matrigel™ is still widely used as an invasion barrier, although different batches may vary in consistency including the levels of growth/chemotactic factors sequestered within them [37].

The most frequent inaccuracy seen in the literature regarding the use of the chemoinvasion assay to measure the ‘‘pure’’ invasive components is the uncoupling of the chemoinvasion assay from a parallel chemotaxis assay, where no coating is applied. Albini *et al* [41] suggest a parallel evaluation of both chemotaxis and chemoinvasion using the same chemoattractants. The comparison between chemotaxis and chemoinvasion allows dissection of the relative contribution of invasion over simple migration.

A schematic representation of the chemotaxis and chemoinvasion assays is represented in Figure 3.8:

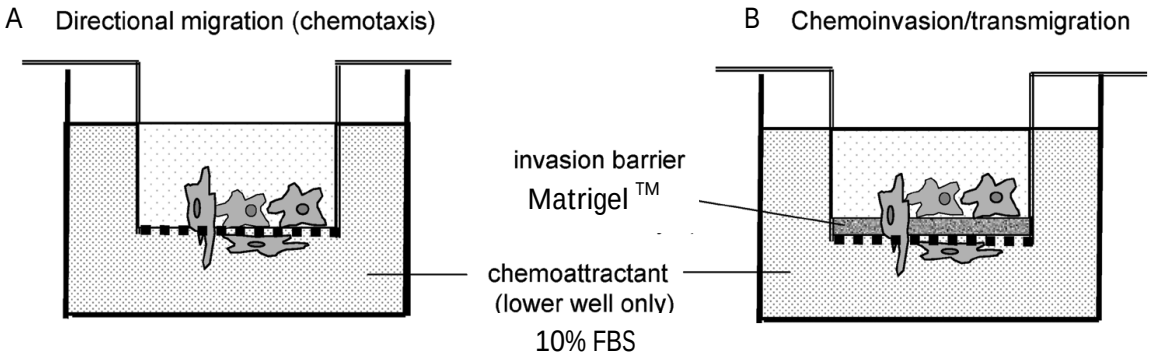


Figure 3.8 - Schematic representation of the chemotaxis (A) and chemoinvasion (B) transwell inserts assay (adapted from [37]).

3.2.5.1. MCF-7 and MDA-MB-231 cells chemotaxis assay

The chemotactic migration of MCF-7 and MDA-MB-231 cells was evaluated in 24-well plates with transwell inserts with transparent PET membranes containing pores of 8 μm (BD Falcon, Biosciences, USA) (Figure 3.9). These membranes allowed the passage of motile cells from the upper chamber to the lower chamber in response to chemotactic gradients. Cells remain attached to the lower surface of the membrane.

A  B 

Figure 3.9- Image of a transwell insert with transparent PET membrane with pores of 8 μm A). Image of a 24-well plate with transwell inserts B) [42].

Cells ($1x10^5$ cells in 200 μL of FBS-free medium) were seeded on the top of the insert and complete medium was placed in the lower chamber of the culture well. The test compounds were added to both chambers and cultures were incubated for 16 h. Non-migrating cells were removed from the top side of the inserts with a cotton swab. Cells that migrated to the underside of the inserts were fixed with cold 96% ethanol (600 μL/well) for 10 min at room temperature and then stained with 0.1% crystal violet (CV) in 10% ethanol (600 μL/well) for 7 min. After staining, the extracellular dye was removed by rising carefully the transwell inserts in tap water. Cells were resuspended in 96% ethanol with 1% acetic acid (700 μL/well) and the absorbance at 595 nm was measured in a Thermo Fisher Multiskan FC microplate reader. The results were expressed as percentages of non-treated control cultures.

3.2.5.2. MDA-MB-231 cells chemoinvasion assay

In this work, the chemoinvasion assay was performed as described for the chemotaxis assessment, but herein the membrane filter was overlaid with Matrigel™ diluted in FBS-free medium (1/30) (Figure 3.8 B), which blocked non-invasive cells from migrating through. This

Matrigel™ chemoinvasion assay has been reported to correlate quite well with metastatic potential for a wide variety of tumor cell types [41].

As referred before, this assay can be used to quantify the invasive potential of most cell types and it can be applied to detect the migratory activity associated with matrix degradation. Therefore, this assay can be used for screening of inhibitors of invasiveness, being selected to this purpuse in this study. Three to five independent experiments were performed.

3.2.6. Intracellular ROS evaluation

Reactive oxygen species (ROS) represent a large group of chemically distinct reactive species (RS) with diverse biological reactivities [43]. To establish a clear relation between a particular cell signaling event and a specific ROS, it is very important to detect and characterize these RS accurately. In fact, several analytical approaches (*e.g.*, EPR, chemiluminescence, fluorescence) have been used to detect ROS [43].

ROS probes, designed to detect specific ROS with a high selectivity, would be desirable, since it is clear that each ROS has its own unique physiological activity. The ideal chemical probe for a free radical or a non-radical product such as hydrogen peroxide, would be highly reactive at low concentrations, specific, sensitive, without other reactivity, non toxic, well-characterized chemically, easy to load into organelles, cells or tissues without subsequent leakage or unwanted diffusion, excretion, or metabolism, readily available, easy to use without too specialized apparatus and, finally, cheap [44].

Dihydro-compounds such as 2`,7`-dichlorodihydrofluorescein (DCFH), is a representative and traditional fluorescent probe that has been widely used to detect ROS generation. DCFH is oxidized by ROS to yield 2`,7`-dichlorofluorescein (DCF) and emits a strong fluorescence, which can be monitored by several fluorescence-based techniques. However, DCFH tend to react with a wide variety of ROS and are not completely photostable [43].

The diacetate derivative 2`,7`-dichlorodihydrofluorescein diacetate (DCFDA) is a chemically reduced form of fluorescein, cell-permeable that accumulates mostly in the cytosol and is thus useful for monitoring oxidative stress in living cells. In fact, DCFDA is the most widely used probe for detecting intracellular H_2O_2 and general oxidative stress [43].

Similar dihydro-compounds such as dihydrorhodamine 123 (DHR) can also be used as fluorescent probes to detect ROS. It is suitable for detecting total oxidative activity in living cells or tissues. DHR is a probe widely used to detect several RS, but is poorly responsive to $O_2^{\bullet -}$, being DHR oxidation more H_2O_2 dependent [44, 45].

Cell-permeable DHR is oxidized to rhodamine 123, a fluorescent product (Figure 3.10) which is lipophilic and positively charged, and tends to accumulate in mitochondria, held there by the membrane potential. As a result, once it is formed, little rhodamine 123 leaks out of cells [44].

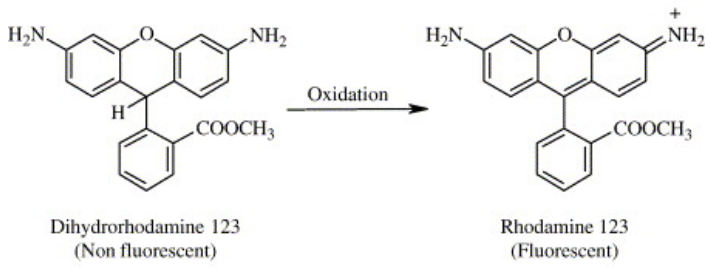


Figure 3.10 – Conversion of dihydrorhodamine 123 to rhodamine 123 [44].

In our laboratory, since we had available the two probes, a preliminary assay of optimization and probe selection was performed, described in section 3.2.6.1.

3.2.6.1. Optimization and probe selection for intracellular ROS evaluation

In order to select the probes DCFDA or DHR and to decide which one was more adequate and with higher sensibility to evaluate intracellular ROS formation in breast cancer cells, the oxidant *tert*-butylhydroperoxide (TBHP) was used in an assay with MDA-MB-231 cells. There is an extensive use of TBHP as an oxidant model [36]. TBHP is a short-chain analogue of lipid hydroperoxides that mimics the toxic effect of peroxidized fatty acids, being thus commonly used as a model of lipid peroxidation [46]. TBHP penetrates cell membranes easily [47] and generates free radicals intermediates, namely alkoxyl and peroxyl radicals in the cytosol [46].

For ROS optimization and probe selection assay, MDA-MB-231 cells were seeded on Matrigel™- coated dishes (1/30 dilution in FBS-free medium) and after 24 h, when cells were ~40 % confluent they were incubated with vehicle, TBHP (50 µM) or TBHP (100 µM) for 1 h at 37 °C in FBS-free medium. Cells were then washed twice with warm PBS and incubated with DHR (10 µM) or DCFDA (10 µM) in FBS-free medium for 20 min at 37 °C. After the probes were removed, in the dark, cells were washed once with FBS-free medium at 37 ºC and covered with a glass cover slip. Washing cells allowed unreacted probe to be removed. Cell image acquisition was performed using a wide field BX51 fluorescent Olympus microscope with a 40x objective using a 460-490 nm/<520 nm excitation/emission filter for

DHR and a 520–550 nm/<580 nm excitation/emission filter for DCFDA [44, 48, 49]. Cell fluorescence and area were determined using ImageJ (National Institutes of Health) [50] for a minimum of 30 cells *per* condition. At least three independent experiments were performed.

3.2.6.2. Intracellular ROS evaluation in MCF-7 and MDA-MB-231 cells

In this work, the intracellular ROS levels in MCF-7 and MDA-MB-231 cells were assessed by fluorescence microscopy using the probe dihydrorhodamine 123 (DHR), which has been selected after the optimization procedure described in section 3.2.6.1.

For ROS assays, MDA-MB-231 and MCF-7 cells were seeded on Matrigel™ (1/30 dilution) coated *Petri* dishes (∅ 35 mm), and after 24 h, when cells were ~40 % confluent, they were incubated with vehicle, doxorubicin (0.1 µM), Cu[15]pyN_5 (100 µM) or doxorubicin and Cu[15]pyN_5 for 16 h at 37 °C in complete cell culture medium. The cells were then washed twice with warm PBS and incubated with DHR (10 µM) in FBS free media for 20 min at 37 °C. After that the probe was removed, in the dark, cells were washed once with FBS-free medium at 37 ºC and covered with a glass cover slip. Cell image acquisition was performed using a BX51 fluorescent Olympus microscope with a 40× objective using a 460-490 nm/<520 nm excitation/emission filter for DHR [48, 49]. Cell fluorescence and area were determined using ImageJ (National Institutes of Health) [50] for 45 cells *per* each condition. Three to four independent experiments were performed. The results were expressed as percentages of non-treated control cultures.

3.2.7. Statistical analysis

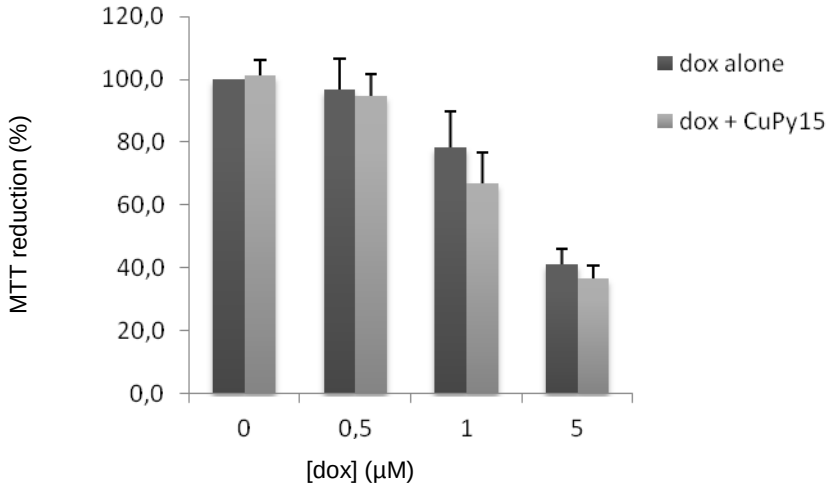
Differences in mean values of the results were evaluated by the *Student's t-test*, after assessing the homogeneity of the variances by the Levene test. All analyses were performed with the SPSS statistical package (version 19, SPSS Inc. Chicago IL).

3.3. Results

3.3.1. Cu[15]pyN_5 and dox combined effects on cell viability

The effect of Cu[15]py15 and dox on the viability of human breast cancer cells was evaluated by the MTT assay [36]. In MCF-7 cells dox was used at concentrations of 0.5, 1, and 5 µM; in MDA-MB-231 cells dox was used at concentrations of 1, 5 and 20 µM. Both cell lines were exposed to the drugs alone or in co-treatment for a 24 h-period (Figure 3.11). Cu[15]pyN_5 was used at concentration of 100 µM [20].

A – MCF-7 cells



B – MDA-MB-231 cells

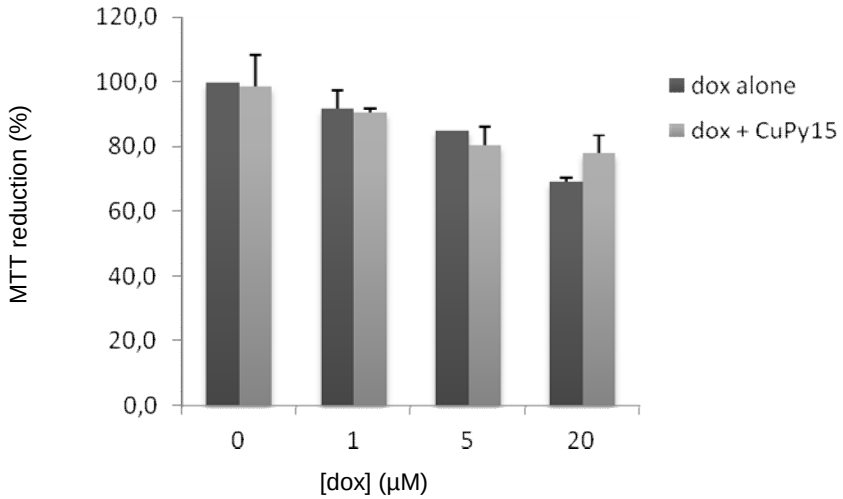


Figure 3.11 - Effect of Cu[15]pyN_5 on the cell viability of human breast cancer cells treated with dox (MTT assay). Cells were incubated with increasing concentrations of dox in the absence or presence of 100 µM Cu[15]pyN_5, for 24 h. Results are expressed as average values ± SD (n = 2–4). (A) MCF-7 cells. (B) MDA-MB-231 cells.

In these two cell lines, the exposure to dox induced a concentration-dependent decrease in cell viability. It was observed that Cu[15]pyN_5 (100 µM) alone was not considerably toxic in MCF-7 cells (Figure 3.11 A, cell viability of 101.1 ± 5.1%) and in MDA-MB-231 cells (Figure 3.11 B, cell viability of 98.8 ± 9.9%). In addition, the combined treatment with dox and with Cu[15]pyN_5 (100 µM) did not significantly alter the cytotoxicity induced by dox, in both cell lines.

An interesting fact is that in the human normal-like epithelial breast cell line MCF-10A, Cu[15]pyN_5 (100 µM) afforded a slight protection against dox cytotoxicity. This protective effect was significant for the dox concentration of 0.5 µM. In this case, the presence of Cu[15]pyN_5 increased cell viability from 70.2% to 84.5% ($p<0.05$) (data not shown).

Based on these results and on previous studies [51], dox (0.1 µM) was chosen as the adequate concentration to use in combination with Cu[15]pyN_5 (100 µM) in the subsequent assays. This level of concentration corresponds to a non-toxic level, when compared with non-treated control cells, which seems to be adequate for further studies, *e.g.* migration/invasion, since higher dox concentrations may lead to cell death and consequently interfere with the assays. This aspect is crucial since as dying cells poorly migrate and invade [41], the utilization of non-cytotoxic concentrations is mandatory when the objective is to test potential inhibitors of migration/invasion. In addition, this concentration is observed in the plasma of patients treated with dox, being thus pharmacologically relevant [52]. Therefore, cell viability studies allowed the selection of concentrations for the subsequent assays.

3.3.2. Impact of Cu[15]pyN_5 on cell migration

Considering the role of cell migration in breast cancer progression and metastases, the impact of the SODm Cu[15]pyN_5 and dox on breast cancer cells migration was determined by two different assays. In this work, breast cancer cells migration was initially assessed by the wound healing assay, optimized according to Liang *et al.* 2007 [38]. This assay analyzes directed migration of the cells by evaluating their movement across an horizontal surface, as a response to clearing of cells in a monolayer, therefore, providing insights into the collectively cell motility [38].

Since the duplication time is about 29 h for both cell lines [53, 54], the 24 h exposure to the compounds was shorter than a cell cycle duration, meaning that cell proliferation wouldn`t interfere with this cell migration assay.

The results obtained for the two breast cancer cell lines are shown in Figure 3.12.

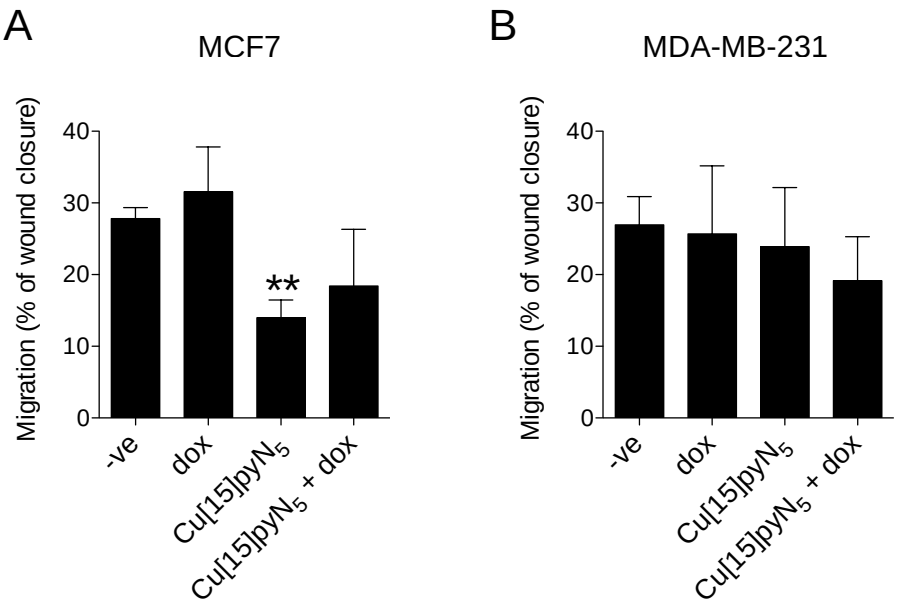


Figure 3.12 - Effect of Cu[15]pyN_5 (100 µM), dox (0.1 µM), or both agents on the migration of MCF-7 (A) and MDA-MB-231 cells (B), evaluated by the wound healing assay. Results (mean ± SD) represent the percentage of wound closure calculated at t = 24h relatively to the initial width (n = 3-4). ** p< 0.01 (*Student's t-test*, relative to untreated cells, -ve).

In the wound healing assay, treatment of MCF-7 cells with Cu[15]pyN_5 (100 µM) led to a significant reduction in cell motility. As depicted in Figure 3.12 A, the % of wound closure decreased to about one-half of that of control cells ($p<0.01$; t = 24 h). No considerable differences were observed in MCF-7 cells exposed to dox (0.1 µM).

In MCF-7 cells, the combined treatment with Cu[15]pyN_5 and dox also reduced cell motility, although this reduction is not statistically significant. While the % of wound closure in controls was 27.8 ± 1.5%, wounds in cultures submitted to this co-treatment exhibited a % closure of 18.4 ± 7.9%.

In MDA-MB-231 cells, only slight decreases in the % of wound closure were observed in cells treated with dox and Cu[15]pyN_5. Co-treatment with Cu[15]pyN_5 and dox also reduced cell motility, although this reduction is not statistically significant. While the % of wound closure in controls was 26.9 ± 3.9%, wounds in cultures submitted to the co-treatment exhibited a % closure of 19.2 ± 6.1% (Figure 3.12 B; n.s.).

3.3.3. Impact of Cu[15]pyN_5 on MCF-7 cells chemotaxis

The impact of the SODm Cu[15]pyN_5 (100 μM) and dox (0.1 μM) on breast cancer cells chemotaxis was assessed by a transwell insert assay, which evaluated directed cell motion and individual cell motility towards an external gradient of FBS [55]. The results obtained for MCF-7 cells are shown in Figure 3.13.

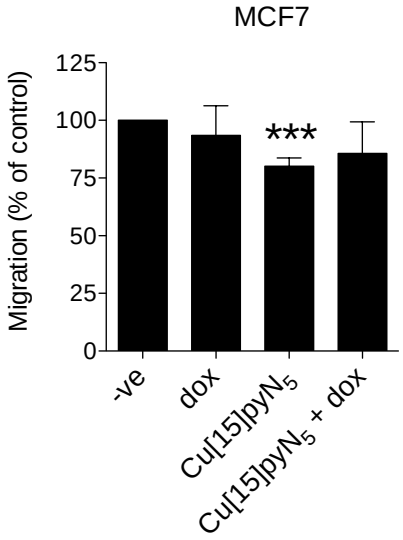


Figure 3.13- Effect of Cu[15]pyN_5 (100 μM), dox (0.1 μM), or both agents on the migration of MCF-7 breast cancer cells, evaluated by chemotaxis using a transwell system with FBS as a chemoattractant. Results are expressed as average values ± SD (n = 5) ***p<0.001 (*Student's t-test*, relative to untreated cells, -ve).

In MCF-7 cells, dox slightly decreased the chemotactic migration to 93.5 ± 12.9% (n.s.). Cu[15]pyN_5 significantly decreased the migration of MCF-7 cells to 80.3 ± 3.8% ($p<0.001$ when compared with non-treated controls). When these cells were simultaneously exposed to dox and Cu[15]pyN_5, the migration was 85.7 ± 13.6% (n.s.).

3.3.4. Impact of Cu[15]pyN_5 on MDA-MB-231 chemotaxis and cell invasion

Chemotaxis of MDA-MB-231 cells was also assessed by the transwell inserts assay [53] (Figure 3.14 A and C). One of the most important properties of metastatic cancer cells is the ability to invade tissues. Therefore, the effect of the SODm Cu[15]pyN_5 (100 μM) and dox (0.1 μM) in MDA-MB-231 cells proteolytic invasion was also evaluated [40, 41] (Figure 3.14 B and C).

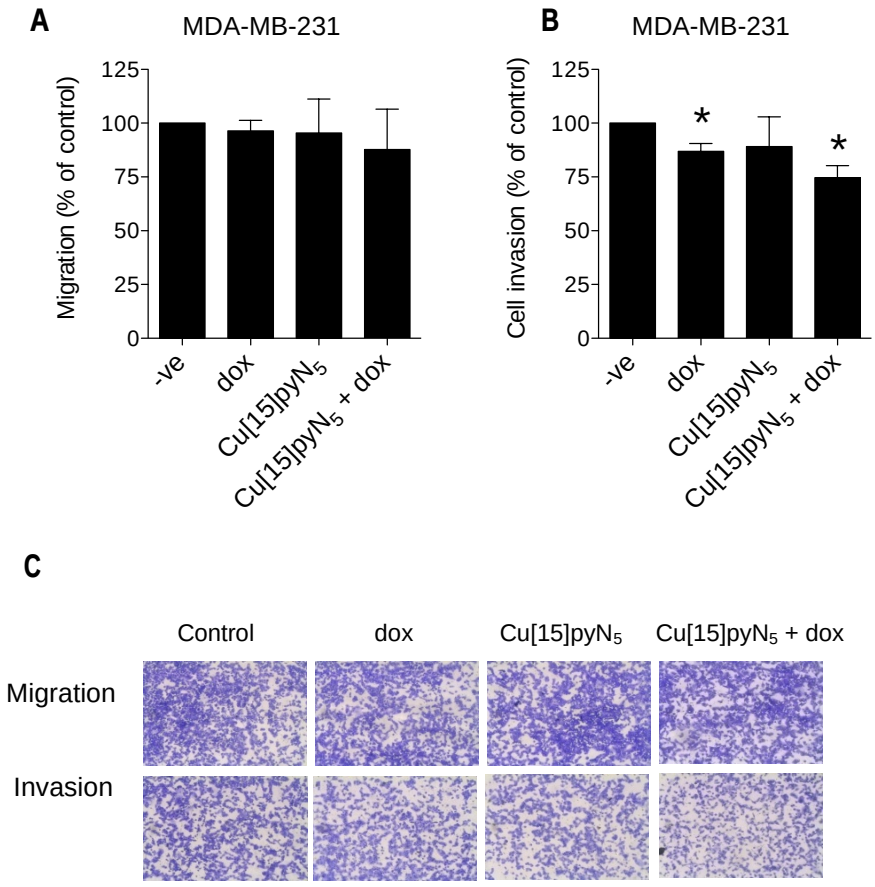


Figure 3.14 - Effect of Cu[15]pyN_5 (100 µM), dox (0.1 µM), or both agents on the migration and proteolytic invasion of MDA-MB-231 cells. Migration was evaluated by chemotaxis (A) using a transwell system with FBS as a chemoattractant. MDA-MB-231 cells were seeded on transwell previously coated with a matrigel layer and allowed to migrate using FBS as a chemoattractant, for proteolytic invasion evaluation (B). Results (mean ± SD) represent the % of migrating or invading cells relative to control (n = 3 - 4). (C) Microscopy images show representative fields of cells that migrated and invaded through the matrigel layer, after their fixation and staining with CV. *p<0.05 (*Student's t-test*, relative to untreated cells, -ve).

Chemotaxis assay in MDA-MB-231 cells (Figure 3.14 A and C) showed that Cu[15]pyN_5 and dox, given separately, induced small decreases in MDA-MB-231 cells migration (95.5 ± 15.6% and 96.4 ± 4.9%, respectively, ns). The co-treatment with both agents reduced cell migration to 87.7 ± 18.8% (n.s.).

Invasive and metastatic cells must cross basement membranes (BMs) in order to disseminate to distant sites. Actually, one of the most important properties of metastatic cancer cells is their ability to invade tissues. Therefore, the effect of the abovementioned treatments in cell invasion was evaluated. Cell invasion experiments were only carried out in MDA-MB-231 cells, due to the non-invasive phenotype of MCF-7 cells (no invasion was observed under our experimental conditions). In fact, regarding the invasive capabilities,

mesenchymal-type cells like MDA-MB-231 cells are much more frequently highly invasive in *Boyden* chamber assays than luminal cells like MCF-7 cells [27]. For the invasion assay, the insert membrane was overlaid with Matrigel™, a natural reconstituted basement membrane widely used as an invasion barrier [40, 41].

Chemoinvasion assay in MDA-MB-231 cells (Figure 3.14 B and C) showed that dox (0.1 μM) treatment decreased the proteolytic invasion of MDA-MB-231 cells to 86.9 ± 3.7 % ($p<0.05$), when compared with non-treated control cells. Cu[15]pyN_5 (100 μM) treatment also decreased cell invasion, although this reduction is not statistically significant (89.2 ± 13.7%; n.s.).

The effect of the combined treatment with both drugs was more pronounced, leading to a reduction in chemoinvasion in more than 20% when compared with non-treated controls (77.5 ± 9.4%, $p<0.05$). In Figure 3.14 C we can clearly observe the significant decrease in the number of invading cells upon the co-treatment with the both compounds, when compared with non-treated control cells.

3.3.5. Cu[15]pyN_5 and dox induce intracellular ROS production in MDA- MB-231 and MCF-7 cells

Regarding the effects observed in migration and invasion of breast cancer cells described in sections 3.3.2 to 3.3.4 and, since SODm Cu[15]pyN_5 is a redox-active compound and dox increases ROS levels, in particular $O_2^{\bullet -}$ [56], alterations in the levels of intracellular ROS could be associated with those effects in migration and invasion. Therefore, the levels of intracellular ROS formation upon exposure of MDA-MB-231 and MCF-7 cells to both drugs, either alone or in combination, were evaluated by fluorescence microscopy.

Initially, an optimization and probe selection procedure was conducted, as described in section 3.2.6.1, in order to select the most adequate and sensible probe between DCFDA and DHR, for intracellular ROS evaluation in breast cancer cells lines.

3.3.5.1. Optimization and probe selection for intracellular ROS evaluation

In order to select between the probes DCFDA and DHR and to decide which one was more adequate and with higher sensibility to evaluate intracellular ROS formation in breast cancer cells, as described in section 3.2.6.1, the oxidant *tert*-butylhydroperoxide (TBHP) was used in MDA-MB-231 cells, as an oxidant model [36].

The results obtained after the optimization procedure are depicted in Figure 3.15.

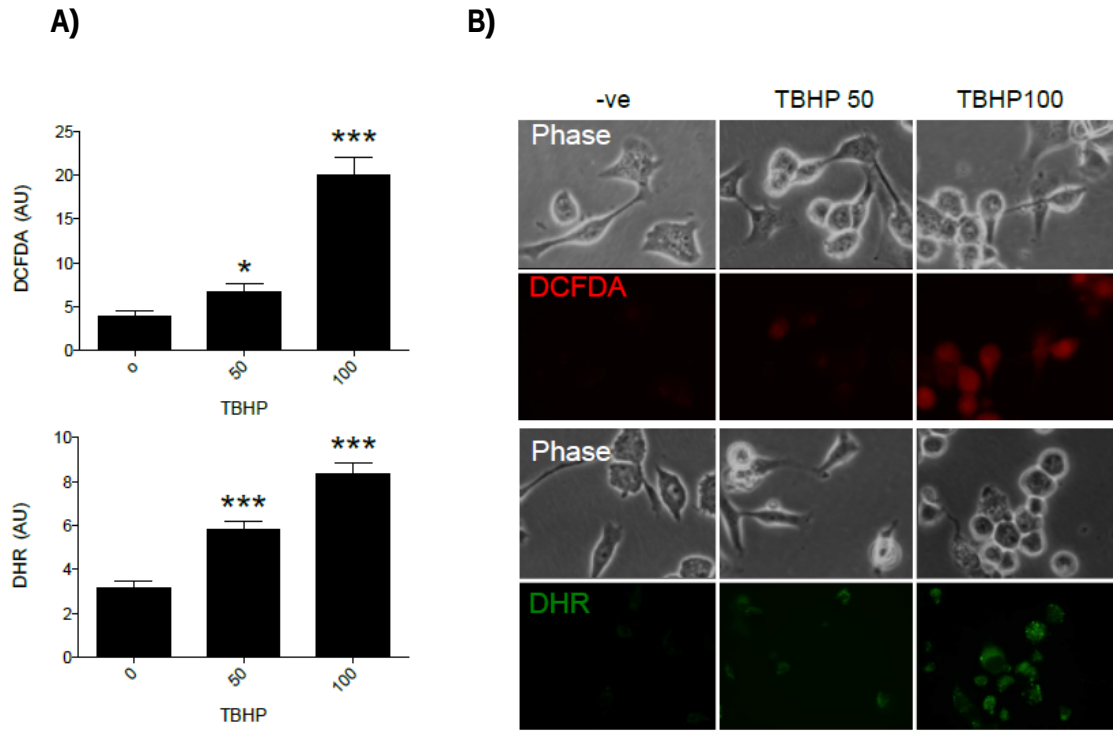


Figure 3.15 – Exposure of MDA-MB-231 cells to cytotoxic concentrations of TBHP leads to an increase in intracellular ROS. Intracellular ROS levels were determined in MDA-MB-231 cells treated with TBHP 50 µM and 100 µM for 1 h. Summary results (means ± SEM from at least 30 cells for each condition) show relative DCFDA or DHR fluorescence. * $p<0.05$ and *** $p<0.001$ (*Student's t-test*, relative to untreated cells). (A). Fluorescence microscopy images show representative cells after 20 min incubation with DCFDA or DHR (B).

High concentrations of TBHP (50 and 100 µM) led to marked morphological changes in MDA-MB-231 cells (round cells), especially for the higher TBHP concentration (Figure 3.15 B), as expected [36].

For both probes, it was observed a significant increase of the ROS levels in MDA-MB-231 cells, being this increase more pronounced for the treatment with TBHP (100 µM) ($p<0.001$ when compared with non-treated controls), which was not surprisingly due to the TBHP oxidant role.

In relation to the TBHP treatment with the lower concentration (50 µM), it was observed a significant increase of the ROS levels in MDA-MB-231 cells, being this increase much more pronounced for the DHR probe ($p<0.001$, when compared with non-treated controls), than for the DCFDA probe ($p<0.05$ when compared with non-treated controls). These differences could be attributed to the fact that DHR is a more sensitive probe at detecting alterations in ROS levels than DCFDA, which is in agreement with several authors [43, 44].

Investigators have routinely used the cell-permeable DCFDA to measure intracellular generation of H_2O_2 and other oxidants or monitor redox signaling changes in cells in response to intra- or extracellular activation with oxidative stimulus [43]. In fact, DCFDA is the most popular of the probes for measure RS in cells, frequently being used to detect "cellular peroxides". However, it is unlikely to do so because it reacts only slowly with H_2O_2 or lipid peroxides [44]. DCFDA is not actually a useful probe for the two commonest ROS in Biology, despite still being widely viewed as such [44]. The DCFDA use could, therefore, lead to false interpretations of findings (*e.g.*, increased peroxidase activity induced by the experimental conditions, or a new redox signaling/redox modification mechanism). The recommendation is that the DCFDA probe cannot be used with confidence to measure intracellular H_2O_2 and other ROS [43].

The DHR oxidation may be mediated by different ROS and provides a notion of the overall cellular oxidant production [45, 56]. However, DHR is considered unreactive towards superoxide anion [44].

Fluorescent product of DHR oxidation, rhodamine123, is a positively charged lipophilic compound that accumulates within mitochondria to a marked degree, with little loss to the extracellular space, thus being an excellent marker for measuring intracellular oxidative stress [44].

Therefore, in the present work DHR probe was selected for evaluate intracellular ROS formation in breast cancer cells MCF-7 and MDA-MB-231 (section 3.3.5.2).

3.3.5.2. Intracellular ROS evaluation in MCF-7 and MDA-MB-231 cells

The levels of intracellular ROS formation upon exposure of MDA-MB-231 and MCF-7 cells to both Cu[15]pyN_5 (100 µM) and dox (0.1 µM), either alone or in combination, were evaluated by fluorescence microscopy with the probe DHR.

Before the assay, the possible auto-fluorescence of both compounds was tested, as described in section 3.2.6.2 except that, in this case, DHR probe wasn`t added. It was observed that for both Cu[15]pyN_5 (100 µM) and dox (0.1 µM) in the excitation/emission wave-length values of the filter used for DHR, the auto-fluorescence levels were less than 5% of those with DHR incubation for 20 min (data not shown). Therefore, it was admitted that both compounds didn`t present considerable auto-fluorescence in the conditions used in this assay.

In order to compare the results obtained for migration and invasion of breast cancer cells upon treatment with both compounds alone or in combination, with the intracellular ROS levels of the cells subjected to the same treatments, a 16 h exposure protocol was used.

The results obtained for MCF-7 and MDA-MB-231 cells upon their treatment with both Cu[15]pyN_5 (100 µM) and dox (0.1 µM), alone or in combination, for 16h, after incubation with DHR are represented in Figure 3.16 A and C.

One representative assay for each cell line is shown in Figure 3.16. All three independent experiments carried out led to coherent results.

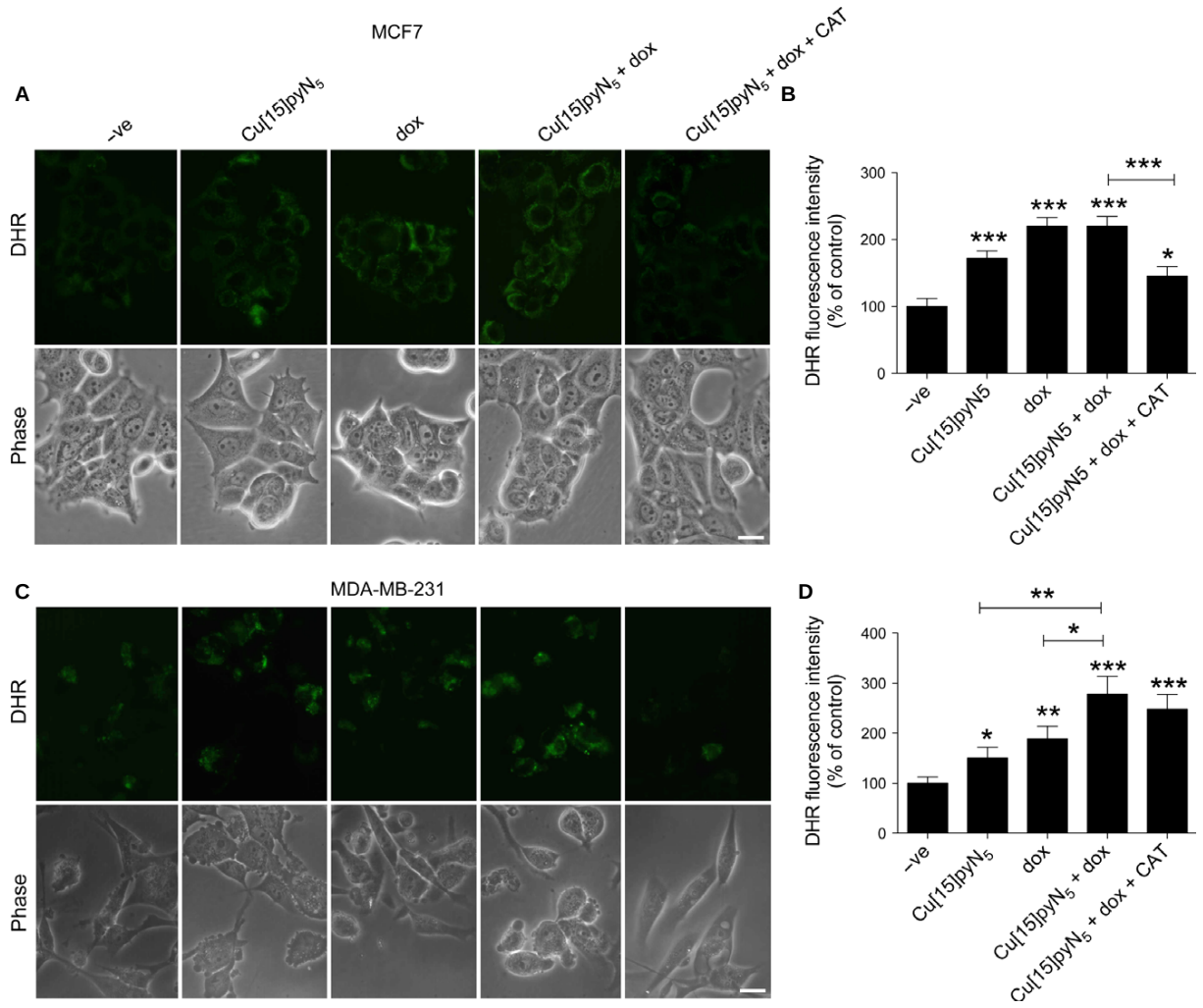


Figure 3.16- Exposure of MCF-7 and MDA-MB-231 cells to non cytotoxic concentrations of Cu[15]pyN_5 and doxorubicin leads to an increase in intracellular ROS. Intracellular ROS levels were determined in MCF-7 (A and B) or MDA-MB-231 (C and D) cells treated with the indicated drugs (doxorubicin (0.1 µM), Cu[15]pyN5 (100 µM) and CAT (50 U/mL) for 16 h. Fluorescence microscopy images show representative cells after 20 min incubation with DHR. Scale bars = 20 µm (A and C). Summary results (means ± SEM from at least 50 cells for each condition) show relative DHR fluorescence (B and D). *p<0.05, **p<0.01, ***p<0.001 (*Student's t-test*, relative to untreated cells, -ve).

As represented in Figures 3.16 A and B, MCF-7 cells treatment with Cu[15]pyN_5 alone, increased the ROS level significantly (Figure 3.16 B, $p < 0.001$ when compared with non-treated control cells). A similar result was also observed for the treatment with dox alone (Figure 3.16 B, $p < 0.001$ when compared with non-treated control cells), but the significant increase in the fluorescence compared with non-treated control cells, was even more pronounced than the observed with Cu[15]pyN_5 alone. Regarding the co-treatment, the results for MCF-7 cells showed a significant increase in the fluorescence for 16 h exposure with both drugs (Figure 3.16 B, $p < 0.001$ when compared with non-treated control cells), being the fluorescence level similar to that observed with dox alone.

In order to investigate if the increase observed in fluorescence could be associated with the formation and accumulation of H_2O_2, the exposure of cells to catalase (CAT) (50 U/mL) simultaneously with the other drugs was also assessed in MCF-7 cells. When MCF-7 cells were simultaneously exposed to Cu[15]pyN_5 (100 µM), dox (0.1 µM) and CAT (50 U/mL), for 16 h period, the level of ROS decreased significantly when compared with those of cells treated only with Cu[15]pyN_5 and dox (Figure 3.16 B, $p < 0.001$). Despite this decrease, the levels of ROS in this case were still higher than those of non-treated control cells (Figure 3.16 B, $p < 0.05$).

Comparing with MCF-7 cells intracellular ROS levels, similar results were obtained with MDA-MB-231 cells.

As represented in Figure 3.16 C and D, the treatment of MDA-MB-231 cells with Cu[15]pyN_5 alone, increased the ROS level (Figure 3.16 D, $p < 0.05$, when compared with non-treated control cells), being this increase not as pronounced as in MCF-7 cells.

A similar result was observed for the treatment with dox alone (Figure 3.16 D, $p < 0.01$, when compared with non-treated control cells), but as observed with Cu[15]pyN_5, this increase was not as pronounced as in MCF-7 cells. However, for MDA-MB-231 cells, the increase in the fluorescence compared with non-treated control cells was more pronounced with dox treatment than with Cu[15]pyN_5.

Regarding the co-treatment with both drugs, results showed a significantly increase in the fluorescence for 16 h exposure (Figure 3.16 D, $p < 0.001$, when compared with non-treated control cells), being the fluorescence level considerably higher than that observed with dox (0.1 µM) alone (Figure 3.16, $p < 0.05$). In addition, compared with Cu[15]pyN_5 alone, the increase in fluorescence level obtained with the co-treatment was even more pronounced (Figure 3.16, $p < 0.01$) for MDA-MB-231 cells.

In line with our findings for MCF-7 cells, the exposure of MDA-MB-231 cells to CAT (50 U/mL) simultaneously with Cu[15]pyN_5 (100 µM) and dox (0.1 µM), for 16 h period, led to a decrease in the level of ROS when compared with cells in co-treatment with Cu[15]pyN_5 and dox (Figure 3.16 D). However, this decrease was not statistically significant, being less pronounced when compared with the decrease observed in MCF-7 cells. Also, when MDA-MB-231 cells were simultaneously exposed to Cu[15]pyN_5 (100 µM), dox (0.1 µM) and CAT (50 U/mL), the levels of ROS increased significantly when compared with those of non-treated control cells (Figure 3.16 D, $p < 0.001$).

3.4. Discussion

Metastasis is a major cause of mortality among cancer patients, including breast cancer patients [57]. The formation of breast cancer metastases requires a cascade of different biological phenomena including loss of cell adhesion, increased motility and invasiveness [58].These different steps constitute potential therapeutic targets to suppress the formation of metastases.

Reactive oxygen species (ROS) have been widely implicated in cancer at different levels [12, 59]. Although there are still many gaps in the understanding of the role of ROS in metastases, redox-active agents could be a beneficial therapeutic approach. In addition, the ability to block the migratory and invasive capacity of cancer cells using redox modulation, offers a new approach in cancer therapy [10, 60]. Therefore, this study aimed to evaluate the role of the SODm Cu[15]pyN_5 in different cell functions related to the metastatic process. Experiments were carried out in MDA-MB-231 (invasive breast cancer cell line) and in MCF-7 cells (non-invasive breast cancer cell line).

Cu[15]pyN_5 (100 µM, 24 h) was not cytotoxic to MCF-7 and MDA-MB-231 cells. The redox properties of Cu[15]pyN_5 were analyzed in a previous work from our group [20].

This copper complex is a superoxide anion scavenger, leading to the formation of H_2O_2. Furthermore, at least *in vitro* the complex may react with the generated H_2O_2 resulting in the production of the deleterious hydroxyl radical [20], a highly reactive ROS with a short lifetime.

In previous work, our group has shown that Cu[15]pyN_5 boosted oxaliplatin toxicity in MCF-7 cancer cells, while it protected normal-like breast ephitelial MCF-10A cells from the toxicity of this drug [20], suggesting a potential for this copper complex to be used in a combined therapy for breast cancer. Therefore, in the present work, the SODm Cu[15]pyN_5 was studied in combination with dox, a first line anticancer drug widely used in breast cancer chemotherapy.

Regarding cell proliferation, Cu[15]pyN_5 had a negligible impact on breast cancer cells treated with dox. Conversely, this complex protected normal-like cells from dox toxicity. This differential effect could be attributed to the differences in basal concentration of ROS and in antioxidant defenses of the cell lines [8, 20].

Along with proliferation, cell migration and invasion were evaluated. In this work, two distinct cell lines were used, varying in the degree of aggressiveness/ invasiveness. Since the basal redox *status* of mammary cells differs with their degree of aggressiveness, redox drugs may operate differently according to the cell type. In fact, previous reports have

demonstrated significant differences in antioxidant enzymes and in basal ROS for MCF-7 and MDA-MB-231 cells.

Kattan *et al* [6] have shown that the expression and activity of SOD1 (Cu,Zn-SOD) was similar in both cell lines, while the levels and activity of SOD2 (MnSOD) was higher in MDA-MB-231 cells. In a similar way, Hecht *et al* [8] referred that SOD1 mRNA levels did not differ among the MCF-7 and MDA-MB-231 cells. In addition, these authors referred that SOD2 mRNA levels were almost 3 times higher in MDA-MB-231 than in MCF-7 cells and SOD3 (EC-SOD) mRNA levels were significantly higher in MDA-MB-231 in comparison to MCF7 cells [8]. Hsieh *et al* [7] also found a higher global SOD activity in MDA-MB-231 cells, which represents the contribution of both SOD1 (cytoplasmic) and SOD2 (mitochondrial) isoform activities [8], when compared with MCF-7 cell line.

Regarding catalase (CAT), these two reports show conflicting results [6, 7]. However, in aggreement with Hsieh *et al* [7] and in opposite way of Kattan *et al* results [6], a more recent study indicates that CAT activity was higher in MDA-MB-231 cells than in MCF-7 cells. In addition, CAT mRNA expression was significantly higher in MDA-MB-231 than in MCF-7 cells [8]. Even so, MDA-MB-231 cells seem to have higher peroxide levels than MCF7, presumably due to higher H_2O_2 content [6]. In accordance, Hecht *et al* [8] observed that the tumor cell line MDA-MB-231 produces larger amounts of intracellular ROS than the tumor cell line MCF-7. In addition, it was also observed a higher amount of extracellular H_2O_2 generation in MDA-MB-231 cells compared with MCF-7 cells.

Cell migration was evaluated by two distinct methodologies – the wound healing assay and the chemotaxis assay. The wound healing assay evaluates the movement of cells across a horizontal surface, providing insights into collective cell motility. Conversely, the chemotaxis assay evaluates the directed cell motion towards an extracellular gradient of bioactive molecules and is indicative of the motility of individual cells [37]. Despite these inherent differences, the results from both methods came to the same finding: Cu[15]pyN_5 significantly decreases MCF-7 cell migration, whereas a minor impact was observed in MDA-MB-231 cell line. These differences may be eventually attributed to the basal differences in SOD activity and in H_2O_2 content between the two cell lines, as described before. In fact, as aforementioned, since MDA-MB-231 cells have higher SOD activity and higher peroxide levels, the potential contribution from Cu[15]pyN_5 to the cellular redox state of these cells is expectedly lower for MDA-MB-231 than for MCF-7 cells.

Cancer invasion requires not only the ability of cells to be motile but also to promote the proteolytic degradation of the extracellular matrix. In fact, cell invasion results from a sum of events that can be evaluated using the chemoinvasion assay. These events include cell

viability, adhesion to matrix, directed movement, production of proteases that allow matrix penetration, and adhesion to a new substrate [41]. The chemoinvasion assay was thus carried out only in MDA-MB-231 cells, since MCF-7 cells are considered non-invasive cells [27]. While the effect of isolated Cu[15]pyN_5 was modest, the combination of the complex with dox significantly reduced cell invasion. The observed effect could be partially ascribed to the modulation of expression and/or activity of matrix metalloproteases (MMPs), since MMPs overexpression is generally associated with breast cancer progression. Different MMPs were previously reported to be expressed by MDA-MB-231 cells, including MMP-1, -2, -3, -7, -9, -10, -13 [61-63]. Expression of the membrane-type MT1-MMP (MMP-14) was also found in MDA-MB-231 cells [61]. MMP-1 is the only MMP capable of degrading all types of mammary gland collagen and plays a key role in the degradation of stromal fibres in several diseases, including breast cancer [63]. MMP-1 levels are increased in a number of metastatic tumors and cell lines [60]. Despite the importance of MMP-1, the gelatinases MMP-2 and MMP-9 have been described as the main orchestrators of ECM remodeling [63] and MMP-9 seems to play an important role in the invasiveness of MDA-MB-231 cells through Matrigel [61]. Therefore, we hypothesize that the combined treatment with dox + Cu[15]pyN_5 could reduce cell invasion, in part, by modulating MMPs.

Several mechanisms have been proposed to explain the redox modulation of migration and invasion, but H_2O_2 seems to be the major signaling molecule involved [64]. However, previous reports on the role of H_2O_2 on cell migration and invasion show conflicting data [64,65].

Contradictory reports are also found for the role of SOD in metastases formation [66-68]. An inverse relationship between the intracellular levels of SOD1 (Cu,Zn-SOD), cell motility and *in vivo* metastatic potential was previously shown [67]. In addition, Kogawa *et al* [68] suggested the use of a combination of dox with recombinant human SOD1 (Cu,Zn-SOD) as a successful strategy to reduce lung metastasis. The role of Cu[15]pyN_5 on cell migration/invasion could be therefore a consequence of its SOD-like activity. As the effect of SOD and H_2O_2 seems to depend on cell type and concentration, a further understanding of the role of SOD in tumorigenesis is still needed [64]. This knowledge will be also important for the development of redox active agents that, by exerting both pro- and anti-oxidative actions, could be useful in cancer chemotherapy [64].

Due to its superoxide scavenging activity, Cu[15]pyN_5 could increase the intracellular levels of H_2O_2. This increase could be more relevant in the presence of dox due to the pro-oxidant effect of this drug [69,70]. Furthermore, dox may provide superoxide anion that could be disproportionated into H_2O_2 by Cu[15]pyN_5. In order to test this hypothesis, the

intracellular levels of ROS were evaluated by the DHR probe. While this probe is considered unreactive to superoxide anion [44], it can be oxidized by several ROS providing a global indication of the level of ROS [45, 56]. Indeed, our data show that ROS are increased when cells were exposed to dox, Cu[15]pyN_5 or both agents. To understand whether H_2O_2 contributed to this increase, the combination of dox, Cu[15]pyN_5 and catalase (CAT) was also studied. The presence of CAT led to a reduction in the level of ROS, indicating that H_2O_2 is, at least partially, responsible for the ROS increase observed. These changes in H_2O_2 could therefore contribute to the alterations in cell migration and invasion aforementioned.

Reineke *et al* [65] showed that treatment of MDA-MB-231 cells with H_2O_2 decreased cell migration by up-regulation of the promyelocytic leukemia protein (PML). In this work, the authors demonstrated that PML-dependent changes in integrin β1 expression were responsible for the inhibitory effect of H_2O_2 on cell migration. Similar results were found for MCF-7 cells [65]. However, given the ambiguous role of H_2O_2, we cannot disregard other mechanisms.

Spoerlein *et al* [71] showed that Cu(II) complexes of (iso-)flavonoids could reduce the migration and the secretion of MMPs by 518A2 melanoma cells. In this work, the authors attributed the antimigratory effects observed to a remodeling of the actin cytoskeleton and alterations of the cellular distribution of β-catenin [71]. Beshir *et al* [72] reported a copper complex with potent antimigratory activity in different cell lines, although no explanation is presented for such observations.

Future work is needed in order to ascertain the molecular mechanisms responsible for the effects in cell migration and invasion described in this Chapter. Due to the diversity and complexity of the mechanisms that may be involved, mechanistically different antioxidant compounds should be evaluated in the presence of Cu[15]pyN_5/dox to further assess the involvement of ROS. Moreover, a detailed characterization of the cellular redox *status* and signaling pathways would be needed to shed light on this issue.

3.5. Conclusions

Overall, the results show that Cu[15]pyN_5 is a redox modulator capable of decreasing cell motility and invasion in breast cancer cells treated with dox. These data reinforce the interest of this complex in combination with standard chemotherapeutic drugs. In addition, data from our Lab suggest that the ligand [15]pyN_5 *per se* displays some antiangiogenic properties in endothelial cells and may lead to the *in situ* formation of the redox active complex Cu[15]pyN_5. Given this hybrid mechanism of action, multiple events related to metastases formation could be targeted. Therefore, future studies are needed to assess whether this rational works properly in more complex systems, namely in animal models of metastatic breast cancer.

3.6. References

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Chapter 4

Role of the SOD mimic MnTnHex-2-PyP in intracellular ROS and breast cancer cell migration and invasion

This Chapter was adapted from:

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Abstract

Breast cancer metastases are a leading cause for cancer mortality. Multiple mechanisms related to metastases undergo redox regulation. Reactive species (RS) have been implicated in the metastatic process, namely in cell proliferation, adhesion, migration and invasion. Mn(III) porphyrins (MnPs) are polyfunctional SOD mimics that interact with different RS and which have demonstrated beneficial effects in cancer treatment in combination with chemo- and radiotherapy regimens. Although MnP could impact different aspects of breast cancer and despite the ongoing clinical trials, only few studies address the effect of such compounds in metastization which means that little is known about the effect of MnPs on metastasis, being therefore essential to understand how MnPs affect this process. To fill this gap, this Chapter focus on the impact of the MnP MnTnHex-2-PyP in endpoints of metastasis-related processes in breast cancer cells MCF-7 (adenocarcinoma; low aggressiveness) and MDA-MB-231 (metastatic cancer; high aggressiveness) cells, either alone (5 μM) or in combination with doxorubicin (dox: 0.1 μM). MnTnHex-2-PyP was not cytotoxic for both cell lines and did not alter the cytotoxicity of dox. Additionally, no differences in Sub-G1 phase were observed. Intracellular RS levels were assessed by fluorescence microscopy. Dihydroethidium (DHE) fluorescence was increased in dox-treated cells, whereas MnTnHex-2-PyP decreased DHE fluorescence. Using dihydrorhodamine 123 (DHR) an increase in RS levels was observed in both cell lines exposed to MnTnHex-2-PyP or dox. This increase was more pronounced upon a co-treatment and was partially reversed by catalase (CAT), suggesting that H_2O_2 accumulation could be partially responsible for the changes observed. MnTnHex-2-PyP or dox alone did not considerably change collective cell migration (wound-healing assay). However, the co-treatment with both compounds significantly decreased the motility of MCF-7 and, in a lesser extent, of MDA-MB-231 cells. For MCF-7 cells, that effect was also partially reversed by CAT, suggesting the involvement of H_2O_2. These data are in agreement with a key role of H_2O_2 in the biological effects of MnPs. Chemotaxis and chemoinvasion were evaluated by the transwell insert assay. In both cell types, MnTnHex-2-PyP or dox alone did not alter chemotactic single-cell migration. However, when cells were simultaneously exposed to both compounds, a significant decrease on chemotaxis was observed. In addition, MnTnHex-2-PyP reduced the dox-induced increase in random migration of MDA-MB-231 cells. The co-treatment with dox and MnTnHex-2-PyP significantly decreased MDA-MB-231 cell invasion. MMP-2 and MMP-9 activities were evaluated by gelatine zymography for these cells, but no significant effects were observed, suggesting that other mechanisms should be responsible for the effects observed in cell invasion. Treatment with either MnTnHex-2-PyP or dox decreased the proteolytic invasion of MDA-MB-231 cells (fluorescent gelatin degradation), although the effect was more pronounced upon co-exposure with both compounds. Moreover, to explore the cellular mechanisms underlying the observed effects,

focal adhesions number and NF-κB activation were also studied. In both cell lines, dox increased the number of FA, being this increase significant for MCF-7 cells. The FA number increased 37% in MDA-MB-231 cells co-treated with dox and MnTnHex-2-PyP^{5+}. In MCF-7 cells, dox and MnTnHex-2-PyP alone increased NF-κB activation by approximately 2-fold, while the co-treatment led to a reduction to levels similar to non-treated cells. In MDA-MB-231 cells, dox and MnTnHex-2-PyP, *per se*, did not change NF-κB activation. Conversely, the co-treatment led to a significant increase.

Although differential effects were observed according to the endpoints analyzed, overall, we present a potential drug that might arrest the progression of breast cancer by different and complementary mechanisms, being the alterations induced by MnTnHex-2-PyP in dox-treated cells consistent with a therapeutically favorable outcome.

Keywords: SOD mimics, breast cancer, redox modulation, cell migration and invasion, manganese(III) porphyrins, doxorubicin

4.1. Introduction

Superoxide dismutase mimics (SODm) are described as a group of synthetic compounds that possess the ability to mimic the functional properties of native superoxide dismutases. As aforementioned in Chapter 1, SODm are catalytical polyfunctional antioxidants, being thus effective not only in the disproportionation of superoxide anion but also in the elimination of other reactive species [1]. Moreover, SODm may also interact with redox domains of several signalling proteins involved in cancer development [1]. Notably, SODm have repeatedly demonstrated beneficial effects in different *in vitro* and *in vivo* experimental models of several human pathologies, including cardiovascular, neurodegenerative, and inflammatory diseases as well as different types of cancer [2,3]. There is growing evidence that SODm have indeed several features that can be valuable for cancer treatment.

In this context, manganese porphyrins (MnPs) have been pointed out as one of the most promising classes of SODm [1]. We have recently addressed the rational for the use of SODm in cancer therapy [4,5]. Due to the differential effects of SOD in non tumor *vs* tumor cells, several reports have demonstrated the usefulness of SODm, including MnPs, either as protectors of normal cells against radio- and chemotherapy or as prototype drugs to impair cancer cell proliferation. As a consequence and as aforementioned in Chapter 1, some SODm are currently being evaluated in cancer clinical trials, in combination with chemo- or radiotherapy regimens [1,4]. Despite all the evidences supporting a role for SODm in cancer therapy, the effect of such compounds in metastasis is still almost unexplored. It is accepted that ROS can regulate key cellular mechanisms involved in cancer cell migration/invasion, including invadopodia formation, MMP activation/expression, focal adhesion dynamics, cell-cell contact, cytoskeleton remodelling, and gene expression [4]. Therefore, SODm may also impact cancer metastasis.

Although elevating SOD enzymes levels generally inhibits tumour invasiveness, some reports show the opposite effect [6], as aforementioned in Chapter 1. In the case of breast cancer, MnSOD can have a dual role in tumorigenic progression [5]. While at an early cancer stage MnSOD can work as a caretaker gene [7], the expression and activity levels of this enzyme have been shown to enhance breast cancer metastatic phenotype [8]. Considering this dual effect of SOD in breast cancer progression along with the previous *in vitro* and *in vivo* studies that suggest the potential use of SODm in breast cancer treatment [5], it is essential to explore the impact of SODm in cell processes related to metastases. This information will be important to exclude potential detrimental effects related to cell migration, in case of a future application of SODm in breast cancer treatment.

In this context, the present Chapter addresses the effect of the cationic *orto*-Mn(III) meso-tetrakis (*N*-*n*-Hexylpyridinium-2-yl) porphyrin MnTnHex-2-PyP (Figure 4.1), a promising SODm [1] in human breast cancer cells with low (MCF-7) and high (MDA-MB-231) aggressiveness. Among MnPs, MnTnHex-2-PyP is considered one of the most promising SODm, since it has demonstrated in pre-clinical trials (non human primates) a very high potency *in vivo* along with a large therapeutic window, being thus considered an adequate prototype of SODm [1,9,10].

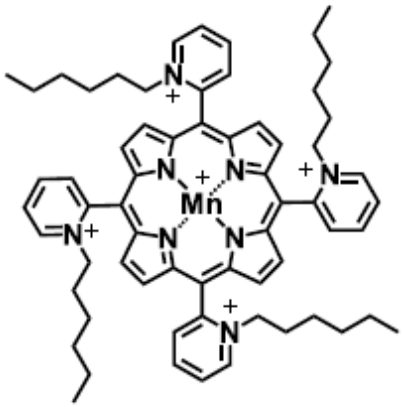

Figure 4.1 – Chemical structure of the MnP *orto*-Mn(III) meso-tetrakis (*N*-*n*-Hexylpyridinium-2-yl) porphyrin (MnTnHex-2-PyP^{5+}) [11].

Because of the macrocyclic effect, MnP are extremely stable with respect to the metal loss, even in concentrated acids. MnTnHex-2-PyP undergoes no demetallation for 3 months in 36% HCl.

In *N*-alkylpyridil MnPs, the length of alkyl chains may be increased to enhance the lipophilicity and in turn, as aforementioned, their bioavailability and effects [9,12]. The hexyl analogue MnTnHex-2-PyP has the optimally balanced bioavailability and toxicity [11], being about 6,500 fold more lipophilic than the ethyl analog, MnTE-2-PyP [1]. Therefore, up to 120-fold lower doses of MnTnHex-2-PyP are needed for the efficacies be identical to those produced by MnTE-2-PyP in several animal models of oxidative stress injury [1,13]. The ability of MnTnHex-2-PyP to cross several tissues (uterus, placenta, fetus and fetal brain) and enter mitochondria is likely to contribute to its efficacy [1,9,14]. Due to their high positive charge, initially speculated to function as a barrier, these compounds accumulate at targeted sites, *e.g.,* mitochondria and nucleus, to levels high enough to afford protection from the damage due to oxidative stress. The cationic charge, driven by the negative mitochondrial membrane potential and attracted by anionic phosphate groups of cellular membranes, may be the prevailing reason for accumulation of MnPs in mitochondria [9].

SODm were previously shown to decrease cancer cells proliferation and boost the anticancer activity of cytotoxic drugs [15-18]. Three different SODm improved the anticancer activity of oxaliplatin in colon carcinoma [18]. MnTnHex-2-PyP delayed tumor growth and increased survival in mice models of glioblastoma multiform and pediatric medulloblastoma [15]. In addition, MnTE-2-PyP was shown to sensitize lymphoma cells to glucocorticoid treatment, presumably via H_2O_2 production [16]. The anti-cancer effects potentiation may be achieved with dosing regimen optimization and with the combination of MnP treatment with irradiation and chemotherapy. MnPs may be advantageous compared with other anticancer drugs, since they can provide radioprotection of normal tissue and have the ability to afford pain management in cancer patients via prevention of chronic morphine tolerance [9,13]. For example, in a preliminary non-human primate study MnTnHex-2-PyP protected lungs from radiation and decreased lung weight, inflammation, edema and epithelial hyperplasia [9]. In addition, MnTnHex-2-PyP and others MnPs have been shown to radiosensitize tumors to radiation therapy [9]. The radioprotection of normal tissue by MnPs in addition to the anti-cancer effects observed [9,19], would allow these drugs to exert dual actions in tumor therapy. Overall, SODm and specifically MnTnHex-2-PyP play a role in different cellular redox pathways, allowing the adjustment of deleterious redox imbalances. Due to the influence of redox balance in cancer and considering the diversity of targets modulated by SODm, as aforementioned, these compounds can be very useful in cancer therapy, in combination with chemo- or radiotherapy regimens.

The innovative aspects of the work presented in this Chapter include the evaluation of the impact of the MnP MnTnHex-2-PyP in several types of cell migration in cells treated with doxorubicin (dox), a widely used chemotherapy drug for metastatic breast cancer, as aforementioned in Chapter 1. In this investigation, SODm exhibited beneficial effects in reducing the migration of dox-treated cells. Furthermore, in order to explore the cellular mechanisms underlying the observed effects, several aspects related to the migratory phenotype were studied in the present Chapter.

4.2. Material and Methods

4.2.1. Chemicals

Dulbecco´s Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin solution, insulin solution from bovine pancreas, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), crystal violet, doxorubicin (dox), catalase (CAT), EDTA, PFA, methanol, RNase A, DAPI, Triton X-100, gelatin from porcine skin, glutaraldehyde (25% commercial solution,) sodium borohydride ($NaBH_4$), tricine, adenosine triphosphate (ATP), Acetyl Coenzyme A (CoA), D-Luciferin, coelenterazine, and TNF-α were purchased from Sigma-Aldrich (St Louis, MO, USA). Dimethylsulfoxide (DMSO), propidium iodide (PI), Poly-D-lysine solution, ethanol, acetic acid, $ZnCl_2$ and $CaCl_2.2H_2O$ were purchased from Merck (Darmstadt, Germany). Acetic acid glacial, nitric acid 65% (w/w) and NaCl were purchased from Panreac (Barcelona, Spain), NaN_3 was purchased from BDH/Merck Laboratory Supplies (Poole, UK). Matrigel™ was purchased from BD Biosciences (San Jose, CA, USA). 40% Acrylamide/Bis solution 37.5:1, sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) and dithiothreitol (DTT) were purchased from Bio-Rad (Hercules, California, USA). APS (ammonium persulfate), Tris Base (Ultra Pure), Protogel Resolving Buffer 4X (1.5 M Tris-HCl/0.4% SDS pH 8.8), Protogel Stacking Buffer (0.5 M Tris-HCl/0.4% SDS pH 6.8), TEMED (N, N, N`, N`-tetramethylenediamine), running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS) and Coomassie Brilliant Blue R-250 were purchased from National Diagnostics (Atlanta, Georgia, USA). Sucrose and $Na_2EDTA.2H_2O$ were purchased from Riedel de Haën (Seeize, Germany). $MgSO_4.7H_2O$ was purchased from Scharlau (Spain). NaOH was purchased from Labkem (Dublin, Ireland). Dox was dissolved in Milli-Q H_2O and stored at -20 ºC. Gelatine from pig skin, Oregon Green 488-conjugated was acquired from Molecular Probes, Life Technologies (Oregon, USA). Dihydrorhodamine 123 (DHR) and dihydroethidium (DHE) probes were purchased from Molecular Probes (Eugene, OR, USA). For these probes, 10 mM stock solutions were prepared in DMSO, aliquoted and stored under nitrogen at – 20 ºC. MnTnHex-2-PyP was synthesized and characterized as described by Batinic-Haberle *et al* (2002) [11]. Mowiol 4-88 and primary antibody anti-vinculin were obtained from EMD Millipore (Burlington, Massachusetts, USA). Primary antibody anti-pFAK Y397 and secondary antibody conjugated to Alexa Fluor 488 were obtained from Invitrogen (Grand Island, NY, USA). pTK-Renilla luciferase and passive lysis buffer 5X were obtained from Promega (Madison, WI, USA). Prestained molecular weight markers (MW: 10 to 260 kDa; Spectra™ Multicolor Broad Range Protein Ladder), Lipofectamine® LTX Reagent, OPTI-

MEM® and PLUS™ Reagent were purchased from ThermoFisher Scientific (Carlsbad, California, USA).

4.2.2. MCF-7 and MDA-MB-231 cells culture conditions

Human breast cancer cell lines MDA-MB-231 and MCF-7 were maintained as described in Chapter 3.

4.2.3. Cell viability assay

The effect of MnTnHex-2-PyP in cell viability, either given alone or in combination with dox, was determined by the MTT assay, as described in Chapter 3 (section 3.2.3). Briefly, 6.5×10^3 cells (for MCF-7) or 5×10^3 cells (for MDA-MB-231) were cultured in 200 μL of complete medium in 96-well plates. The cells were grown for 48 h and then exposed to different concentrations of dox (0.5-5 μM for MCF-7 cells and 0.5-20 μM for MDA-MB-231 cells), alone or in combination with MnTnHex-2-PyP (5 μM), for a 24 h-period. MTT reduction assay was performed as previously described [20]. Two to ten independent experiments were performed and four replicate cultures were used for each condition.

4.2.4. Cell cycle analysis

In this Chapter, the effect of MnTnHex-2-PyP on cell cycle distribution and cell death was analysed by Fluorescence-Activated Cell Sorting (FACS) analysis using PI staining of fixed cells. The theoretical basis is explained in detail in Annex.

MDA-MB-231 and MCF-7 cells were seeded in 6-well plates in complete growth medium. Twenty-four h latter cells were exposed to vehicle, dox (0.1 μM), MnTnHex-2-PyP (5 μM), or both drugs for 16 h at 37 °C in complete medium. On the harvesting day (~70% confluence), both floating and adherent cells were collected using 5 mM EDTA in PBS. Cells were washed with cold PBS and fixed with cold 80% ethanol (PI cannot pass through intact cell membranes). Cells were resuspended in PBS with 1% FBS and after RNase A-treatment (50 μg/mL) (to prevent RNA staining) and PI (25 μg/mL) staining for 15-20 min, cell DNA content was analysed using a FACSCalibur flow cytometer (BD). Data acquisition and analysis were performed using CellQuest software (BD) and FlowJo (Tree Star, San Carlos, Calif.), respectively.

4.2.5. Intracellular ROS measurement

Intracellular ROS levels in MCF-7 and MDA-MB-231 cells were assessed by fluorescence microscopy using the probe dihydrirhodamine 123 (DHR), which has been selected after the optimization procedure described in Chapter 3. As mentioned in Chapter 3, DHR probe is widely used to evaluated general RS formation, is reactive to H_2O_2 but considered unreactive to $O_2^{\bullet -}$ [21,22].

In addition, we have also assessed the intracellular generation of $O_2^{\bullet -}$, using the cell permeable probe dihydroethidium (DHE), frequently used as a probe for $O_2^{\bullet -}$. DHE is oxidized by $O_2^{\bullet -}$ to the fluorescent product 2-hydroxyethidium (Figure 4.2) [21,23].

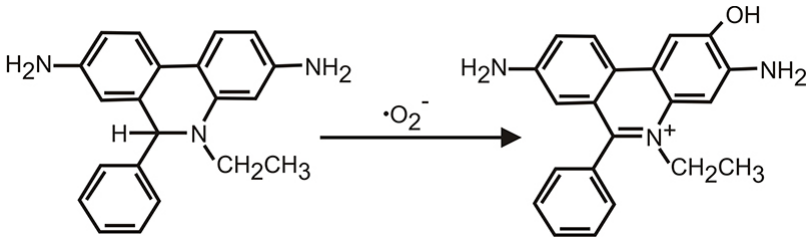

Figure 4.2 – Reaction of dihydroethidium (DHE) with $O_2^{\bullet -}$, originating the fluorescent product 2-hydroxyethidium [21].

DHE probe is much more reactive than DCFDA or DHR toward $O_2^{\bullet -}$. In fact, it appears to be one of the more specific fluorescent probes for $O_2^{\bullet -}$ [21]. Although its reaction with other ROS may interfere with the fluorescence peak [23-25], the fluorescence intensity reflects approximately the intracellular levels of $O_2^{\bullet -}$ [23]. In addition, it is considered to be quite insensitive to H_2O_2 [20,22,26].

Therefore, the intracellular ROS levels were assessed by fluorescence microscopy using the two different probes DHE and DHR, as described in Chapter 3. Briefly, MDA-MB-231 and MCF-7 cells were seeded on Matrigel™- coated (1/30 dilution in FBS-free medium) dishes and after 24 h, when cells were ~40 % confluent they were incubated with vehicle, dox (0.1 µM), MnTnHex-2-PyP (5 µM) or both drugs for 1 h or 16 h at 37 °C in FBS-free medium. For the DHR assays, CAT (50 U/mL) was used alone or in combination with both drugs. Cells were then washed twice with warm PBS and incubated with DHR or DHE (10 µM) in FBS-free medium for 25 min at 37 °C. Cell image acquisition was performed using a wide field BX51 fluorescent Olympus microscope with a 40x objective using a 460-490 nm/<520 nm excitation/emission filter for DHR and a 520–550 nm/<580 nm excitation/emission filter for DHE [27,28]. Cell fluorescence and area were determined using ImageJ (National Institutes of Health) [29] for a minimum of 45 cells per condition. Three to four independent experiments were performed.

4.2.6. *In vitro* wound healing assay

The *in vitro* wound healing assay was optimized according to Liang *et al.* (2007) [30] and performed as described in Chapter 3. Briefly, MCF-7 cells and MDA-MB-231 cells were seeded in 24-well plate with an inoculum of 2×10^5 cells per well and cultured in complete media for 24 h. After this, medium was removed and each well was scratched using a 200 µL pipette tip, leaving a gap of approximately 0.8 mm in width. Cells were washed twice with PBS to remove the detached cells and cell debris. Cells were kept in FBS- and insulin-free media containing the test compounds. The distance between the two limits of the scratch was monitored using a Motic AE 2000 inverted microscope with an objective of 10x at different time points: 0, 9, and 24 h after compounds addition. Images were collected using a Moticam 2500 (*v.d.* Figure A.2 in Annex) and measurements were performed using Motic Images Plus V2.0 software. Zero h was considered as 0% of wound closure. At each time point one photo of each scratch was taken and three representative measures were performed. Each assay was performed with intern triplicates and at least 4 independent experiments were performed per condition.

4.2.7. Chemotaxis and chemoinvasion assays

The chemotactic migration of MCF-7 and MDA-MB-231 cells was evaluated as described in Chapter 3. Briefly, cells ($1x10^5$ cells in 200 µL of FBS-free medium) were seeded on the top of the insert and complete medium was placed in the lower chamber of the culture well. The test compounds were added to both chambers and cultures were incubated for 16 h. Non-migrating cells were removed from the upper side of the inserts with a cotton swab. Cells that migrated to the underside of the inserts were fixed with cold 96% ethanol and stained with 0.1% CV in 10% ethanol (*v.d.* Figure A.3 in Annex) Cells were ressuspended in 96% ethanol with 1% acetic acid and the absorbance at 595 nm was measured in a Thermo Fisher Multiskan FC microplate reader. Three to five independent experiments were performed.

The chemoinvasion assay in MDA-MB-231 cells was performed as described in Chapter 3.

4.2.8. Random cell migration assay

Time-lapse microscopy and cell tracking was performed to determine the migration phenotypes in each cell line, *i.e.*, their random migration profiles. Individual random cell migration of MDA-MB-231 and MCF-7 cells was evaluated as previously described by Saraiva *et al* (2013) [31]. Briefly, MDA-MB-231 or MCF-7 cells were seeded at low density (30% confluent) on Matrigel^{TM}-coated 12-well plates (with homogeneous cell seeding, *v.d.* Figure A.4 in Annex). Drugs were added and cells were allowed to adhere for 4 h at 37 ºC. Individual cells were imaged at 10-min intervals for 12 h (cells were imaged O/N, for 12h every 10 min) with a wide-field microscope (Observer.Z1; CarlZeiss) contained within an environmental chamber at 37 °C using a 10x objective and a camera (AxioCam HRm; Carl Zeiss). Six videos were recorded for each well of each condition. Migration tracks were generated using the ImageJ Manual Tracking plugin, and at least 10 cells per video were followed (*v.d.* Figure A.5 in Annex) and tracks were analyzed using an in house-written Mathematica 7 notebook (provided by G. Dunn, King`s College London, London, England, UK) to calculate migration rates and movement persistence.

4.2.9. Gelatin zymography assay

The protease activities of secreted MMP-2 (gelatinase A) and MMP- 9 (gelatinase B) in the culture medium of MDA-MB-231 cells were analysed using a gelatin zymography assay, similarly as previously described by Hu (2010) [32]. This procedure allowed the detection of proteolytic activity at the specific molecular weights of the active MMPs [33], being useful for the assessment of these two key MMPs, which have potent gelatin-degrading activity [32]. MDA-MB- 231 cells (~$7x10^5$ cells) were seeded on 25 cm^2 T-flasks coated with Matrigel^{TM} (diluted 1/30 in serum-free medium) and allowed to grow for 24 h. Cells were incubated for 24 h with fresh FBS-free medium containing vehicle (control cells), MnTnHex-2-PyP (5 µM), dox (0.1 µM) or both drugs. After that, the conditioned medium was collected, cleared by centrifugation (4 ºC, 400x*g*, 5 min) to remove floating cells and debris and concentrated using Vivaspin concentrators (500 µL, 30 kDa cut-off, Sartorius Stedim Biotech, Germany) (*v.d.* Figure A.6 in Annex). After that, the protein content was measured using the NanoDrop (Microplate Reader Spectrostar Omega BMG Labtech). Concentrated supernatants with equal amount of total protein (30 µg) were mixed with an equal volume of 2× sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) under non-reducing conditions, and were incubated at room temperature for 10-15 min

before electrophoresis. Then, the concentrated denaturated samples were resolved in a 10% SDS–polyacrylamide gel (PAGE) copolymerized with 0.1% (w/v) gelatine as substrate, for ~120 minutes with constant 100 V voltage, within running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS) diluted 1:10 in dH_20. Prestained molecular weight markers (MW: 10 to 260 kDa; Spectra^{TM} Multicolor Broad Range Protein Ladder, *v.d.* Figure A.7 in Annex) were also run with the samples (10 µL). The gel was gently washed three times 20 min in 2.5% Triton X-100 solution (in dH_2O) at room temperature, to remove SDS and renatured the enzymes. During electrophoresis, the SDS causes the MMPs to denature and become inactive. Then, after electrophoresis, when MMP-2 and MMP-9 are separated by molecular weight, the gels were washed to replace SDS by Triton X-100. After that, the gels were incubated in developing buffer (50 mM Tris Base, pH 7.5, 200 mM NaCl, 5 µM $ZnCl_2$, 5 mM $CaCl_2$, 0.02% NaN_3) for the enzymatic reaction to proceed at 37 °C for 22-24 h. Gels were subsequently stained with 0.2% Coomassie brilliant blue R-250 for 1 h and destained with destaining solution (40% methanol, 10% acetic acid in dH_2O). To test if the white bands observed on the blue background (as in Figure A.8 in Annex) indicated zones of gelatin digestion corresponding to the presence of really activated MMPs, gels were also incubated with 7.8 mM EDTA in developing buffer. It the presence of EDTA and due to its metal chelating properties, if the white bands disappeared, that means they were from MMPs and not from non metallic proteinases. In this work, it was observed in all gels that the white bands disappeared (data not shown) therefore, it was admitted that they resulted from gelatin digestion corresponding to the presence of activated MMPs and, in this case, on the basis of their molecular weight they were activated MMP-2 and MMP-9. The gel images were captured with ChemiDoc XRS (Bio-Rad, USA) and the semi-quantitative densitometry of MMP-2 and MMP-9 bands was performed using ImageJ [29]. The pixel density was determined after background subtraction and used to calculate the integrated density of the selected band. Three to four independent experiments were performed.

4.2.10. Fluorescent-gelatin degradation assay

A critical step related to initiation of cellular invasion is the focalized proteolytic degradation of ECM components in the epithelial or endothelial basement membrane. Some *in vitro* analysis performed in tumor cells has demonstrated that ECM degradation is accomplished by structures designated by invadopodia [34], which contribute to cancer cells invasion and metastasis [35]. Visualization of invadopodia-mediated ECM degradation of cells by fluorescent microscopy using dye-labeled matrix proteins coated on the surface of

glass coverslips has emerged as the predominant technique for evaluating the degree of matrix proteolysis and cellular invasive potential [34]. The fluorescent gelatin degradation assay was performed as previously described by Martin *et al* [34], a method generating fluorescently-labeled glass coverslips utilizing a commercially available Oregon Green-488 gelatin conjugate. In addition, this procedure permits accurately and reproducibly, to monitor invadopodia activity, as well as testing anti-invasive compounds on extracellular matrix degradation [34].

The first part of this procedure consisted in the glass coverslips coating (*v.d* Figure A.9 in Annex), including the optimization step in order to select the most adequate cover slip coating being this optimization procedure explained in detail in Annex.

The next step was seeding cells followed by cell fixation.

4.2.10.1. Incubation with MDA-MB-231 cells and fixation

Prior to use, glass coverslips were sterilized with 70% ethanol for 15 min at room temperature, in the dark, then transferred to a sterile 24-well plate, washed with sterile PBS (at this time, coverslips can be stored in PBS protected from light at 4ºC for at least 2 months). To be used, glass coverslips were incubated in complete medium for, at least, 1h before use. Then, 1.0 mL of MDA-MB-231 cells suspension ($4x10^4$ cells to obtain a 30-40% confluency) were cultured on cross-linked fluorescent conjugated matrix with vehicle, dox (0.1 µM), MnTnHex-2-PyP (5 µM), both drugs or both drugs with CAT (50 U/mL), for 16 h at 37 °C. The media was aspirated and the cells were washed twice, quickly, with ice-cold PBS (this was carried out on ice). Cells were fixed with cold 4% PFA + 250 mM HEPES (pH 7.4) in PBS, at room-temperature for 20 minutes, washed twice with PBS and incubated for 5 minutes in ammonium chloride NH_4Cl (50 mM in PBS) (to quench free aldehydes, which can auto-fluorescence). After washing with PBS, glass coverslips were dipped in dH_2O for 20 seconds (to prevent formation of PBS crystals) and place on a slide prepared with a 10 µL drop of mounting media Mowiol 4-88, containing DAPI to reveal nuclei. After 30 minutes at room temperature, the glass coverslips were then placed at 4˚C overnight.

The final step of this protocol was imaging and quantifying the invadopodia formation and activity in MDA-MB-231 cells.

4.2.10.2. Imaging and quantifying invadopodia activity in MDA-MB-231 cells

Slides were imaged with a BX51 fluorescent Olympus microscope with a 40x objective equipped for detection of Oregon Green-488 and DAPI. Gelatin degradation causes loss of fluorescence with black spots on the fluorescent background and, therefore, it was detected in the "green" channel as dark areas over the green background. To quantitate the degradation activity of invadopodia, 10 randomly selected fields, usually containing 15 to 25 cells, were imaged with a 40x objective for each determination. The degradation area in black and white thresholded images was determined by using ImageJ and normalized for the number of cells (number of nuclei in each image as measured from the DAPI channel in the same field, Figure 4.3) for measuring "area fraction" (the percent of area that corresponds to degradation) on a given image. In each analysis, the mean value of the control cells was set at 100% and the relative values of the cells treated with the compounds were then calculated. Data are mean ± SE of three independent experiments.

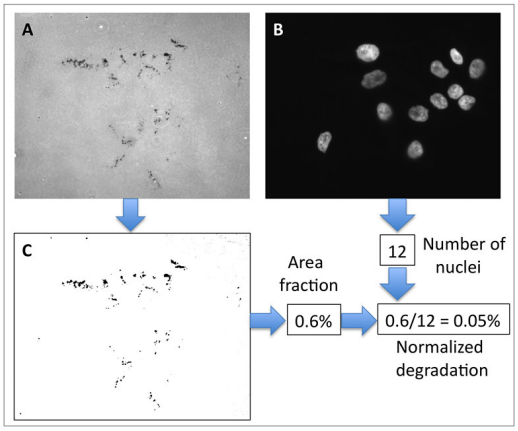

Figure 4.3 - Quantification of invadopodia activity. Images from gelatin (green channel) and nuclei (blue channel) referent to the same microscopy field are used. The original image from the green channel (A) is processed using Image J (C) to obtain the area fraction value and the original image from the blue channel (B) is used to calculate cell number. These two values are used to obtain the "normalized degradation" value of each microscopy field from a sample (reproduced from [35]).

4.2.11. Focal adhesions number

Focal adhesions (FA) consist of a dynamic group of proteins with structural and regulatory roles in the cell adhesion process [36]. FA are stable cell–substrate interactions that evolve from a focal complex which contain focal adhesion kinase (FAK), integrins, talin, vinculin, paxillin and many other proteins that match to the actin filament network [37]. The effect of

the drugs under study in the FA number in MDA-MB-231 and MCF-7 cells were evaluated, since they are important molecular components of FA [36].

MDA-MB-231 or MCF-7 cells were seeded in Matrigel^{TM} (diluted 1/30 in serum free media) – coated coverslips so that they would reach 30-40% confluence 40 h later. Drugs were added to cells 24 h post seeding and were left to incubate for another 16 h. Cells were fixed with cold 4% PFA, incubated at room-temperature for 20 minutes and washed twice with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and blocked with 10% Foetal Calf Serum (FCS) in PBS (blocking buffer) for 30 minutes. After that cells were stained with primary antibodies anti-vinculin (1:500) and anti-pFAK Y^{397} (1:80) in 100 µL of blocking buffer, incubated for 60 minutes and washed three times in blocking buffer (for a total of 30 minutes). Then cells were incubated with secondary antibody conjugated to Alexa 488 for 45 minutes. After that, cells were washed once with blocking buffer for 10 minutes and four times in PBS for 5 minutes each. The cover slips were dipped in distilled water for 20 sec (to prevent formation of PBS crystals), placed on to a slide prepared with a 35 µL drop of mounting media Mowiol 4-88 containing DAPI, left for 30 minutes at room temperature to set and then then placed at 4˚C overnight. Cells were imaged by confocal microscopy using a 63x oil objective and a microscope (LSM 5 PASCAL; Carl Zeiss). Images were acquired using LSM image browser software (Carl Zeiss) and the number of FA per cell was counted.

4.2.12. NF-κB gene dual-reporter assay

The NF-κB activation in MCF-7 and MDA-MB-231 cells upon treatment with dox and/or MnTnHex-2-PyP was evaluated using a dual luciferase-based gene reporter assay (*v.d.* Figure A.14 in Annex), described in detail in Annex.

The first step to perform this assay was the production of *TK Renilla* and *Firefly Luciferase*-NF-κB plasmids, as described in Annex. Then, transfection conditions were optimized using an EGFP fluorescence assay, as described also in Annex, being the optimized NF-κB gene dual-reporter assay performed as described below.

MDA-MB-231 cells ($3x10^4$ cell/100 µL complete medium) and MCF-7 cells ($1.5x10^4$ cell/100 µL complete medium) were seeded in 96-well plates for 24 h (should be 60% confluent in the next day for transfection). Before transfection the medium was replaced and incubated at 37 ºC for 1h. Cells were transfected with 60 ng/well of firefly luciferase NF-κB reporter plasmid and 20 ng/well of pTK-Renilla luciferase (pRL-TK) as transfection control, using Lipofectamine® LTX Reagent (0.45 µL/well) and PLUS^{TM} Reagent (0.1 µL/well) according to the manufacturer`s protocol and as described in section 4.2.12.2. After the

diluted DNA plasmids were added to the diluted LTX Reagent (1:1 ratio), the mixture was carefully pipetted 2-3 times and left to incubate at room temperature for 25 min for MCF7 cells or 30 min for MDA-MB-231 cells. Then, the transfection mixture was added to the cells drop wise and the cells were incubated at 37 ºC. Forty-eight hours after transfections, cells were treated with dox (0.1 µM), MnTnHex-2-PyP (5 µM), both drugs or both drugs with CAT (50 U/mL), for 16 h at 37 °C (1 h before adding compounds the medium was changed and incubated at 37 ºC). Cells non transfected were used as assay control, cells transfected without compounds were used as negative controls and cells transfected and stimulated with TNF-α (50 ng/mL) for 9 h were used as positive controls of transfection (before TNF-α treatment, the medium was replaced to medium with 0.1% FBS and incubate at 37 ºC for 15 min). After the 16 h or 9 h treatment, cells were harvested with 100 µL Passive Lysis Buffer 1X (diluted in dH_2O from Promega Passive Lysis Buffer 5X) and plates with cell lysates were freeze at – 80 ºC. The firefly luciferase and *Renilla* luciferase activities were both measured in cell lysates (10 µL/well) using a Synergy™ HTX Multi-Mode Microplate Reader. For the dual luciferase assay it was used, per well, 50 µL of a home made luciferase substrate (20 mM Tricine, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM $Na_2EDTA.2H_2O$, 33.3 mM dithiothreitol (DTT), 530 µM ATP, 270 µM Acetyl Coenzyme A (CoA), 0.47 mM D-Luciferin, 5 mM NaOH and 0.348 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$) and 50 µL of coelenterazine solution (1 mg/mL in absolute EtOH) diluted 1:500 in PBS at room temperature, as Luc and *Renilla* substrates, respectively. The relative stimulation of reporter-gene expression was calculated by normalizing firefly luciferase activity with *Renilla* luciferase activity in cell lysates and present as relative luciferase units. In all cases, data shown are representative from at least three independent experiments, each comprising five replicates.

4.2.13. Statistical analysis

All assays were performed in at least three independent experiments. Quantitative data are presented as means ± SD. Statistical analysis was performed using student's t test or one-way ANOVA (analysis of variance). Differences were considered significant at *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.3. Results

4.3.1. Impact of MnTnHex-2-PyP and dox on cell viability and cell death

The effect of MnTnHex-2-PyP and dox, alone or in co-treatment, on the viability of human mammary cells was evaluated by the MTT assay. Dox was used at 0.5, 1 and 5 μM in MCF-7 and in MDA-MB-231 cells at 1, 5 and 20 μM (Figure 4.4 A and B).

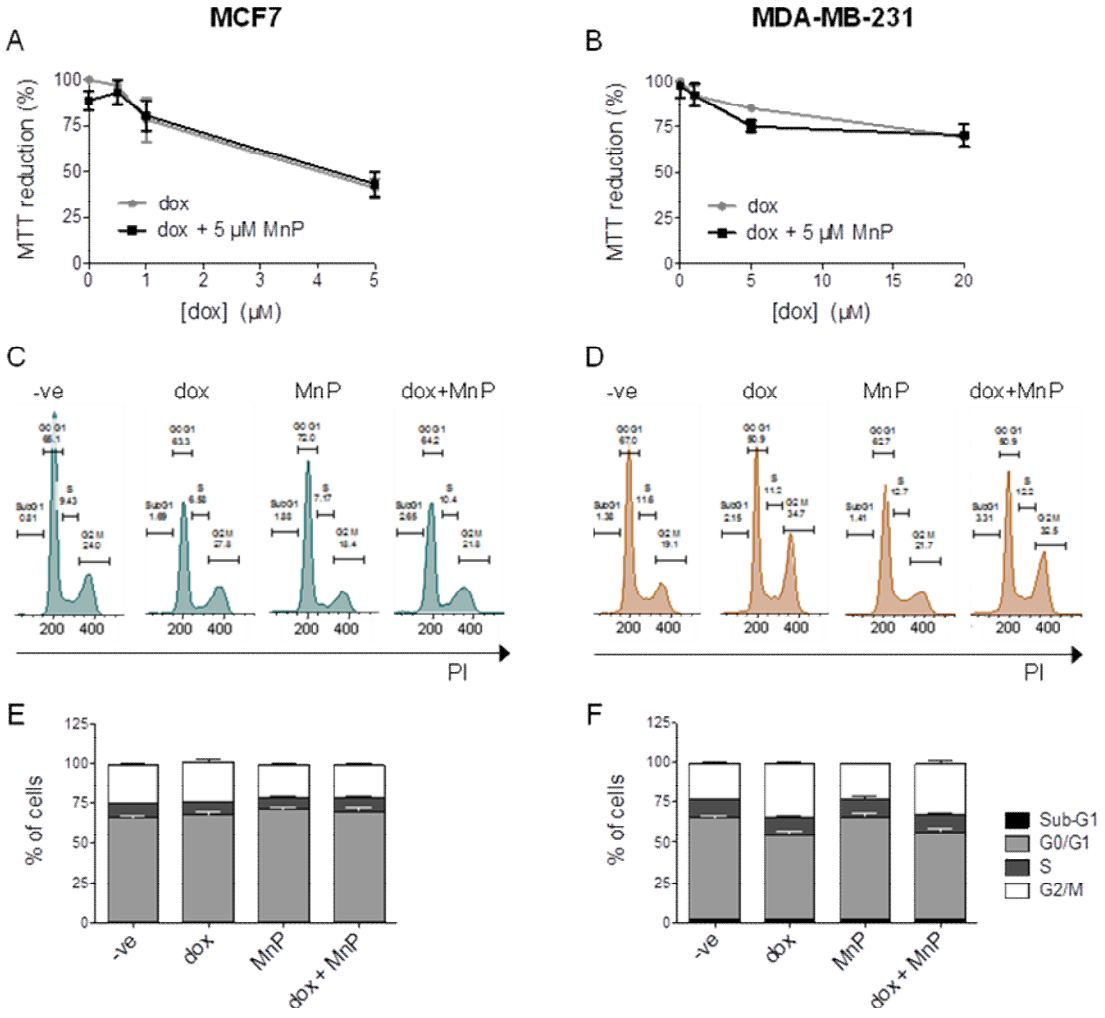

Figure 4.4 – Treatment with MnP and low concentrations of dox does not induce cell death. MCF-7 (A, C and E) and MDA-MB-231 (B, D and F) cell viability and cell death induction following exposure to the indicated MnP and dox concentrations for 16 h, were evaluated by an MTT assay (A and B) and a DNA content assay after cell fixation (C-F), respectively. Histograms show representative MCF-7 and MDA-MB-231 DNA content profiles following exposure to dox (0.1 μM), MnTnHex-2-PyP (5 μM) or both, fixation and propidium iodide (PI) stain (C-D). Summary results from cell viability (A and B) and DNA content assays (E and F) are represented as means ± SD from three independent experiments.

A concentration-response decrease in cell viability was observed in the two breast cancer cell lines. It was observed that MnTnHex-2-PyP alone at the biologically relevant concentration of 5 μM [38] was not considerably toxic in MCF-7 and especially in MDA-MB-231 cells (Figure 4.4 A, 88.4 ± 5.1% and Figure 4.4 B, 97.5 ± 6.8%, respectively). However, MDA-MB-231 cells were more resistant to dox toxicity. For example, under our experimental conditions, exposure to 5 μM of dox decreased cell viability more drastically in MCF-7 than in MDA-MB-231 cells ($p < 0.001$). Dox alone (0.1 μM) exhibited a concentration-response decrease in cell viability in both cell lines, which was not altered by the addition of MnTnHex-2-PyP (5 μM). Interestingly, in human normal-like breast cells (MCF-10A) MnTnHex-2-PyP (5 μM) afforded a slight protection against dox cytotoxicity, increasing cell viability from 56.2 ± 2.3 % (n=3) to 64.1 ± 4.9 % (n=2) (data not shown).

Cell viability studies allowed the identification of non-toxic levels of the compounds. Since dying cells poorly migrate and invade [39], the use of clinical relevant [40,41] and non-cytotoxic concentrations is essential when testing potential inhibitors of migration/invasion [39,42]. Based on these results, concentrations of 0.1 μM of dox and 5 μM of the MnP MnTnHex-2-PyP were selected for the subsequent experiments.

To confirm that the concentrations abovementioned did not induce cell death, the percentage of cells in sub-G1 was investigated, in order to evaluate an apoptotic population upon treatment with the indicated compounds (Figure 4.4 C-F). Identification of a sub-G1 cell population is frequently performed to estimate the frequency of apoptotic cells [43] with fractional DNA content [44]. Therefore, the impact of MnTnHex-2-PyP (5 μM) alone or in combination with dox (0.1 μM) in MCF-7 and MDA-MB-231 cell cycle was investigated by assessing the cell DNA content using PI stain in fixed cells, following a 16 h exposure to drugs. Representative histograms obtained by flow cytometric analysis are depicted in Figure 4.4 C and D and representative graphs obtained by flow cytometric analysis of the cells are shown in Figure 4.4 E and F. No differences were found in the Sub-G1 population in either cell line treated for 16 h with 0.1 μM of dox and/or 5 μM of the MnTnHex-2-PyP (Figure 4.4 C-F), suggesting that treatment conditions are not inducing cell death. MnTnHex-2-PyP alone (5 μM; 16 h) led to a cell cycle distribution similar to that of control cells. Under our experimental conditions, no major overall differences in sub-G1 and G2/M were observed between control and treated cells. However, MDA-MB-231 cells treated with dox presented a G2/M population increase (*** $p < 0.001$) compared with MCF-7 cells, which is consistent with previous reports [45].

4.3.2. MnTnHex-2-PyP and dox modulate intracellular ROS levels

Since the biological effects of the MnP MnTnHex-2-PyP are probably associated with the modulation of the cellular redox status and since dox has been shown to induce ROS [26], the impact of the drugs in the intracellular levels of ROS was assessed by microscopy using two complementary oxidant-sensitive fluorescence probes (DHE and DHR), after 1 h or 16 h of drug exposure (Figure 4.5). As referred previously in Chapter 3 (section 3.2.6.2), cell-permeable DHR is widely used to evaluated general RS formation, is reactive to H_2O_2 in peroxidase-containing cells [21], but considered unreactive to $O_2^{\bullet -}$ [21,22]. The base of this assay consists in the oxidative conversion of DHR to its corresponding two-electron oxidized fluorescent product, rhodamine (Figure 3.10, section 3.2.6.2) [21]. As aforementioned in section 4.2.5, DHE is a cell permeable probe that reacts with $O_2^{\bullet -}$ to form the fluorescent product 2-hydroxyethidium [21]. The oxidation of DHE is mostly superoxide dependent and is considered to be quite insensitive to H_2O_2 [20,22,26].

Before the assay, as performed for the compounds studied in Chapter 3, the possible auto-oxidation of both MnTnHex-2-PyP and dox was tested. This experiment was performed as described in Chapter 3 (section 3.2.6.2), except that, in this case, the probes weren`t added. For both compounds, in the excitation/emission wave-length values used for DHR and DHE, the fluorescence levels without probes addition were less than 5% of those with DHR or DHE incubation for 20 min (data not shown). Therefore, it was admitted that both compounds didn`t have auto-oxidation in the conditions used in this assay.

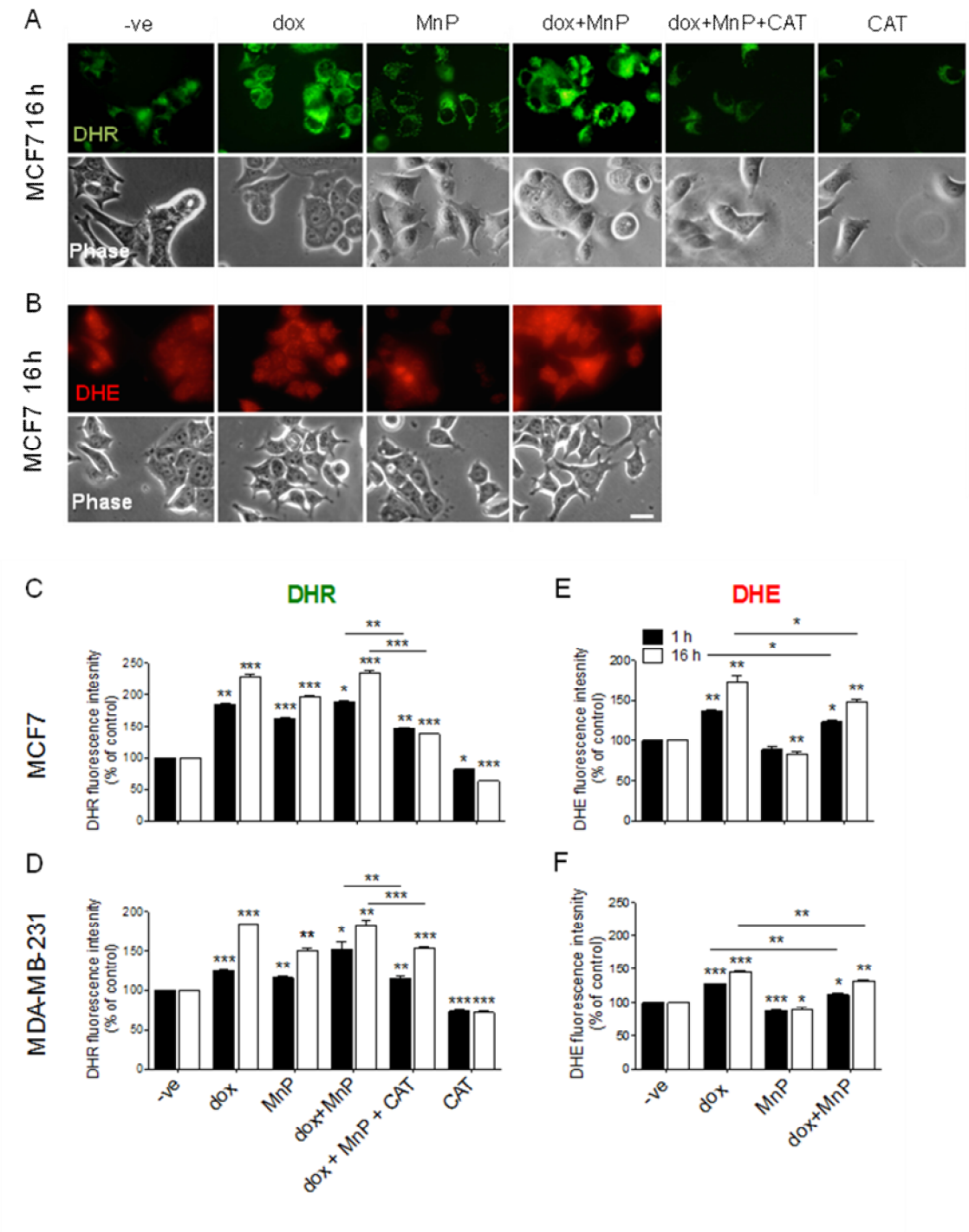

Figure 4.5 – MnTnHex-2-PyP and dox lead to an increase in intracellular ROS. Intracellular ROS levels were determined in MCF-7 (A, B, C and E) or MDA-MB-231 (D and F) cells treated with the indicated drugs (dox (0.1 µM), MnTnHex-2-PyP (5 µM) and CAT (50 U/mL)) for 1h or 16 h. Fluorescence microscopy images show representative MCF-7 cells after 25-min incubation with DHR and DHE (A and B). Scale bars = 20 µm. Summary results (means ± SD from three independent experiments) show relative DHR and DHE fluorescence (C to F). *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA with Tukey's test, relative to untreated cells, - ve).

In the representative fluorescence microscopy images of MCF-7 cells after 25-min incubation with DHR and DHE probes (Figure 4.5 A and B), it is perfectly clear that DHR probe has tropism for mitochondria, while DHE probe is not specific to a given organelle, hence the more diffuse pattern observed in Figure 4.5 B.

MCF-7 cells incubated with dox (0.1 μM), MnTnHex-2-PyP (5 μM) or both drugs, exhibited a significant increase in ROS levels assessed by the DHR probe, which was more pronounced after 16 h of drug exposure. Similar results were found with MDA-MB-231 cells. However, the ROS increase in cells treated only with MnTnHex-2-PyP or only with dox was not as pronounced as in MCF-7 cells, when compared with non-treated control cells.

In order to understand if the increase observed in fluorescence could be associated with the formation and accumulation of H_2O_2, the exposure of cells to CAT alone (50 U/mL) or simultaneously with the other drugs was assessed. When cells were exposed only to CAT (50 U/mL), for 1h or 16h period, the level of ROS decreased when compared with the one of non-treated cells (Figure 4.5 A, C and D). When these cells were simultaneously exposed to MnTnHex-2-PyP, dox and CAT, the levels of ROS decreased significantly when compared with those of cells treated only with MnTnHex-2-PyP and dox (Figure 4.5 C and D, *** $p < 0.001$), demonstrating that higher ROS was in part due to enhanced H_2O_2 accumulation.

DHE fluorescence was significantly increased in dox-treated cells, in both cell types and both incubation periods, being the increase more pronounced in the 16h treatment, especially for MCF-7 cells. This increase was partially reverted by MnTnHex-2-PyP (Figure 4.5 B, E and F), suggesting an increase in $O_2^{\bullet -}$ in dox-treated cells, as previously described [26,42,46]. The intracellular ROS alterations induced by the treatments were similar in both cell lines.

As described in section 4.2.5, cells were incubated with compounds for 1h or 16 h at 37 °C in FBS-free medium. In order to evaluate if the presence of FBS in the medium could influence the results obtained, the assay was also performed as described in section 4.2.5, but using medium with 10% FBS.

The results obtained are depicted in Tables 4.1 and 4.2, for MDA-MB-231 and MCF-7 cells, respectively, treated with MnTnHex-2-PyP, dox and CAT, for 16 h, after 25 min incubation with DHR:

Table 4.1 – Intracellular ROS levels (% RFU) for MDA-MB-231 cells in FBS 10% medium exposed to MnTnHex-2-PyP (5 µM), dox (0.1 µM) and CAT (50 U/mL), for 16 h, after 25 min incubation with DHR (n=3, means± SD)

Ve	dox	MnP	dox+MnP	dox+MnP+CAT
100±0.0	182.20±5.72	146.11±4.22	219.91±8.31	150.79±6.58

Comparing the results obtained with FBS 10% medium (Table 4.1) with those without FBS (Figure 4.5), they are similar regarding MDA-MB-231 cells treated with MnTnHex-2-PyP and dox alone and in co-treatment with the presence of CAT. The only difference was observed in the oxidation levels in MDA-MB-231 cells in co-treatment with both drugs, where they were higher. Routes to the formation of highly reactive $OH^{\bullet}$ radicals involving *Fenton* chemistry catalyzed by Fe(II) or Cu(I) [21], as described in Chapter 1, could explain, in part, the higher oxidation values in the co-treatment, since FBS has in its composition Fe (II) ion.

Table 4.2 – Intracellular ROS levels (% RFU) for MCF-7 cells in FBS 10% medium exposed to MnTnHex-2-PyP (5 µM), dox (0.1 µM) and CAT (50 U/mL), for 16 h, after 25 min incubation with DHR (n=2, means± SD)

Ve	dox	MnP	dox+MnP	dox+MnP+CAT
100±0.0	249.29±1.97	236.31±6.53	253.15±3.55	147.97±1.53

Comparing the results obtained with FBS 10% medium (Table 4.2) with those without FBS (Figure 4.5), they are similar regarding MCF-7 cells treated with dox alone. For MCF-7 cells exposed to MnTnHex-2-PyP alone, to co-treatment with both drugs and to co-treatment with both drugs in the presence of CAT, the oxidation levels observed were slightly higher, probably due to the involvement of *Fenton* chemistry catalyzed by Fe(II).

Regarding the incubation with the second probe, DHE, the effects of FBS 10% in the medium as also evaluated in MCF-7 cells exposed to MnTnHex-2-PyP, dox alone or in co-treatment, for 16 h, after 25 min incubation with DHE probe. The results obtained with FBS 10% were very similar to those without FBS, for all the conditions (data not shown).

4.3.3. Impact of MnTnHex-2-PyP and dox on cell migration

Since the changes observed in intracellular ROS may be associated with differences in migration/invasion and considering the role of cell migration in breast cancer progression and metastases, the impact of MnTnHex-2-PyP and dox on breast cancer cells migration was determined by three different assays: the wound healing assay (Chapter 3, section 3.2.4), the transwell inserts assay [47,48] (Chapter 3, section 3.2.5) and the time-lapses microscopy random migration assay (section 4.2.8). The summary of the results obtained with the three different assays are depicted in Figure 4.6.

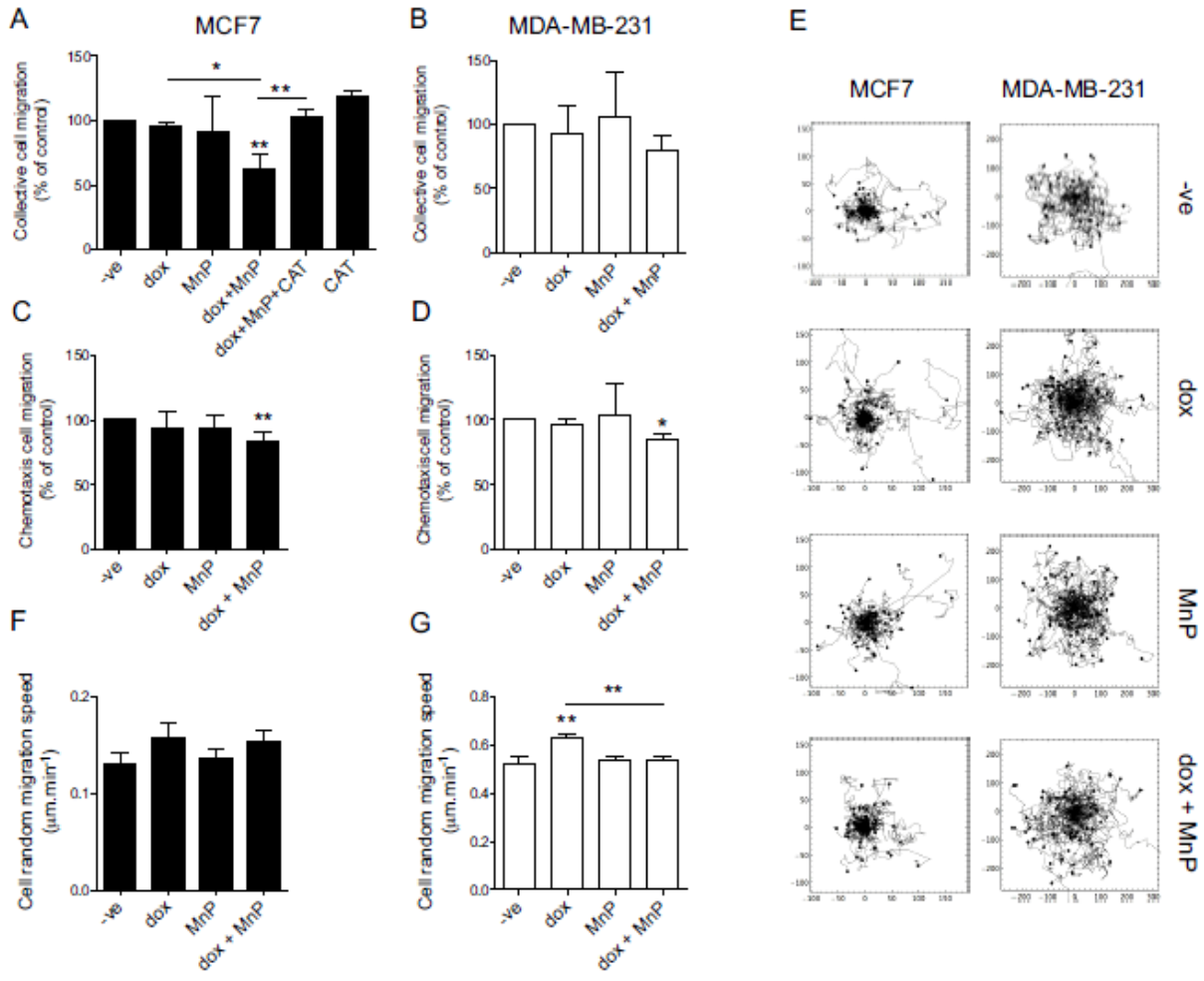

Fig 4.6 – MnTnHex-2-PyP and dox can reduce chemotaxis and random and collective cell migration. Collective cell migration, chemotaxis and random migration of MCF-7 (A, C and F) or MDA-MB-231 (B, D and G) cells treated with the indicated drugs (dox (0.1 µM) and MnTnHex-2-PyP (5 µM) for 16 h) were measured. Collective cell migration was measured by the wound healing assay (A and B), chemotaxis was measured using a transwell system with FBS as chemoattractant (C and D) and random cell migration on matrigel was measured using time lapse microscopy (F and G). Tracks of individual migrating cells (n = 60 for each condition) used to measure random cell migration are shown in E. Results are shown as means ± SD. *p < 0.05, **p < 0.01 (Student's t-test, relative to untreated cells, - ve).

Collective cell migration was assessed by the wound-healing assay (Figure 4.6 A and B). MnTnHex-2-PyP or dox, given alone did not significantly change cell migration. However, the co-treatment led to a reduction in cell motility. This decrease was more pronounced in MCF-7 cells, in which the % of wound closure decreased to 63 ± 9% of control (** $p < 0.01$, when compared with non-treated controls). A similar trend was observed for MDA-MB-231 cells treated with dox, MnTnHex-2-PyP or in co-treatment, although much less pronounced (Figure 4.6 B, ns).

To explore if H_2O_2 increased levels are associated with the reduction of collective cell migration observed upon the co-treatment with dox+MnP, experiments using CAT were performed. The addition of CAT (50 U/mL) significantly reverted this inhibitory effect of dox+MnP (Figure 4.6 A, ** $p < 0.01$, when compared with cells in co-treatment), suggesting a role for H_2O_2 in mediating this migration phenotype.

In addition, in MCF-7 cells it was also observed that the decrease in the wound closure in the co-treatment was also statistically significant when compared with dox alone (Figure 4.6 A, * $p < 0.05$).

Chemotaxis was evaluated by a transwell inserts assay using FBS as chemoattractant. In both cell types MnTnHex-2-PyP or dox alone did not alter chemotaxis (Figure 4.6 C, D). However, when cells were simultaneously exposed to both compounds it resulted in a significant decrease in chemotactic migration of MCF-7 and MDA-MB-231 cells to 83.4 ± 7.9% (Figure 4.6 C, ** $p < 0.01$, when compared with non-treated controls) and to 84.3 ± 4.4% % (Figure 4.6 D, * $p < 0.05$, when compared with non-treated controls), respectively.

Single cell random migration was evaluated by time-lapse microscopy. As expected, the migration speed of MDA-MB-231 cells was higher than that of MCF-7 cells (Figure 4.6 E-G, *** $p < 0.001$), being cell speed defined as the average of all instantaneous speed for all cells [49]. No significant changes were observed in MCF-7 cells random migration when exposed to MnTnHex-2-PyP, dox or both drugs (Figure 4.6 E and F). For MDA-MB-231 cells, dox promoted the random migration of these cells (Figure 4.6 G, ** $p < 0.01$, when compared with non-treated controls), increase significantly reverted by MnTnHex-2-PyP addition (Figure 4.6 E and G, ** $p < 0.01$, when compared with dox-treated cells). In the different conditions tested, no changes in migration persistence were observed (data not shown).

4.3.4. Dox and MnTnHex-2-PyP reduce MDA-MB-231 cell invasion

Cellular invasion into local tissues is a relevant process in development and homeostasis. Malregulated invasion and subsequent cell movement occur in several pathological processes, including inflammation and tumor cell metastasis [34]. Since a critical feature of metastatic cancer cells is their ability to invade tissues, the effect of the MnP, MnTnHex-2-PyP (5 μM) and dox (0.1 μM) in cell invasion was evaluated [39]. Cell invasion experiments were only carried out with MDA-MB-231 cells, due to the non-invasive phenotype of MCF-7 cells [50] (no invasion was observed under our experimental conditions; data not shown).

Cell invasion was assessed using a transwell inserts assay with FBS as a chemoattractant, as described in Chapter 3 (section 3.2.5.2) and the results are depicted in Figure 4.7.

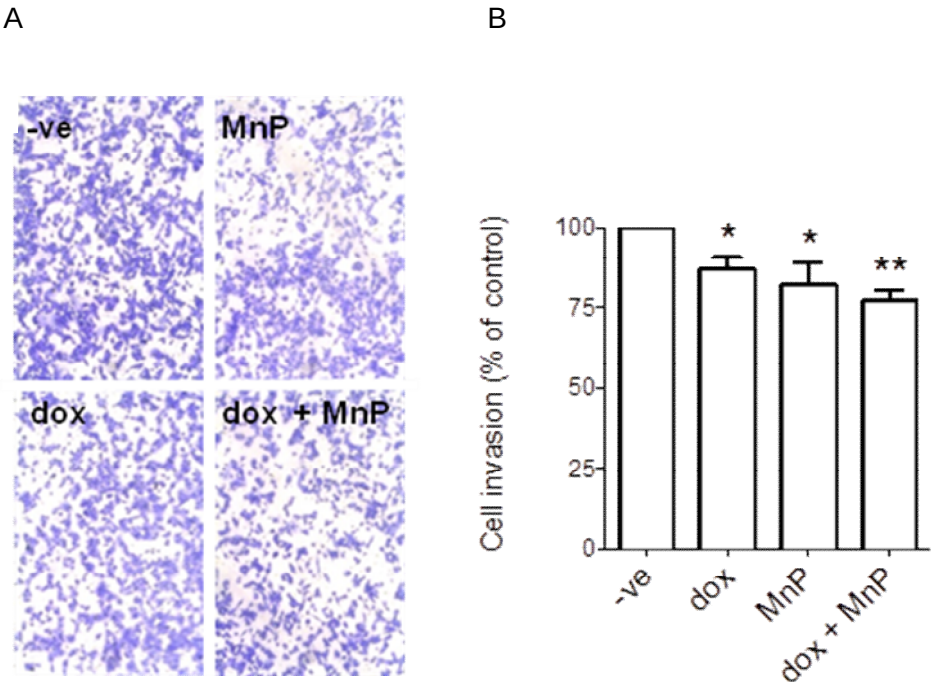

Figure 4.7 – Treatment with MnTnHex-2-PyP and dox reduces MDA-MB-231 cell invasion. MDA-MB-231 cells were seeded on matrigel coated transwells and were allowed to invade for 16 h in the presence of the indicated drugs (dox (0.1 μM), MnTnHex-2-PyP (5 μM)) (A). The percentage of invading cells is summarized in B. At least three independent experiments are shown as means ± SD. *p < 0.05, **p < 0.01 (Student's t-test, relative to untreated cells, - ve).

Chemoinvasion assay in MDA-MB-231 cells showed that MnTnHex-2-PyP (5 μM) and dox (0.1 μM) individually decreased invasion of MDA-MB-231 cells (Figure 4.7 B: 82.3 ± 6.8 % and 86.9 ± 3.7 %, respectively, * p < 0.05, when compared with non-treated controls). The co-treatment with both drugs significantly decreased MDA-MB-231 cells invasion to 77.3 ± 3.1% of controls (Figure 4.7 B: ** p < 0.01, when compared with non-treated controls). In

Figure 4.7 A it is clearly observed the significant decrease in the number of invading cells upon the co-treatment with both compounds, when compared with non-treated control cells.

Given the important role of several metalloproteinases (MMPs) in the invasion mechanisms, in particular, MMP-2 and MMP-9, and due to the results abovementioned observed in the chemoinvasion assay, the activities of the referred MMPs were evaluated by gelatin zymography for MDA-MB-231 cells. Representative results from four independent experiments are shown in Figure 4.8.

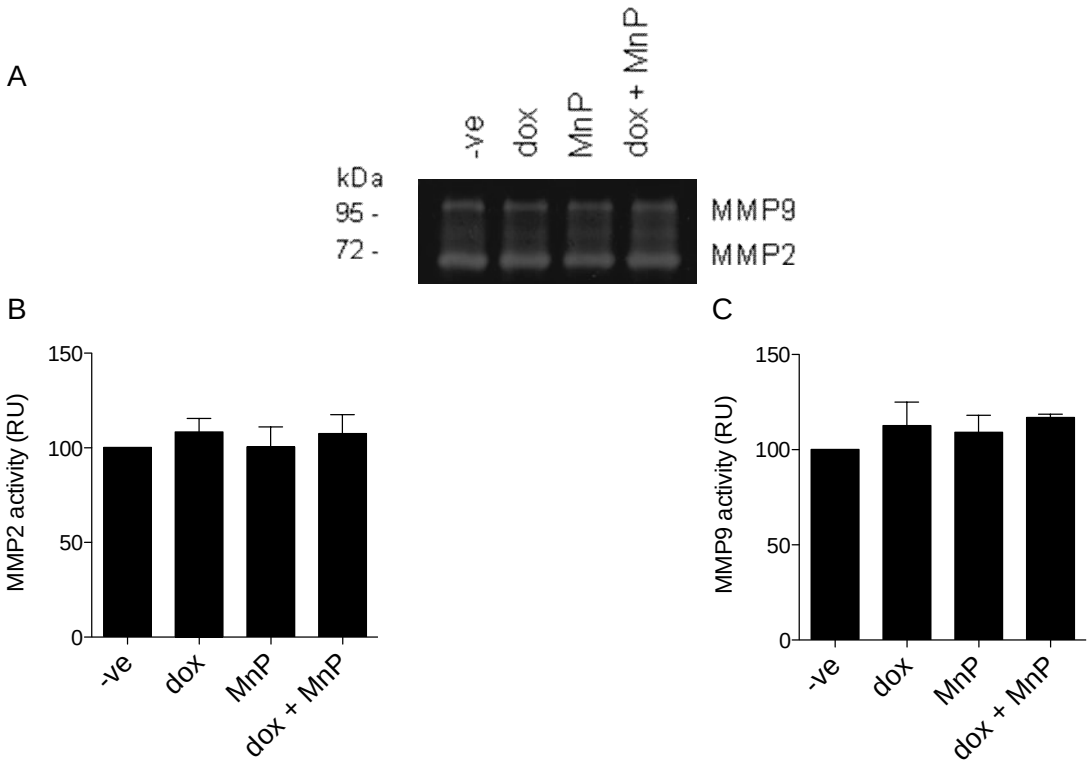

Figure 4.8 – Treatment with MnTnHex-2-PyP and dox does not alter MMP-2 and MMP-9 activities in MDA-MB-231 cells. The activity of MMP-2 and MMP-9 from supernatants of MDA-MB-231 cells treated with the indicated drugs (dox (0.1 μM), MnTnHex-2-PyP (5 μM) for 16 h were detected by gelatin zymography (A). MMP activity is summarized in B (MMP-2) and C (MMP-9). MMP activities from at least three independent experiments are shown as means ± SD. The results were expressed as percentages of non-treated control cells.

No significant effects were observed in MMP-2 activity (Figure 4.8 A, B) or in MMP-9 activity (Figure 4.8 A, C) upon MDA-MB-231 cells exposure for 24 h to MnTnHex-2-PyP (5 μM) and dox (0.1 μM) given alone or in co-treatment, suggesting that other mechanisms should be responsible for the effects observed in cell invasion.

4.3.5. The co-treatment with MnTnHex-2-PyP and dox reduces MDA-MB-231 cell extracellular proteolytic activity

ECM`s focalized proteolytic degradation in both epithelial or endothelial basement membrane is a critical step in initiating cellular invasion. In tumor cells, ECM degradation is performed by ventral actin-rich membrane protrusive structures, named invadopodia [34]. As aforementioned, cancer cells elaborate invadopodia which, in turn, mediate cell attachment and remodeling of the ECM. In fact, these structures contribute to the capability of cancer cells to invade and metastatize [35]. The invasion of cancer cells can be a consequence of the proteolytic degradation of ECM or from amoeboid cell migration through the ECM components [51]. Therefore and due to the fact that no significant effects were observed in MMP-2 and MMP-9 activities in MDA-MB-231 cells exposed to these compounds (section 4.3.4), a fluorescent-gelatin degradation assay was performed, as described in section 4.2.10, to explore the contribution of the proteolytic degradation of ECM to the reduction in cell invasion observed in the chemoinvasion assay. Therefore, this assay allowed to evaluate the ability of MDA-MB-231 cells to form invadopodia and corresponding gelatin matrix proteolysis and also to access the effect of MnTnHex-2-PyP (5 μM) and dox (0.1 μM), given alone or in co-treatment in that proteolytic degradation.

The results obtained are depicted in Figure 4.9.

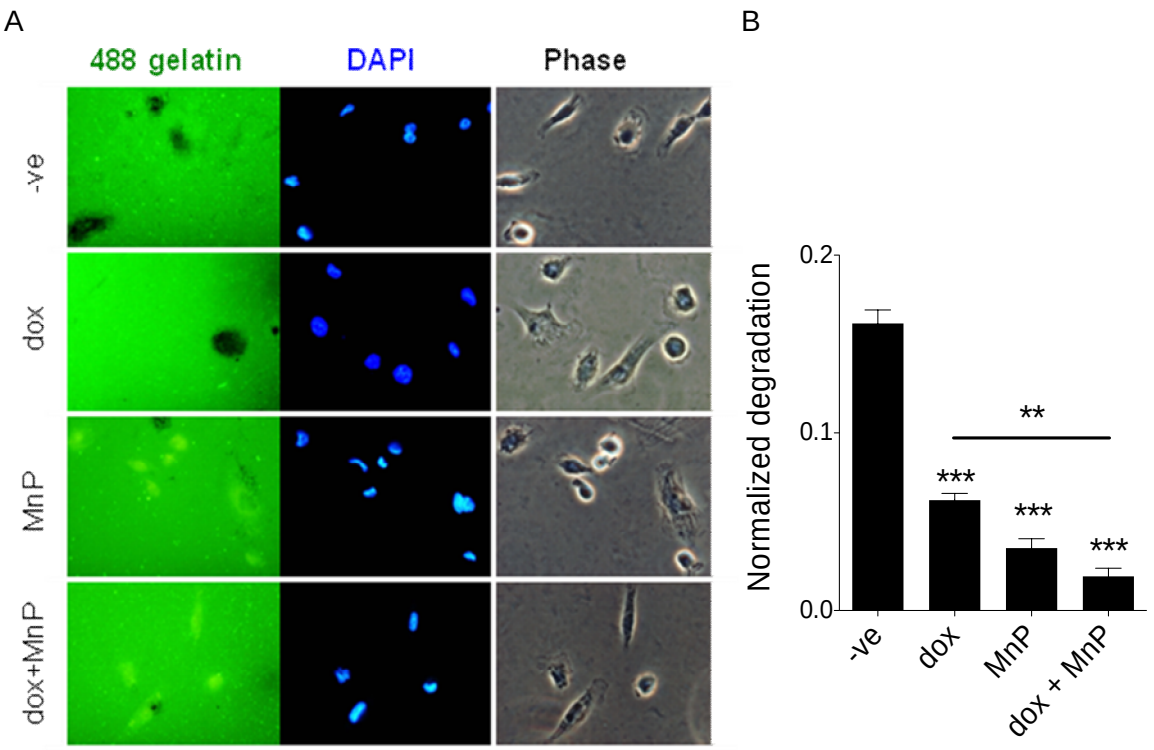

Figure 4.9 – Treatment with MnTnHex-2-PyP and dox reduces MDA-MB-231 cell extracellular proteolytic activity. The extracellular proteolytic activity was measured using a fluorescent gelatin degradation assay: MDA-MB-231 cells were plated on Oregon Green 488-conjugated gelatin coverslips, were allowed to proteolytically invade for 16 h in the presence of the indicated drugs (dox (0.1 µM), MnTnHex-2-PyP (5 µM)), then fixed with 4% PFA and coverslips were mounted with Mowiol 4-88 containing DAPI (A). Normalized gelatin degradation from at least three independent experiments are shown as means ± SD (B), **p < 0.01, ***p < 0.001 (Student's t-test, relative to untreated cells, - ve).

In Figure 4.9 A, degradation of fluorescent gelatin was detected as nonfluorescent black spots at the cell matrix contacts, due to proteolytic removal of the fluorescent gelatin. Results show that the treatment with dox or MnTnHex-2-PyP alone significantly reduced gelatin degradation of MDA-MB-231 cells to 38.4% and 21.7% of controls, respectively (Figure 4.9 B: *** p < 0.001).The result obtained for dox is in accordance with previous data [52]. Treatment with both drugs together had a significantly greater impact on degradation inhibition (11.8% of control; Figure 4.9 B: *** p < 0.001). In addition, the higher decrease in gelatin degradation of MDA-MB-231 cells induced by MnTnHex-2-PyP in combination with dox compaired with that induced by dox alone, was also statistically significant (Figure 4.9 B: ** p < 0.01).

4.3.6. Impact of MnTnHex-2-PyP and dox on focal adhesions number

Focal adhesions (FA) represent, as aforementioned in section 4.2.11, a dynamic group of proteins with structural and regulatory functions in the cell adhesion process [36]. Focal adhesion kinase (FAK) and vinculin are two important proteins of the focal adhesion complex, whose altered expression and/or distribution is correlated with altered cell adhesion and motility [53,54]. In addition, FAK is a non-receptor protein tyrosine kinase that is a "multifunctional regulator" of cell signalling within the tumor microenvironment. FAK localizes to cellular FA or cell contacts with the ECM to regulate several processes involved in cancer progression, namely cell motility, spreading, adhesion, migration, invasion, anoikis (survival), proliferation and angiogenesis. In fact, FAK is a critical signaling molecule involved in breast tumor metastasis [55]. Vinculin is used as a general marker of FA, as this protein is known to associate with all types of cell matrix adhesions in a wide range of cell types. Considering the differences in cell migration (section 4.3.3) observed after treatment with the compounds under study and to further understand the mechanisms behind the differences, the influence in the number of FA was evaluated in the two cell lines. The results obtained are depicted in Figure 4.10.

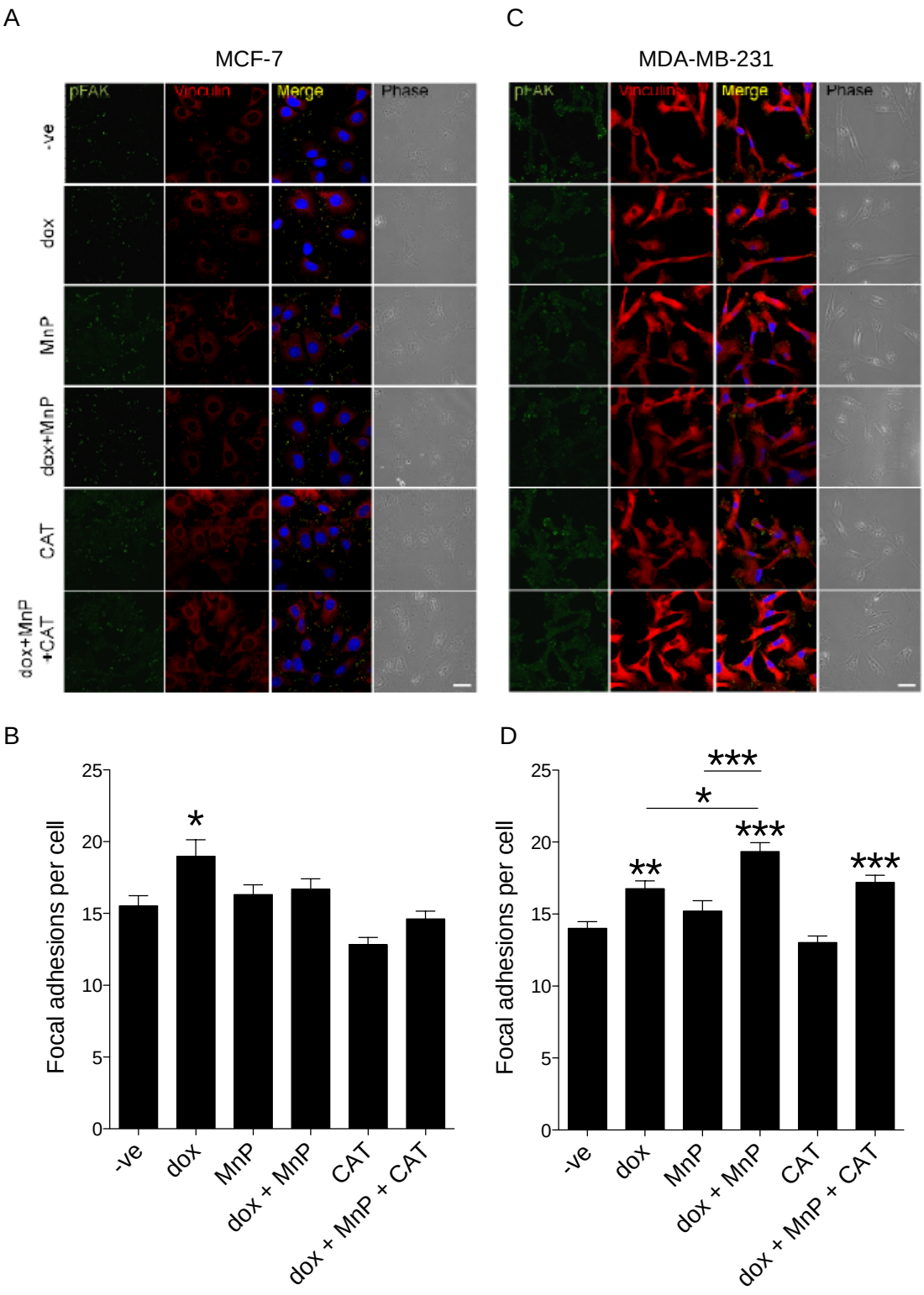

Figure 4.10 – Effect of MnTnHex-2-PyP and dox on the number of FA. Confocal images show MCF-7 (A) and MDA-MB-231 (C) cells treated with the indicated drugs for 16 h, fixed and stained with anti pFAK and anti vinculin. Images are typical of three independent experiments. Scale bars, 20 µm. Summary results (means ± SEM from #90 cells for each condition) show numbers of FA per cell, determined by counting pFAK positive spots (B and D). *p < 0.05, **p < 0.01 (one-way ANOVA with Tukey's test, relative to untreated cells, - ve).

In MCF-7 cells, the exposure to MnTnHex-2-PyP (5 µM) alone or in combination with dox (0.1 µM) did not alter the number of FA (Figure 4.10 A, B). However, when these cells were incubated with dox (0.1 µM) alone it was observed a significant increase in the number of FA per cell (Figure 4.10 A, B: * $p < 0.05$). The exposure of MCF-7 cells to CAT (50 U/mL) alone lead to a decrease in the number of FA although not significant (Figure 4.10 A, B, ns).

For MDA-MB-231 cells it was observed a similar trend. The exposure to MnTnHex-2-PyP or to dox alone, led to an increase in the number of FA more expressive in MDA-MB-231 cells treated with dox alone (Figure 4.10 C, D: ** $p < 0.01$). However in MDA-MB-231 cells and in an opposite way compared with MCF-7 cells, the co-treatment led to a significant increase in the number of FA per cell (Figure 4.10 C, D; *** $p < 0.001$ versus controls), suggesting a possible mechanistic link between the observed changes in cell area (data not shown) and in the number of FA. The increase in the number of FA observed for the co-treatment was also significant when compared with cells treated only with dox (Figure 4.10 D: * $p < 0.05$) or only with MnTnHex-2-PyP (Figure 4.10 D: *** $p < 0.001$).

For MDA-MB-231 cells in co-treatment and in an opposite way compared with MCF-7 cells, the simultaneously exposure to CAT (50 U/mL) leads to a significant increase in the number of FA when compared with controls (Figure 4.14 C, D: *** $p < 0.001$).

While treatment of MCF-7 cells with dox+MnP only increased the number of FA by 7% when compared with control, an increase of 37% was observed in MDA-MB-231 cells. Comparing the impact of this treatment in both cell lines, a significantly difference was clear (*** $p < 0.001$).

4.3.7. Impact of MnTnHex-2-PyP and dox on NF-kB activation

Due to the results obtained regarding intracellular ROS and since ROS and alterations in intracellular levels of H_2O_2 modulate various signaling pathways, such as the ones of the transcription factor NF-kB [56], it was important to evaluate the effects of the compounds under study on the transcriptional activity of NF-κB, which is highly relevant for cell migration and invasion [57-60]. In addition, it is known that a direct interaction between NF-κB and MnPs can occur [1,61,62] which reinforces the importance of this evaluation. Therefore, the NF-κB activation in MCF-7 and MDA-MB-231 cells upon treatment with dox (0.1 µM) and/or MnTnHex-2-PyP (5 µM) was evaluated using a dual luciferase-based gene reporter assay, as described in section 4.2.12. The transfection conditions optimization was performed through an EGFP fluorescence assay, as described in Annex. After the optimization of the technique, the NF-κB activation in MCF-7 and MDA-MB-231 cells upon treatment with dox

and/or MnTnHex-2-PyP was evaluated using a dual luciferase-based gene reporter assay, as described in section 4.2.12. The results obtained for the two cell lines are depicted in Figure 4.11.

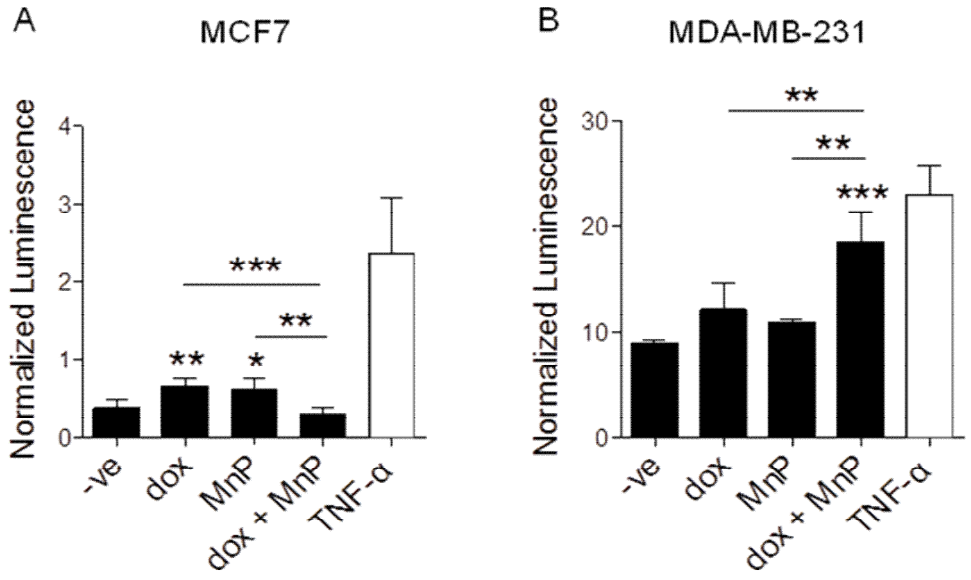

Figure 4.11 – Effect of MnTnHex-2-PyP and dox treatment on NF-kB-dependent transcription. MCF-7 (A) and MDA-MB-231 (B) cells transfected with a firefly luciferase reporter plasmid under the control of an NF-kB-dependent promoter and a *Renilla* luciferase transfection control were treated with the indicated drugs for 16 h. Data are from one experiment representative of at least three, each performed in 5 replicates are presented as means ± SD, *p < 0.05; **p < 0.01; *** p < 0.001 (one-way ANOVA with Tukey's test), compared with untreated cells (-ve).

Results showed that in MCF-7 cells, dox (0.1 μM) and MnTnHex-2-PyP (5 μM) alone increased NF-kB activation by approximately 2-fold, while the co-treatment led to a reduction to levels similar to those of non-treated cells (Figure 4.11 A). In addition, the reduction observed with the co-treatment was significant when compared with the levels of NF-kB activation for MCF-7 cells treated with dox or MnTnHex-2-PyP alone (Figure 4.11 A: *** $p < 0.001$ and ** $p < 0.01$, respectively).

In MDA-MB-231 cells, dox and MnTnHex-2-PyP, *per se*, did not change NF-kB activation. Conversely, the combined treatment led to a significant increase (Figure 4.11 B: *** $p < 0.001$, when compared with non-treated cells). This finding is in accordance with the study of Shah *et al.* [63], who demonstrated that the addition of a MnP to MDA-MB-231 enhanced H_2O_2 levels leading to an increase in NF-kB activity.

4.4. Discussion

SODm are currently being tested in different clinical trials, in combination with chemo- or radiotherapy, due to their capability of boosting anticancer treatments, while protecting non-tumor tissues from ROS-mediated side effects [4,61,62]. However, only very scarce data is available regarding the impact of SODm in cell migration and invasion, which are determinant features of cancer progression and prognosis. Regarding native SOD enzymes, their effects in cancer metastases are still unclear, as aforementioned in Introduction (section 4.1). In many conditions, including the advanced breast cancer, SOD seems to promote cancer progression and aggressiveness [5,6]. This fact raises some concerns on the use of SODm in cancer treatment, justifying the need to comprehend the effects of SODm in cellular processes related to the formation of metastases.

O'Leary *et al.* [64] showed that the SODm GC4419 significantly decreases the invasive capacity of pancreatic ductal adenocarcinoma cells. Tong *et al.* [65] have shown that MnTE-2-PyP (30 μM) reduced the migration and invasion of prostate cancer cells. Recently, an *in vivo* study conducted by Chatterjee *el al.* [66] showed that MnTE-2-PyP did not affect the metastatic progression of PC3 cells in an orthotopic prostate tumor model. On the other hand, in a mouse D-245MG glioma xenograft model, down-regulation of metastatic pathways was observed upon treatment with MnP + radiation vs radiation only [61].

Regarding breast cancer, our group has previously shown that a macrocyclic copper(II) complex with superoxide scavenger activity decreased MCF-7-directed cell migration and, in combination with dox, reduced the invasion of MDA-MB-231 cells [42] (Chapter 3). Shah *et al.* [63] reported that the SODm EUK134 reduced the chemotaxis of MCF-7 and MDA-MB-231 cells. However, these results were observed at cytotoxic concentrations of EUK134. In the same report, MnTM-4-PyP was also studied. This MnP enhanced the chemotaxis of MDA-MB-231 cells, while in MCF-7 only a minor decrease in chemotactic migration was observed [63].

The few available studies are insufficient to draw conclusions on the impact of SODm in breast cancer metastases. Therefore, we herein studied the potential impact of the SODm MnTnHex-2-PyP in cancer cells migration in the breast cancer models MCF-7 (non-invasive) and MDA-MB-231 cells (invasive). The SODm MnTnHex-2-PyP was also studied in combination with dox, a widely used drug in breast cancer chemotherapy, as aforementioned in Introduction (section 4.1). Importantly, these studies were carried out at concentrations that did not impair cell viability. Although the use of low concentrations may lead to less pronounced effects, this is a technical requirement for an accurate evaluation of changes in the migratory phenotype, excluding the influence of cytotoxicity. In fact, regarding cell

proliferation results, MnTnHex-2-PyP had a negligible impact on breast cancer cells treated with dox. Conversely, in a previous work of our group, the SODm MnTnHex-2-PyP protected MCF-10A cells from dox toxicity, in a similar way that was observed for the SODm Cu[15]pyN_5, as aforementioned [17]. This differential effect could be attributed to the differences in basal concentration of ROS and in antioxidant defenses of the two cell lines [17,67-69].

In this work cell cycle distribution upon treatment with dox and MnTnHex-2-PyP was analysed to ensure that under our experimental conditions cells were not undergoing apoptosis or other types of cell death that could indirectly affect migration and invasion results. The low percentage of Sub-G1 cells obtained after exposure to all conditions did not justify the use of Annexin V/PI staining method [70]. Therefore, our results show that not only our compounds but also the doses used in this work, are not significantly inducing apoptosis. Under our experimental conditions, no major overall differences in sub-G1 and G2/M were observed between control and treated cells. These observations suggest that none of the results concerning cell migration and invasion are due to a less viable cell population. Moreover, the concentrations used in this work are biologically relevant. A pharmacokinetic study of this MnP carried out in mice found plasma and tissue concentrations in the order of magnitude of low micromolar [38]. Regarding dox, the concentration used herein is in the range of steady-state plasma concentrations (25-250 nM) observed in patients after standard bolus infusion [40,41].

Due to its superoxide scavenging activity, the catalytic polyfunctional redox-active SODm MnTnHex-2-PyP could increase the intracellular levels of H_2O_2. This increase could be more relevant in the presence of dox, since as a pro-oxidant drug, dox has been shown to induce ROS, providing the superoxide anion [70-72] that could be disproportionate into H_2O_2 by MnTnHex-2-PyP [26,73]. In order to test this hypothesis and since the compounds under study are redox active, the intracellular levels of ROS in the two cell lines, were evaluated by fluorescence microscopy using DHE and DHR probes. Our data regarding DHR probe show that ROS are increased when cells were exposed to dox, MnTnHex-2-PyP or both. The increase observed was more pronounced with both agents, which could be easily explained taking into account the pro-oxidative action of dox. In addition, it was also observed that this increase was more pronounced in MCF-7 cells than in MDA-MB-231 cells, which could be a consequence of the higher peroxide levels found in MDA-MB-231 cells [67]. Indeed, previous reports have demonstrated significant differences in both antioxidant enzymes and in basal ROS for MCF-7 and MDA-MB-231 cells [67-69], as aforementioned in Chapter 3 (Discussion). The differences referred may thus contribute to protecting cells from oxidative stress and, partially, justifying the different effects observed on both cell lines. To understand

whether H_2O_2 contributed to the increase in intracellular ROS observed in co-treatment, the combination of dox, MnTnHex-2-PyP and catalase (CAT) was also studied, since MnPs lacks catalase-like activity [74]. It was observed that CAT presence reduced the accumulation of ROS, being an indicative that H_2O_2 is, at least partially, responsible for the ROS increase observed. In fact, it was observed that in peroxidase-containing cells, H_2O_2 oxidizes DHR [75,76].

In the present work, regarding DHE probe, it was observed that the exposure of both cells to MnTnHex-2-PyP led to a significantly decrease in the fluorescence intensity from DHE oxidation product, being this reduction explained by the dismutation of $O_2^{\bullet -}$ exerted by the MnP MnTnHex-2-PyP. Similar results were also observed in previous work of our group, with the same MnP and with MnTE-2-PyP and MnTM-4-PyP which had reduced the intracellular levels of ROS in V79 cells, to values lower than those presented by control cells [20,26]. Similar results were obtained by other authors with MnTM-4-PyP [25] and with others MnPs [78]. In an opposite way, when both cells were exposed to dox alone it was observed a significant increase in fluorescence, being this increase more pronounced in MCF-7 cells. The increase observed in fluorescence levels may be a consequence of the pro-oxidative action of dox, which contributes to $O_2^{\bullet -}$ generation. In addition, since MDA-MB-231 cells have higher levels and activity of MnSOD than MCF-7 cells [79], the rate of $O_2^{\bullet -}$ dismutation probably is higher in MDA-MB-231 cells and, therefore, the difference between fluorescence levels of non-treated control cells and those exposed to dox alone was not so high in these cells. Regarding the co-treatment of both cells, it was observed a similar increase in fluorescence, but in a lesser extent when compared with the treatment with dox alone. As a consequence of the SOD-like activity of MnTnHex-2-PyP and in combination with dox, resulted in lower fluorescence levels, compared with the levels for the treatment with dox alone, although higher than in non-treated control cells.

Merging the results obtained in our work for both probes in the two cell lines, the intracellular ROS levels observed are compatible with the SOD-like activity of MnP. In fact, the MnP MnTnHex-2-PyP decreased superoxide and increased H_2O_2, which is in accordance with previous studies [63,65]. In addition, cells co-treated with MnP and dox showed increased ROS levels, at least partially due to an increase in H_2O_2. Previous studies have suggested that both $O_2^{\bullet -}$ and H_2O_2 are relevant for the regulation of cell migration [57]. While H_2O_2 seems to be a key signaling molecule in this process, the exact impact of H_2O_2 is still unknown and may vary with the cell type, concentrations, and specific conditions, justifying the contradictory reports found in the literature [80-83].

As different types of cell migration have been described in breast cancer [37,84], our study also addressed different types of this process. Cell migration was evaluated by three

distinct methodologies – the wound healing assay, the chemotaxis assay and the random migration assay. The wound assay evaluates the movement of cells across a horizontal surface, providing insights into collective cell motility. Conversely, the chemotaxis assay evaluates the directed cell motion towards an extracellular gradient of bioactive molecules and is indicative of the motility of individual cells [47]. The random migration assay evaluates individual random cell migration. Despite these inherent differences, the results from both wound healing and chemotaxis assays came to the same finding: the co-treatment significantly decreases MCF-7 cell migration, whereas a minor impact was observed in MDA-MB-231 cells, but more pronounced in the chemotaxis assay results. These differences may be eventually attributed to the basal differences in SOD activity and in H_2O_2 content between the two cell lines. As aforementioned, MDA-MB-231 cells have both higher SOD activity and higher peroxide levels. Therefore, the potential contribution from MnTnHex-2-PyP to the cellular redox state is expectedly to be lower than for MCF-7 cells. In fact, in MCF-7 cells the decrease in cells migration was significantly reverted by CAT, which means that probably H_2O_2 could contribute to the decrease in cell migration observed in this work. Regarding individual cell random migration no significant changes were observed in MCF-7 cells random migration when exposed to MnTnHex-2-PyP dox or both drugs. For MDA-MB-231 cells, dox promoted the random migration of these cells, effect which was significantly reverted by this MnP.

Therefore, MnTnHex-2-PyP when used as a single agent, did not impact on collective, chemotactic or random cell migration. Regarding dox, an increase in random migration was observed for MDA-MB-231 cells. Comparing with non-treated cells, the co-treatment with dox and MnTnHex-2-PyP exhibited beneficial effects by reducing collective cell migration and chemotaxis.

The changes in intracellular ROS and, particularly in H_2O_2 described in the present work may be associated with the differences in migration observed. In both cell lines, the reduction in migration observed in the co-treatment was probably due to the higher level of H_2O_2, as a consequence of the $O_2^{\bullet -}$dismutation by MnTnHex-2-PyP. In a same way, Reineke *et al* [83] showed that treatment of MDA-MB-231 cells with H_2O_2 decreased cell migration by up-regulation of the promyelocytic leukemia protein (PML). These authors demonstrated that PML-dependent changes in integrin β1 expression were responsible for the inhibitory effect of H_2O_2 on cell migration. Similar results were found for MCF-7 cells [83]. However, given the ambiguous role of H_2O_2, other mechanisms could not be disregarded.

Cell invasion is a particular type of cell migration particularly relevant in the metastization process. Cancer invasion requires not only the cell capability of being motile but also to promote the proteolytic degradation of the ECM. Cell invasion results from a combination of events that can be evaluated, partially, using the chemoinvasion assay. These events

include cell viability, adhesion to matrix, directed movement, production of proteases that allow matrix penetration, and adhesion to a new substrate [39]. Matrigel invasion chambers provide an *in vitro* tool to evaluate cell invasiveness. Only MDA-MB-231 cells displayed a significant invasive potential in this invasion assay [85]. Therefore, the chemoinvasion assay was carried out in MDA-MB-231 cells. While the effect of MnTnHex-2-PyP alone was modest, the combination of the MnP with dox significantly reduced cell invasion.

Several mechanisms have been proposed to explain the redox modulation of migration and invasion, but H_2O_2 appears to be the main signaling molecule involved [8]. In addition, changes in intracellular ROS may be associated with the differences in migration and invasion observed and described in the present work. However, previous reports on the role of H_2O_2 on cell migration and invasion show conflicting data [8,83]. Contradictory reports are also found for the role of SOD in metastases formation [6,86,87]. For example, Tanaka *et al* [86] had shown the existence of an inverse relationship between the intracellular levels of Cu,Zn-SOD, cell motility and *in vivo* metastatic potential. In addition, Kogawa *et al* [87] suggested the use of a combination of dox with recombinant human Cu,Zn-SOD as a successful strategy to reduce lung metastasis. The role of MnTnHex-2-PyP on cell migration/invasion could be therefore a consequence of its SOD-like activity. As the effect of SOD and H_2O_2 seems to depend on the cell type and concentration, is really necessary a better understanding of the role of SOD in tumorigenesis [8]. In addition, that knowledge will be also important regarding the development of redox active agents that, by exerting both pro- and anti-oxidative actions, could be useful in cancer chemotherapy [8].

In other way, the effect observed in the chemoinvasion assay could be assigned to the modulation of expression and/or activity of matrix metalloproteinases (MMPs). These proteinases are a family of at least 28 zinc dependent endopeptidases [88] which digest ECM in normal processes such as embryogenesis, reproduction and tissue remodeling, and in disease processes such as arthritis and cancer metastasis [89]. In fact, MMPs are a wide family of proteolytic enzymes secreted by both tumor and microenvironmental cells that are involved in many phases of cancer progression, namely angiogenesis, invasiveness, and metastasis [90]. MMPs can be subdivided into four groups depending on their substrate specificity and domain organizations: interstitial collagenases, gelatinases (type IV collagenases), stromelysins and membrane-type MMPs (MT-MMPs) [85,88].

MMPs are directly linked with invasion and metastasis through complete ECM breakage [91]. The MMPs are multifunctional enzymes and have complicated and sometimes opposing roles in the development of various diseases, such as cancer [90]. In fact, depending on the type of MMP and whether it is from a cancer cell or a stromal cell, some MMPs either promote or suppress cancer progression [89]. It is probable that cancer cells are able to stimulate MMPs production by fibroblasts in a paracrine fashion [88]. Moreover, some MMPs

from both cancer cells and stromal cells have been shown to promote cancer invasion, and MMPs have both cancer-promoting and cancer-suppressing roles [89]. In fact, it is known that many members of the MMP family are involved in tumorigenesis and cancer progression, however the precise function of the various members of MMP family have proven to be much more complex to elucidate than anticipated in the early phase of MMP research [90].

As aforementioned in Chapter 3 (Discussion), different MMPs were previously reported to be expressed by MDA-MB-231 cells, namely MMP-1, -2, -3, -7, -9, -10, and -13 [85]. Some of these MMPs have been identified in the extracellular medium of breast cancer cells in culture, in particular MMP-1, -2 and -9 for MDA-MB-231 cells [85]. Expression of the membrane-type MT1-MMP (MMP-14) was also found in MDA-MB-231 cells [85]. MT1-MMP`s expression level was found correlated with proMMP-2`s activation and also with the presence of lymph node and distant metastases [85]. In what concerns breast cancer, overexpression of several MMPs has been described, being generally associated with breast tumor progression: MMP-2, MMP-9, MMP-11, MMP-13 and MT1-MMP (MMP-14) [85]. MMP-1 is the only MMP capable of degrading all types of mammary gland collagen, playing a key role in the degradation of stromal fibres in several diseases, including breast cancer [91]. MMP-1 levels are increased in a number of cell lines and also in metastatic tumors [92]. Despite the importance of MMP-1, the gelatinases MMP-2 and MMP-9 have been described as “the main orchestrators of ECM remodeling” [91] and MMP-9 seems to have a relevant role in the invasiveness of MDA-MB-231 cells through Matrigel [85]. So, given the important role of several MMPs in the invasion mechanisms, and due to the results observed in the chemoinvasion assay, the activities of the MMP-2 and MMP-9 were evaluated by gelatin zymography for MDA-MB-231 cells. However, our results showed that no significant effects were observed in MMP-2 or MMP-9 activities upon MDA-MB-231 cells exposure to MnTnHex-2-PyP and dox given alone or in combination. This result indicates that probably other(s) MMP(s) that are expressed in MDA-MB-231 cells, as referred earlier, could be responsible for the invasion capability of MDA-MB-231 cells observed in the chemoinvasion assay and whose activity was reduced in MDA-MB-231 cells treated with both MnTnHex-2-PyP and dox. In agreement with this, several reports suggest a more relevant role for MT-MMPs in collagenolysis than for other MMPs [91]. In fact, upregulation of MT1-MMP is associated with poor prognosis in cancer patients however it is not clear how MT1-MMP becomes elevated in tumors. In clinical specimens, in cancer cells located at the invasive front of the tumors, it was observed enhanced expression of MT1-MMP. In addition, it was observed that the reduction of MT1-MMP levels was enough to lessen cancer cell migration and invasion. Further, this reduction in MT1-MMP levels was also sufficient to reduce *in vivo* invasion and angiogenesis [93]. In this way, it is possible that MT1-MMP which is found in

MDA-MB-231 cells, as aforementioned, could be one of the MMP responsible for the invasive capacity of MDA-MB-231 cells observed in the chemoinvasion assay. In addition, there are evidences that MMPs appear to be essential in early tumor progression (local invasion and micrometastasis) but have little impact once metastatic cells are already established [91]. In accordance with those evidences, MMP-3 which is expressed in MDA-MB-231 cells is known to play a crucial role in the EMT of breast tumors [91]. Kim and collaborators [94] suggested that TGF-β1 also induces invasion in premalignant breast cancer cells (MCF-10A), by upregulation of MMP-2 and MMP-9. Subsequent reports also indicated that MMP-2 and MMP-9 are essential in the TGF-β1-increased invasion of MCF-10 cell series in a 3D *in vitro* model [95,96], which reinforces the higher importance of MMP-2 and MMP-9 in early phases of invasion than in established metastatic cells.

More extensive investigation has shown that the role of proteases in the metastatic process is much more complex than initially thought, contributing to this complexity the host who plays a key role too. An early example of this was found when attempting to associate MMP expression with invasion, *in situ* hybridization, showed that most carcinomas did not make their own MMPs, but convinced the local stromal cells (fibroblasts and inflammatory cells) to do so. Therefore, the tumor cells bound host produced MMPs for invasion [39].

As referred earlier, cell invasion is a particular type of cell migration relevant in the metastization process, in particular proteolytic cell invasion. Regarding cell invasion, focalized proteolytic degradation of ECM components in the epithelial or endothelial basement membrane is a critical step in its initiating. In tumor cells, ECM degradation is fulfilled by “ventral actin-rich membrane protrusive structures” named invadopodia [34]. Invadopodia are structures which are elaborated by cancer cells to mediate both cell attachment and remodeling of the ECM [35]. Invadopodia are selectively found in invasive cancer cells, being important for the degradation of the ECM [88]. They are almost exclusive to cancer cells of epithelial origin and are constructed on an actin core surrounded by scaffolding proteins, MMPs and kinases. In fact, the capability of tumor cells to form invadopodia directly correlates with their ability to invade into local stroma and associated vascular components [34]. A variety of invasive tumor cells, including mammary adenocarcinoma, are able to form invadopodia when they are cultured in physiologic substrates [97]. Invadopodia are thought to function in cancer cells migration *in vivo* through the physical barriers of the ECM existent in the tumor microenvironment that is necessary for cancer invasion and metastasis [97]. Invadopodia have two distinct aspects: structural by organizing the cellular actin cytoskeleton to form membrane protrusions and functional by using proteolytic enzyme(s) for ECM degradation [98]. While invadopodia are critical for the initial breach of the basement membrane and exposure to stromal ECM, it is necessary

invadopodia maturation into larger protrusive extensions which enables invasion into the surrounding stroma [88].

Visualization of invadopodia-mediated ECM degradation of cells by fluorescent microscopy using dye-labeled matrix proteins coated into glass coverslips has emerged as the most prevalent technique for evaluating the degree of matrix proteolysis and cellular invasive potential [34]. The procedure described provides the ability to monitor invadopodia activity, with reproducibly and accuracy, as well as for testing anti-invasive compounds on ECM degradation [34]. Therefore and due to the fact that no significant effects were observed in MMP-2 and MMP-9 activities in MDA-MB-231 cells exposed to the compounds, the ability of MDA-MB-231 cells to form invadopodia and degrade matrix and also to access the effect of MnTnHex-2-PyP and dox given alone or in co-treatment in this proteolytic degradation, a fluorescent-gelatin degradation assay was performed. It was observed that the drugs under study reduced proteolytic MDA-MB-231 cell invasion, especially in combination.

It is reported that the matrix degradation activity of invadopodia is mostly mediated by the focal concentration of MT1-MMP at the surface of invadopodia [97]. Indeed, in the breast cancer cell line MDA-MB-231, MT1-MMP was identified as the key “invadopodial enzyme” which is responsible for gelatin matrix degradation [98]. In addition, MT1-MMP is highly expressed in different invasive cancers, promoting cell migration, invasion and metastasis both *in vitro* and *in vivo* [98]. Albrechtsen *et al* [99] reported an association between ADAM12, a disintegrin and metalloproteinase, with MT1-MMP which results in gelatin degradation in human breast cancer cells. The authors also referred the formation of invadopodia clusters containing both ADAM12 and MT1-MMP. Moreover, their results demonstrated that MMP-2 was not the proteinase responsible for the ADAM12-induced gelatin degradation [99]. This fact is also in accordance with the results obtained in our work regarding no existence of effect of compounds in MMP-2 activity, although the drugs under study reduced proteolytic MDA-MB-231 cell invasion, especially in combination. Although both MMP-1 and MMP-2 can modulate breast cancer behavior *in vivo*, silencing both proteases does not affect MDA-MB-231 invasion. In an opposite way, MT1-MMP knockdown strongly inhibits the capability of the breast cancer cells to infiltrate or degrade surrounding breast tissue [51]. In the work referred it is demonstrated that cancer cells have an absolute requirement for the membrane-anchored metalloproteinase MT1-MMP for invasion, and that protease-independent mechanisms of cell migration are only plausible when the collagen network do not have the covalent cross-links characteristic of normal tissues [51]. Therefore, taking in account all of the results abovementioned, probably MT1-MMP and others MMPs could be responsible for the degradation capability of MDA-MB-231 cells observed in the present work. Due to the diversity and complexity of the mechanisms that may be involved,

future work will be needed to unravel exactly what molecular mechanisms are responsible for the effects in cell migration and invasion described in our work.

Although contradictory data can be found in the literature [52,100,101], several reports demonstrate that dox might promote migratory and invasive phenotypes. Regarding breast cancer, Bandyopadhyay *et al.* [102] have described that dox increased cell migration and invasion in breast cancer cells, and it induced lung metastasis of human breast cancer cells. In addition, Niu *et al.* [60] observed a marked increase in MDA-MB-231 cells migration and invasiveness upon treatment with dox. Indeed, these effects might be critical in cells that have been exposed to lower drug concentrations and therefore remain viable after chemotherapy. Importantly, we herein showed that the addition of MnP counteracted most of the dox-induced effects, suggesting potential clinical benefits of combining dox with MnP. In other endpoints, MnP and dox showed a synergistic effect.

Several cellular and extracellular events are involved in ROS production and can both directly or indirectly impact on mechanisms implicated in different types of cell migration, contributing therefore to the invasiveness and poor prognosis of several cancers. As aforementioned in Introduction (section 4.1), these mechanisms include invadopodia formation, MMP activation/expression, focal adhesion dynamics, cell-cell contact, cytoskeleton remodeling, and gene expression regulation [103,104].

Metastatic cancer cells develop altered affinity for ECM, and some of these changes are mediated by altered expression of integrins [105]. Integrins are the principal mediators of cell adhesion to ECM, being responsible for the link between ECM and actin cytoskeleton at cellular structures called focal adhesions (FA) [55,106]. FA represents a dynamic group of proteins in the cell adhesion process with structural and regulatory functions [36].

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic protein tyrosine kinase that is localized to cellular FA or cell contacts with the ECM, which plays a role in the regulation of cell spreading, adhesion, migration, anoikis (survival), proliferation, differentiation and angiogenesis, processes that are all involved in cancer progression [36,54,105,107]. Therefore, FAK can be considered as a “multifunctional regulator” of cell signalling into the tumor microenvironment.

FAK and vinculin are two important proteins of the FA complex [36] and their altered expression and/or distribution is correlated with altered cell adhesion and motility [53,54]. Vinculin is an actin-binding protein, when overexpressed reduces cell migration and its downregulation enhances cell motility [53]. Canonical FAK signaling is associated to FA`s formation and turnover [55]. In various tumors FAK promotes, as abovementioned, cell motility, invasion, survival and proliferation through kinase-dependent and kinase-independent mechanisms [55]. In fact, FAK has intricate roles in tumor invasion, growth and metastasis [55], being another critical signaling molecule involved in breast tumor

metastasis. Overexpression of FAK in a large fraction of breast cancers is discussed in several different studies, being also documented the *fak* gene amplification in both human breast cancer cells and tumor tissues. These studies also reported that in breast cancer specimens, increased FAK expression and activity are frequently associated with poor prognosis and correlate with progression to metastasis [36,54,107]. As changes in cell shape and morphology are mediated by alterations in actin cytoskeleton, results suggest that exposure to MnTnHex-2-PyP may impact such structure [108]. It has been shown that ROS can induce FAK, leading to its membrane localization, an important feature of FAK function, critical for its biological function [109]. Previous studies suggest that H_2O_2 acts as a messenger molecule during growth factor and integrin receptor signalling [110]. These data are in agreement with a central role of H_2O_2 in the cytoskeleton rearrangement that provides cells to contact with ECM proteins and complete cytoskeletal architecture [110]. Therefore, and considering the results obtained in cell area (data not shown), it was important to evaluate the effect of the SODm MnTnHex-2-PyP on FA number. Due to the results referred, considering that the formation of FA ensure points of contact between the cell and the substrate, and knowing that the number of FA increases with cell area [111], it was important to evaluate the effect of the compounds under study in the FA number. Regarding dox, an increase in the number of FA per cell was observed. Our results also showed an increase in the number of FA per cell observed, in MDA-MB-231 cells treated with dox and MnTnHex-2-PyP in combination. This result was in accordance with the increase in cell area observed in this cell line with the co-treatment (data not shown). In addition, the changes observed in H_2O_2 could contribute to the alterations and results mentioned in our work, emphasizing the role of H_2O_2 in cell adhesion dynamics. In fact, as aforementioned, several studies report significant and rapid increases in intracellular ROS, namely intracellular H_2O_2, during cell adhesion to ECM proteins [57,110]. However, how precisely H_2O_2 and $O_2^{\bullet -}$ promote the expression of adhesion molecules is not clear [57]. Chiarugi *et al.* suggest that both integrin signalling modulation, as well as cell adhesion through FA formation by ROS, are mediated, at least partially, by an up-regulated FAK. They propose a model of redox regulation of FA formation in which ROS play a key role in the transduction of the signals engaged by cell adhesion [110]. FAK depletion decreases the abundance of tyrosine-phosphorylated proteins at FA, while simultaneously increases their levels at invadopodia [55]. Regarding the relation of FA and MMPs, there are evidence for a complex with FAK that targets MT1-MMP (MMP-14) to FA and promotes the presentation of MT1-MMP at the membrane surface [55]. MT1-MMP function has also been proposed to require FAK signaling through an alternative pathway involving activation of Krüppel-like factor 8 during *in vivo* metastasis of human breast cancer cell xenografts in mice [55]. These aspects could be correlated with our results regarding gelatin degradation capability of MDA-MB-231 cells and

a possible role of MT1-MMP, as well as with the decreased degradation capability in cells exposed to MnTnHex-2-PyP and dox simultaneously.

In our work the differences observed in cell migration of non-treated cells, compared with those in cells exposed to the co-treatment with dox and MnP, might be partially explained by the alterations detected in cell area (data not shown) and FA number.

ROS-scavenging mechanisms mitigate the expression of many pro-inflammatory genes which are known to be redox-sensitive [56]. NF-kB was the first transcription factor (TF) shown to be redox-regulated [112,113]. NF-kB/Rel is a family of dimeric TF distinguished by the presence of a 300-amino acid region, termed the Rel homology region, which determines much of its function. Classical NF-kB is a heterodimer constituted of a RelA (p65) and p50 subunit. In most cells, NF-kB/Rel proteins are isolated in the cytoplasm in an inactive form by protein-protein interaction with specific IkB inhibitory proteins [114,115]. In resting cells, NF-κB is predominantly found in the cell cytoplasm with an oxidized p50 cysteine 62. Upon activation, NF-κB translocates into the nucleus where p50 cysteine 62 is reduced by APE1/Ref-1, thereby allowing NF-κB DNA binding. APE1/Ref-1 can act as a redox factor by directly reducing p50 and as a redox chaperone by promoting the reduction of p50 by TRX or GSH [10].

NF-kB not only modulates cellular responses to oxidative stress but also controls hundreds of genes involved in regulation of inflammation, immunity, development, apoptosis, cell proliferation, survival, and transformation [56,112-114]. NF-kB misregulation in cells is associated with several diseases, namely autoimmunity, chronic inflammation and cancer [10]. In fact, apart from directly influencing the transcription of genes, NF-kB may increase the expression of other genes involved in cell proliferation, differentiation, apoptosis, invasion, and metastasis [115].

ROS, including the freely diffusible and long-lived H_2O_2 molecule, can act as second-messengers by oxidizing cysteine residues and impact on cell migration-relevant kinases, phosphatases and transcription factors [92,116]. In fact, H_2O_2 has been involved in the regulation of NF-kB and the deregulation of its associated pathways is characteristic of multiple malignancies [56,113]. H_2O_2 cannot be simply defined as a NF-kB inducer but should instead be considered as a fine-tuning modulator of NF-kB activation pathway by other agents [56].

More specifically and as aforementioned, activation of NF-kB pathway protects cancer cells from apoptosis and has been associated to tumor progression and metastasis representing, therefore, an interesting target to suppress carcinogenesis [63,113]. In addition, NF-kB is a redox-regulated TF highly relevant for cell migration and invasion [57-60]. It is known that the direct reactions of MnPs with thiols of TF, such as NF-κB or Keap1/Nrf2, and/or associated to other species, may present important contributions to the

therapeutic efficacy of MnPs. In fact, previous studies showed that MnP can modulate NF-kB activity [61,62] by a direct pro-oxidative effect in their subunits that affects DNA-binding properties [10]. In addition to direct reactions, the indirect impact of MnP on NF-κB by scavenging RS, that otherwise would have signaled its activation, cannot be set aside. In fact, MnP can modulate NF-kB activity also indirectly by increasing H_2O_2 production [63]. The dose-dependence and the exact mechanism behind NF-kB regulation by MnPs are still under investigation. Probably an impact of MnPs on MAPK exists with possible involvement in the cross-talk between NF-κB and Nrf2 [61,62].

Regarding the role of NF-kB in cell adhesion and metastasis described by several studies [117,118], in the present work it was also important to understand the impact of the compounds under study on NF-kB signalling and transcription activity. It is known that MnTnHex-2-PyP is reactive towards redox-active thiols of cellular signaling proteins involved in transcription, such as NF-kB and oxidation of thiols of NF-kB would result in its inactivation. Via such action, MnTnHex-2-PyP would reduce secondary oxidative stress and in turn would indirectly suppress levels of ROS or RNS. This is commonly accepted as anti-oxidative therapeutic effects [1]. Both direct scavenging of ROS/RNS and indirect reduction of their levels in order to affect cellular transcription may be involved in the MnPs effects observed [1,9].

Therefore, in the present work, the NF-κB activation in MCF-7 and MDA-MB-231 cells upon treatment with the compounds under study was evaluated using a dual luciferase-based gene reporter assay. The differential results observed in the two cell lines may be attributed to the inherent differences in peroxide levels and in antioxidant enzymes of MCF-7 and MDA-MB-231 cells [42,67,68]. NF-κB activation has a dual and opposite dependence on oxidative events and the redox state of the cell, since the translocation of NF-κB is favored by oxidative events in the cytosol while binding to DNA requires a reductive environment in the nucleus [56]. Among human breast cancer cell lines, MDA-MB-231 contains a higher level of NF-kB DNA binding activity than MCF-7 [115], since in the MDA-MB-231 cells, NF-kB is constitutively activated, contributing to the aggressive phenotype of these cells [114,115].

Globally, although we have obtained differential results, depending on cell line and migration type, the alterations induced by MnP in dox-treated cells were consistently towards a therapeutically favorable effect on cell phenotype. Therefore, these data contribute to substantiate the usefulness and safety of SODm-based treatments in breast cancer therapy.

4.5. Conclusions

Globally there are some conclusions regarding the study in the present Chapter:

- MnPs are SOD mimics with potential therapeutic applications in cancer
- Treatment with MnP+dox decreased collective cell migration, chemotaxis and invasion
- MnP also reduced the dox-induced increase in random migration of MDA-MB-231 cells
- Combination of MnP with dox revealed therapeutically favorable effects

Overall, the results show that the MnP MnTnHex-2-PyP is a redox modulator capable of decreasing cell motility and invasion in breast cancer cells treated with dox. Importantly, we herein showed that the addition of MnP counteracted most of the dox-induced effects, suggesting potential clinical benefits of combining dox with MnP. In other endpoints, MnP and dox showed a synergistic effect. These data reinforce the interest of this complex in combination with standard chemotherapeutic drugs.

Future studies are needed to assess whether this rational works properly in more complex systems, namely in animal models of metastatic breast cancer.

4.6. References

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Chapter 5

FINAL DISCUSSION, CONCLUDING REMARKS AND FUTURE PROSPECTS

Metastasis is the principal cause of death in cancer patients since 90% of deaths result from metastases formation. Concerning metastatic breast cancer (MBC), it is still a deadly disease, being considered the main cause for treatment failure [1,2] and therefore a major challenge in clinical treatment. The detailed molecular mechanisms driving breast cancer cells migration/invasion and metastasis are not totally understood. Thus, their study continues to be a significant research area, in which this thesis is included. Researchers have identified treatment modalities that occasionally mitigate metastatic tumor growth, namely estrogen antagonists, radiation and chemotherapy; however, no treatment that permanently eradicates metastases exists at present [3].

The role of ROS in cancer migration/invasion and metastasis remains controversial due to the complexity of ROS signaling in this context since their effects on signal transduction depend on the type of ROS, dose, production site, exposure duration and cell type [4]. For instance, while H_2O_2 seems to be a key signaling molecule in these processes, its exact impact is still unknown, somehow explaining the contradictory reports found in the literature [5-7]. In addition, although ROS seem to be relevant for cancer invasiveness and metastasis, there are only few studies in breast cancer focusing on the redox regulation of invasiveness and metastasis, being therefore one of the main goals of this thesis.

Although preclinical and clinical studies have demonstrated a beneficial role for SOD enzymes in many pathological conditions, including cancer, their clinical use has several handicaps (Chapter 1). Therefore, synthetic SOD mimetics (SODm) with drug-like properties have emerged as pharmaceutical candidates, being a promising approach to treat a variety of diseases, including cancer. A very important aspect related to SOD enzymes is their role in cancer invasiveness, which is considered controversial [8-10] since in advanced breast cancers their expression is often up-regulated contributing to metastatic phenotype [11] and, in an opposite way, MnSOD is often down-regulated in early cancer stages [9,12]. Considering this dual effect along with the previous *in vitro* and *in vivo* studies suggesting the potential use of SODm in breast cancer treatment [9], it was pertinent at the beginning of this PhD to explore the impact of SODm on cell processes related to metastasis, one of the main aims of this thesis. Therefore, this thesis explored two classes of SODm, the manganese(III) porphyrins and the copper macrocyclic complexes, to better understand the role of SODm redox modulation in metastasis, mainly in cell migration and invasion processes. Human cell lines representative of breast adenocarcinoma with low aggressiveness (MCF-7) and metastatic breast cancer (MDA-MB-231) were used as useful *in vitro* models for the purpose of this work. In addition, since breast cancer tissue has higher levels of oxidative stress than normal breast tissue, these cell lines vary in their inherent oxidative stress levels and antioxidant defenses, being therefore appropriate models to mimic the *in vivo* situation.

In this thesis, it was studied the MnP MnTnHex-2-PyP, a reference SODm and the Cu(II)-macrocycle complex Cu[15]PyN$_5$, in some determinant events of metastases formation since these SODm were not studied yet in this context as aforementioned in Chapters 1 and 2. In addition, as key redox modulators, SODm can alter the effects of anticancer drugs on cell migration/invasion and, since SODm role in this context is still almost unexplored, this clearly justified our work. SODm could be very important and useful in breast cancer therapy in combination with chemo and radiotherapy, thus increasing the efficacy of such treatments in cancer cells, while mitigate drug side effects and associated toxicity issues (Chapter 1). Therefore, some SODm including MnPs are currently being evaluate in cancer clinical trials in combination with chemo or radiotherapy regimens [8,13-15]. This is the rational of our work, since this thesis evaluated the role of the two SODm alone or in combination with doxorubicin (dox), in the migration/invasion, intracellular ROS levels and other features related to the migratory/invasive phenotype in the two human breast cancer cell lines selected.

The specific choice of these two SODm was based on some relevant aspects. Regarding Cu[15]pyN$_5$, in previous studies of our group it enhanced the anticancer properties of the anticancer drug oxaliplatin in breast cancer cells, while it protected non-tumoral breast cells from its cytotoxicity, suggesting a potential for this Cu(II) complex in combined therapy for breast cancer [16]. Therefore, the effect of Cu[15]pyN$_5$ and/or dox in MCF-7 and MDA-MB-231 cell lines was evaluated in the present study. The choice of studying a MnP was based on the fact that cationic Mn(III)Ps are among the most efficacious SODm and redox-active experimental therapeutics for the treatment of diseases associated with disturbed cellular redox environment (Chapter 1). Several studies have shown remarkable protective effects of MnPs in a variety of oxidative stress models [8,13-15] and several reports attest the *in vivo* therapeutic efficacy of MnPs as SODm [15]. Thus, some MnPs are currently being evaluated in clinical trials to test their efficacy, particularly as radioprotectors of normal tissue during cancer treatment [8,13-15]. Despite all the highlighted aspects, a thorough knowledge on MnPs effects on migration/invasion and invasiveness mechanisms is still lacking. Therefore, in this thesis and to fill this gap, the MnP MnTnHex-2-PyP was selected since it is a promising SODm [13] with a more favorable bioavailability profile, contributing thus to a better therapeutic window [13-15]. In addition, MnPs may be effective when used in combination with cytotoxic treatment such as chemotherapy, radiation and possibly immunotherapy [15].

Considering the model of anticancer drug selected, dox was chosen mainly due to its clinical relevance as a first line anticancer drug in MBC and also for generating ROS, such as $O_2^{\bullet -}$. However, the onset of severe cardiotoxic effects, mostly attributed to oxidative stress is a major limitation of dox clinical use (Chapter 1). Therefore, these aspects justified its use in

combination with the two SODm, since this approach could have differential effects similarly to what was observed in previous work of our group with Cu[15]pyN_5 and oxaliplatin [16], highlighting the potential of this strategy for MBC chemotherapy. In addition, recent data indicates that MnPs have therapeutic potential in cardiac disorders [15] and being so, the combination of SODm and dox could be advantageous in clinical setting by protecting patients from the cardiotoxic effects exerted by dox.

After compounds selection, the next step was to select their concentrations. All studies were carried out at concentrations that did not impaired cell viability (Chapters 3 and 4). Although the use of low concentrations may lead to less pronounced effects, this is a technical requirement that is recommended for an accurate evaluation of changes in the migratory phenotype, excluding the influence of cytotoxicity bias. In fact, as dying cells poorly migrate and invade [17], the use of non-cytotoxic concentrations is a requisite when testing potential inhibitors of migration/invasion. Moreover, the concentrations used in this work were considered biologically relevant [18-20]. Regarding the MnP, a pharmacokinetic study carried out in mice found plasma and tissue concentrations in the order of magnitude of low micromolar [18]. In addition, in previous work of our group, 5 μM of MnTnHex-2-PyP was the lowest concentration that exhibited a maximum reversal of cell viability against the cytotoxicity induced by TBHP in V79 cells [16]. Cu[15]pyN_5 was used at a concentration of 100 μM, since in previous work of our group, it did not showed marked cytotoxicity up to 100 μM in V79 cells [16]. The difference in the SODm concentrations is a consequence of their inherent potencies, being MnP more potent than the macrocyclic Cu(II) complex. In the present work, Cu[15]pyN_5 (100 μM) was not toxic for both cell lines and MnTnHex-2-PyP (5 μM) was not considerably toxic in MCF-7 and, especially in MDA-MB-231 cells. In addition, both SODm did not altered dox cytotoxicity pattern. Conversely, in previous work, both SODm protected human normal-like breast cells from dox toxicity. This differential effect exerted by both SODm could be attribute to differences in basal ROS concentration and antioxidant defenses of these cells (Chapters 3 and 4). For dox, the concentration used (0.1 μM) is in the range of steady-state plasma concentrations (25–250 nM) observed in patients after standard bolus infusion, being thus pharmacologically relevant [19,20].

Considering that SODm are redox-active compounds with $O_2^{\bullet -}$ scavenging activity and that dox generates $O_2^{\bullet -}$, it was logical to evaluate intracellular ROS levels in both cell lines exposed to SODm and/or dox, using DHE e DHR probes (Chapters 3 and 4). Regarding DHR probe, data showed that RS levels increased in both cell types exposed to MnTnHex-2-PyP, Cu[15]pyN_5 or dox. The increase was more pronounce upon co-treatment and partially reverted by CAT indicating that H_2O_2 is, at least, partially responsible for the increase observed. In fact, due to its $O_2^{\bullet -}$ scavenging activity both SODm could increase intracellular levels of H_2O_2, being more relevant in the presence of dox which provides $O_2^{\bullet -}$ anion [21-23],

that in turn, could be disproportionate into H_2O_2 by both SODm [24]. DHE fluorescence was significantly increased in dox-treated cells, being more pronounced for MCF-7 cells. This increase was partially reverted by MnTnHex-2-PyP, suggesting higher $O_2^{\bullet -}$ levels in dox-treated cells, as previously described [25,26]. The intracellular ROS alterations induced by treatments were similar in both cell lines. Merging the results for both probes in the two cell lines, the intracellular ROS levels observed are compatible with SOD-like activity of both SODm. In addition, an important aspect was the demonstration of H_2O_2 involvement.

Cancer cells can use a large number of migration and invasion mechanisms, both individual (mesenchymal and amoeboid) and collective cell migration strategies. In mesenchymal migration, cells adhere to substrate releasing ECM degrading enzymes, such as MMPs. Amoeboid migration occurs when cell adhesion to substrate is weak and cells move by “squeezing” through tissues without proteolytic cleavage of ECM [27-29]. In alternative, cancer cells may expand in multicellular groups that maintain cell-cell interactions, depending on their morphology [27-29]. Interestingly, cancer cells have the ability to switch between different modes of cell migration, since in many tumors both single cells and collectives are simultaneously present. For example, Friedl showed that cells with proteolysis inhibited ceased to move in a ‘‘mesenchymal’’ fashion and adopted amoeboid motion [27], which demonstrates cancer cells “plasticity”. The molecular mechanisms underlying different types of cancer cell migration, as well as the transition between them, the microenvironment factors promoting these transitions and this plasticity, still needs to be further elucidated. Several studies revealed that cancer cells adapt to different environmental conditions assuming different morphologies and migration characteristics to stay motile [30]. Nowadays, it is not well known how cell morphology affects the ability or tendency of cancer cells to migrate individual or collectively. However, cells with amoeboid-like morphology usually tend to migrate individually or in “streams”, while epithelial cells tend to migrate collectively [30]. Epithelial tumors with high or intermediate levels of differentiation, such as breast cancer, commonly use collective cell migration [27,30]. Considering the abovementioned information, it was very important to evaluate the impact of the two SODm in several types of cell migration in the breast cancer cell lines exposed to SODm and/or dox. In fact, the innovative aspects of this work include the evaluation of the impact of Cu[15]pyN_5 and MnTnHex-2-PyP in several types of cell migration in breast cancer cells treated with dox. Therefore, cell migration was evaluated by two distinct methodologies – the wound healing assay (collective cell migration) and the chemotaxis assay (individual cell migration) for both SODm (Chapters 3 and 4). Wound healing assay has also the advantage of mimicking, in some extent, the collective migration of cells *in vivo* [31], thus justifying its selection. For MnTnHex-2-PyP, a random cell migration assay was also performed.

Cu[15]pyN_5 significantly decreased MCF-7 cells directed migration, an effect also observed for co-treatment, although less pronounced. In MDA-MB-231 cells, only slight decreases were observed by treatment with both agents alone or in combination. Likewise, results with MnTnHex-2-PyP were also very promising, since MnTnHex-2-PyP or dox did not considerably changed collective cell migration, however, co-treatment significantly decreased MCF-7 motility and, in a lesser extent, that of MDA-MB-231 cells. To explore if H_2O_2 increased levels were associated with the reduction of cell migration observed upon co-treatment, experiments using CAT were performed with MnTnHex-2-PyP, only in MCF-7 cells, due to the more expressive reduction observed in their cell migration. CAT significantly reverted the inhibitory effect of co-treatment suggesting a role for H_2O_2 in mediating this migration phenotype, which is in agreement with a key role of H_2O_2 in the biological function of MnPs [13-15], being this an important result of the present thesis.

Directed individual cell migration towards an external gradient of FBS, *i.e.*, chemotaxis was studied by the transwell inserts assay, the most widely used for evaluating cells chemotactic migration. Cu[15]pyN_5 decreased significantly MCF-7 cells directed migration, but not that of MDA-MB-231 cells, while co-treatment induced a small reduction in chemotatic migration of both cells. In both cell types, MnTnHex-2-PyP or dox did not alter chemotactic migration of individual cells. However, co-treatment significantly reduced cell migration, being the effect more expressive in MCF-7 cells. Despite the inherent differences, results from both assays came to the same finding.

Summarizing, Cu[15]pyN_5 significantly decreases MCF-7 cell migration, whereas a minor impact was observed in MDA-MB-231 cells. Regarding MnTnHex-2-PyP, co-treatment significantly decreases MCF-7 cell migration, whereas a minor impact was observed in MDA-MB-231 cells but more pronounced in chemotaxis assay results. These differences may be, eventually attributed to the basal differences in SOD activity and H_2O_2 content between the two cell lines, as described before. MDA-MB-231 cells have higher SOD activity and higher peroxide levels, so the potential contribution from both SODm to the cellular redox state of these cells is expectedly lower than for MCF-7 cells. In fact, in MCF-7 cells the decrease in cells migration was significantly reverted by CAT, which means that probably H_2O_2 could contribute to the decrease in cell migration observed in this work. In addition, the changes in intracellular ROS and particularly in H_2O_2 described in this thesis may be associated with the differences observed in migration. In both cell lines, the reduction in migration for co-treatment was probably due to the higher level of H_2O_2, as consequence of the $O_2^{\bullet -}$ dismutation by both SODm. As mentioned in Chapter 4, other authors also observed an inhibitory effect of H_2O_2 on migration of MCF-7 and MDA-MB-231 cells [7]. However, given the ambiguous role of H_2O_2, other mechanisms could not be disregarded.

The third method used was the random migration assay, which evaluates individual random cell migration related to the intrinsic ability of cells to migrate exploring their local environment, being mesenchymal migration the main mode of motility [32]. No significant changes were observed in MCF-7 cells random migration when exposed to MnTnHex-2-PyP, dox or both drugs. For MDA-MB-231 cells, dox promoted their random migration, which was significantly reverted by MnTnHex-2-PyP addition, other important result of this thesis. Summarizing, MnTnHex-2-PyP when used as a single agent, did not impact collective, chemotactic or random cell migration. Regarding dox an increase in random migration was observed for MDA-MB-231 cells that was reduced by MnTnHex-2-PyP. Comparing with non-treated cells, co-treatment with dox and MnTnHex-2-PyP exhibited beneficial effects by reducing collective cell migration and chemotaxis. The combination of our results concerning intracellular ROS levels and breast cancer cells migration was important in order to shed light about the H_2O_2 participation in cell migration and its possible inhibitory effect on breast cancer cells migration. Other very important aspect was that the changes observed in H_2O_2 levels could therefore, also contribute to alterations in cell invasion, being this evaluation a crucial issue of this thesis.

Cell invasion is a specific type of cell migration relevant for metastasis. In this way, collective cell movement could be a primary mechanism for invasion and metastasis by highly differentiated tumors, influenced by tumor microenvironment [27]. However, it is not currently clear if migration mode influences later metastasis [30]. The process by which cohesive groups of cancer cells invade into surrounding stroma is collective invasion, which occurs in a variety of solid tumor types such as human breast tumors [33]. Cancer cell invasion requires not only cells being motile but also their ability to promote ECM proteolytic degradation. Thus, cell invasion is a combination of events such as adhesion to matrix, directed movement, production of proteases for matrix penetration and adhesion to a new substrate, being this process evaluated, partially by the chemoinvasion assay [17]. This *in vitro* assay is widely used to quantify the invasive potential of most cell types and detects migratory activity associated with matrix degradation [17]. Therefore it can be used for the screening of putative invasiveness inhibitors, being thus selected with this purpose in mind in the present work. The cell invasion assay was carried out only with MDA-MB-231 cells, due to the non-invasive phenotype of MCF-7 cells [34]. In accordance, no chemoinvasion was observed for MCF-7 cells under our experimental conditions (data not shown). MnTnHex-2-PyP, Cu[15]pyN_5 and dox individually decreased cell invasion of MDA-MB-231 cells. While the effect of Cu[15]pyN_5 or MnTnHex-2-PyP was modest, the combination of each SODm with dox significantly reduced MDA-MB-231 cells proteolytic invasion, which is a very important finding of the present thesis. As abovementioned, H_2O_2 appears to be the main signaling molecule involved in redox modulation of cell migration/invasion [11]. Thus,

changes in intracellular ROS levels may be associated with the differences in migration and invasion described in our work. However, as mentioned previous reports on the role of H_2O_2 on cell migration/invasion show conflicting data [7,11]. The role of both Cu[15]pyN_5 and MnTnHex-2-PyP on cell migration/invasion could be therefore a consequence of their SOD-like activity. As the effect of SOD and H_2O_2 seems to depend on cell type and concentration, a further understanding of the real role of SOD in tumorigenesis is still needed [11].

ROS can regulate key cellular mechanisms involved in cancer cell migration/invasion including MMPs activation/expression, invadopodia formation, focal adhesions (FA) dynamics, cytoskeleton remodelling and gene expression [4]. Therefore, SODm may impact those cellular mechanisms related to metastasis. Given the important role of several MMPs to invasion mechanisms, in particular MMP-2 and MMP-9 [35] and due to the results observed in the chemoinvasion assay, we hypothesized that co-treatment could reduce cell invasion, in part by modulating MMPs. Thus, the activities of those MMPs were evaluated for MDA-MB-231 cells exposed to MnTnHex-2-PyP and/or dox. However, no significant effects were observed in MMP-2 or in MMP-9 activity, suggesting that other mechanisms should be responsible for the effects observed in cell invasion or, probably, other(s) MMP(s) expressed in MDA-MB-231 cells [35,36] could be responsible for the invasion capability observed, whose proteolytic activity was reduced in cells in co-treatment. In agreement, several reports suggest a more relevant role for MT-MMPs, in particular MT1-MMP than for other MMPs [35]. In addition, there are evidences that MMPs appear to be essential in local invasion and micrometastasis having little impact once metastatic cells are already established [35]. Moreover, MT1-MMP levels reduction appears to be sufficient to decrease cancer cell migration/invasion and to reduce *in vivo* invasion and angiogenesis [37]. Being so, it is possible that MT1-MMP could be one of the MMP responsible for the invasive capacity observed in our work.

Some *in vitro* analysis revealed that ECM degradation is accomplish by membrane structures, the invadopodia [38], that mediates ECM breakdown through MMPs action [39], contributing to cancer cells ability to invade and metastasize. Visualization of invadopodia-mediated ECM degradation by fluorescent microscopy using dye-labeled matrix proteins, coated on the surface of glass coverslips has emerged as the predominant technique for evaluating matrix proteolysis degree and cellular invasive potential [38]. Since no significant effects were observed in MMP-2 and MMP-9 activities in MDA-MB-231 cells exposed to the compounds, a fluorescent-gelatin degradation assay was performed in order to evaluate the effect of SODm and/or dox in invadopodia-mediated ECM degradation. Additionally, this allowed to explore the contribution of ECM proteolytic degradation for the reduction of cell invasion observed, meaning to understand better if MDA-MB-231 cells invasion mechanism was more proteolytic or amoeboid-type. Treatment with MnTnHex-2-PyP or dox decreased

the proteolytic invasion of cells, being the effect much more pronounced upon co-treatment. This result was very important regarding this thesis aims and showed that MDA-MB-231 cells invasion was much more proteolytic than amoeboid-type. Matrix degradation as a result of invadopodia activity is mediated, mainly by focal concentration of MT1-MMP at the surface of invadopodia [40], being this MMP identified as the key “invadopodial enzyme” responsible for gelatin matrix degradation in MDA-MB-231 cells [41]. Also an association between ADAM12 with MT1-MMP, resulting in gelatin degradation in human breast cancer cells has been reported [42]. This is in accordance with our results regarding no effect of compounds in MMP-2 and MMP-9 activity, although the drugs under study reduced proteolytic MDA-MB-231 cell invasion, especially in combination. Therefore, taking into account all the results mentioned, probably MT1-MMP and/or other MMPs could be responsible for the degradation ability of MDA-MB-231 cells observed in our work. However, the molecular mechanisms subjacent to invadopodia recruitment are also still not well understood. Due to the diversity and complexity of the mechanisms that may be involved, future work will be need in order to understand more clearly which molecular mechanisms are responsible for the effects in cell migration/invasion described in our study. Regarding future prospects, it would be interesting to investigate whether MnTnHex-2-PyP and/or dox could be able to reduce MT1-MMP expression and activity in MDA-MB-231 cells.

Metastasis is a multistep process including several cellular changes that affect cytoskeleton dynamics and expression of adhesion molecules [4]. Although the mechanisms that regulate these processes are not well understood, ROS may cause changes in cell adhesion by regulating cell spreading [43,44]. In addition, ROS can also regulate FA dynamics and cytoskeleton remodelling related to cell motility [4]. ROS have shown to induce focal adhesion kinase (FAK) localization in the membrane at FA, being FAK a critical signaling molecule involved in breast tumor metastasis [45]. Results obtained by our group showed that co-treatment with dox and MnTnHex-2-PyP did not impact cell spread (data not shown), but lead to an increased cell area in MDA-MB-231 and MCF-7 cells, reverted by CAT (data not shown), meaning that probably H_2O_2 may be stimulating adhesion in both cell lines. These results are in agreement with those obtained for intracellular ROS for cells in co-treatment. The results point to an influence of MnTnHex-2-PyP in combination with dox in cell area (data not shown) but not in FA dynamics. Due to the results regarding cell area, since the formation of FA ensure points of contact between cell and the substrate and knowing that the number of FA increase with cell area [46], it was also important to investigate if MnTnHex-2-PyP can modulate molecular mechanisms related to FA dynamics. Therefore, the number of FA was evaluated in both cell lines exposed to MnTnHex-2-PyP and/or dox. In MCF-7 cells, the exposure to MnTnHex-2-PyP and/or dox did not alter the number of FA per cell. However, dox treatment led to a significant increase in this number.

For MDA-MB-231 cells it was observed a similar trend for exposure to MnTnHex-2-PyP or dox, being the increase more expressive for cells treated only with dox. However, in MDA-MB-231 cells compared to MCF-7, co-treatment led to a significant increase in the number of FA, being 37% for MDA-MB-231 cells and only 7% for MCF-7. Comparing the impact of co-treatment in both cell lines, a significantly difference was clear, suggesting a possible mechanistic link between the changes observed in cell area (data not shown) and in the number of FA observed in MDA-MB-231 cells in co-treatment. Regarding the relation of FA and MMPs, there is evidence for a complex with FAK that targets MT1-MMP to FA and promotes the presentation of MT1-MMP at the membrane surface [45]. During *in vivo* metastasis of human breast cancer cells xenografts in mice it was proposed that MT1-MMP function requires FAK signaling [45]. These aspects could be related to our results regarding gelatin degradation capability and a possible role of MT1-MMP, as well as with the decreased degradation ability observed in MDA-MB-231 cells exposed to SODm and dox. In addition, in our work the differences observed in cell migration of non-treated cells compared with those in cells exposed to co-treatment, might be partially explained by the alterations detected in cell area (data not shown) and in FA number.

Trying to understand tumor cells invasion mechanisms is challenging, since there are many coordinated molecular changes that occur prior to and during invasion, being many of the cellular phenotypes involved controlled by complex and inter-related signaling networks [30]. The identification of the signaling pathways that control a “metastasis signature” would be an effective way to control distant spread of tumor cells. One important aspect regarding breast cancer is that the molecular characteristics that determine malignant cell behavior remain only partially understood. In addition, it is known that ROS activate several transcriptional factors, namely NF-κB and the NF-κB signaling pathway, which is constitutively activated in aggressive breast cancer cells. In fact, H_2O_2 has been involved in NF-κB regulation and the deregulation of the associated pathways is characteristic of multiple malignancies [47,48]. Apart from directly influencing the transcription of genes, NF-κB may indirectly increase the expression of other genes involved in cell proliferation, apoptosis, invasion and metastasis [49]. As SODm, MnPs undergo intricate interactions with numerous redox sensitive pathways, such as those involving NF-κB, thereby impacting cellular transcriptional activity [14,15]. Due to the results obtained regarding intracellular ROS and since ROS and alterations in intracellular levels of H_2O_2 modulate several signaling pathways, namely those of NF-κB [47], it was important to evaluate the effects of MnTnHex-2-PyP and/or dox on the transcriptional activity of NF-κB, highly relevant for cell migration/invasion [43, 50-52]. Additionally, it is known that NF-κB and MnPs can interact directly [13-15], which reinforces the importance of this evaluation. In MCF-7 cells, dox and MnTnHex-2-PyP alone increased NF-κB activation (2-fold), while co-treatment led to a

reduction to levels similar to those of non-treated cells. In MDA-MB-231 cells, dox and MnTnHex-2-PyP, *per se*, did not change NF-κB activation. Conversely, co-treatment led to a significant increase, which is in accordance to Shah *et al.* [53], who demonstrated that the addition of a MnP to MDA-MB-231 cells enhanced H_2O_2 levels leading to an increase in NF-κB activity. The differential results observed in the two cell lines might be attributed to their inherent differences in peroxide levels and in antioxidant enzymes, as abovementioned. In addition, NF-κB activation has a dual and opposite dependence on oxidative events and also on the redox state of the cell, since NF-κB translocation in the cytosol is favored by oxidative events, while binding to DNA requires a reductive environment in the nucleus [47]. MDA-MB-231 cell line contains a higher level of NF-κB DNA binding activity than MCF-7 [49], since in MDA-MB-231 cells NF-κB is constitutively activated, contributing to these cells aggressive phenotype [49,54]. The role of NF-κB in cellular metabolism is complex, since studies have showed that it may act as both oncogene and tumor suppressor [14,15]. The story with NF-κB is similar to that of MnSOD, as it protects normal cell during pre-carcinogenic stage, becoming oncogenic when cells redox environment gets disturbed and carcinogenesis "kicks in" [14,15]. Previous studies showed that MnP can modulate NF-kB activity [14,15] by a direct pro-oxidative effect in their subunits that affects DNA-binding properties [56]. In addition to direct reactions, the indirect impact of MnP on NF-κB by scavenging RS, that otherwise would have signaled its activation, cannot be set aside. In fact, MnP can modulate NF-κB activity indirectly by increasing H_2O_2 production [53]. Scavenging of RS or indirect reduction of their levels affecting cellular transcription, may be involved in the MnP effects observed in this work [13-15]. Several sets of data including redox proteomics, point to the oxidation of NF-κB to be at the core of MnPs activity. However, the dose-dependence and the exact mechanism behind NF-κB regulation by MnPs are still under investigation [13-15].

Putting together the results obtained in the present thesis, some conclusions may be drawn. Overall, results showed that Cu[15]pyN_5 is a redox modulator capable of decreasing cell motility and invasion in breast cancer cells treated with dox, thus reinforcing the interest of the copper(II) complex in combination with standard chemotherapeutic drugs. In addition, data from our group suggest that the ligand [15]pyN_5, *per se*, displays some antiangiogenic properties in endothelial cells and may lead to the *in situ* formation of the redox-active complex Cu[15]pyN_5. Therefore, combination of Cu[15]pyN_5 with dox may be beneficial in breast cancer treatment as it could help to reduce cancer cell migration and invasion. Regarding the MnP, globally results showed that like Cu[15]pyN_5, MnTnHex-2-PyP is a redox modulator capable of decreasing cell motility and invasion in breast cancer cells treated with dox, since co-treatment decreased collective cell migration, chemotaxis and cell invasion. MnTnHex-2-PyP also reduced dox-induced increase in random migration of MDA-MB-231 cells. Importantly, our work showed that the addition of MnTnHex-2-PyP counteracted most

of the dox-induced effects, while in other endpoints both compounds showed a synergistic effect. As for Cu[15]pyN_5, these data reinforce the interest of this MnP in combination with standard chemotherapeutic drugs, like dox. Although differential effects were observed according to the endpoints analyzed and the cell line overall, this MnP might arrest the progression of breast cancer by different and complementary mechanisms, being the alterations induced by MnTnHex-2-PyP in dox-treated cells consistent with a therapeutically favorable outcome. Therefore, this SODm has potential therapeutic application in cancer.

There are several therapeutic opportunities for SODm in Oncology. SODm can increase the therapeutic index of chemo- and radiotherapy by promoting their efficacy in cancer cells, while counteracting major drug and radiation toxicity issues, protecting normal cells. This is a very important aspect related to the differential effect of both Cu[15]pyN_5 and MnTnHex-2-PyP in breast cancer cells (boosting chemotherapeutic drug efficacy) and in normal breast cells (protective effect). Chemotherapy regimens frequently use high doses to reduce the number of tumor cells, however leading to considerable side effects. Therefore, a therapeutic strategy that allows effective treatment without the side effects of chemotherapy is necessary, which could be achieved with the combination of a SODm with an anticancer drug like dox. Additionally, since both Cu[15]pyN_5 and MnTnHex-2-PyP can improve dox efficacy, this could allow a reduction in the anticancer drug doses, thus contributing for decreasing side effects. Furthermore, the sensitization of cancer cells by SODm can be an advantageous strategy to overcome drug resistance, important especially in metastatic breast cancer disease. These important aspects highlight the therapeutic opportunities of MnPs and Cu(II) complexes with SOD-like activity in Oncology, supporting the possible future application of SODm in clinical setting as chemotherapy sensitizers.

In what concerns future prospects, more studies are needed to assess whether this rational works properly in more complex systems, namely in three-dimensional (3D) breast cancer cultures of the two cell lines, which closely recreate the *in vivo* situation of breast tumors, being particularly useful for investigating signaling pathways in a physiologically relevant context. Co-cultures of breast cancer cells with other cells from the tumor microenvironment (*e.g*. fibroblasts) should also be included in the further set of experiments. In addition, it should also be assessed in animal models of metastatic breast cancer, such as MDA-MB-231 cells xenografts in mice, since studies that simultaneously explore differential effects in the same animal model are still lacking [14,15], which are essential for understanding the intricate interactions of metal-based drugs, like SODm with complex cellular networks of normal and cancer cells/tissues. SODm compounds have already and continue to prove their high therapeutic potential and future research in this field will certainly push it fast towards their clinical use. However, future work is needed to fully comprehend the nature of SODm beneficial effects and in particular of MnPs. It is also very important to

have a better understanding of the mechanisms underlying anticancer effects, namely in what concerns to the redox modulation of transcription factors beyond NF-κB, that may be involved [14,15]. Regarding MnPs, rather than reacting with small reactive species, evidences has pointed to their interactions with other transcription factors, such as, Nrf2 and AP-1 [14,15]. Such interactions seem to be involved in the healing of normal tissue and suppression of tumor growth. Therefore, for future prospects, it should be interesting to investigate if the SODm MnTnHex-2-PyP and/or dox can influence the activity of those transcription factors in MCF-7 and MDA-MB-231 cells. In addition, since it is reasonable to anticipate some type of impact of MnPs on MAPK with possible involvement in the cross-talk between NF-κB and Nrf2 [14,15], the effect of MnP and/or dox in MAPK signaling should also be investigated. Additionally, it was reported that members of the MAPK family (ERK1/2, p38 and JNK) can modulate MMPs activation, thus justifying once more the interest in this investigation. Therefore, these future studies are fundamental to clarify how the cross-talk of those pathways results in suppression of tumor growth and survival of a normal cell/tissue. In fact, understanding the molecular mechanisms related to SODm effects is very important not only in chemotherapy, but also in the diversity of diseases in which SODm may have clinical applications.

Considering the complexity and redundancy of the redox and cell migration pathways, an integrated approach that includes redoxome and *in vivo* tumor redox *status* analysis might constitute a step forward to unravel more the redox basis of cancer cell migration. This additional knowledge could contribute to explore an eventual correlation between redox *status* and metastasis risk, with subsequent potential applications in cancer therapeutics. For future prospects, the influence of the SODm MnTnHex-2-PyP and/or dox in cellular redox *status* should be evaluated in terms of antioxidant enzymes activity, *e.g.*, GPx and glutathione *status*, *e.g.*, total glutathione content (GSHt), its oxidized form (GSSG) and reduced glutathione (GSH). The increasing knowledge of redox mechanisms, along with the availability of optimized SODm, will be decisive for moving SODm into clinic. Regarding optimized SODm, all the assays referred as future prospects should also be performed with the butoxyethyl analog MnTnBuOE-2-PyP, one of the MnPs being evaluated in clinical trials (Chapter 1).

In what concerns cell migration/invasion there are questions that remain to be answered. However, the endpoints evaluated in this thesis were adequate to give more insights on the effects of SODm in cell migration/invasion and invasiveness in the context of breast cancer. Globally, this thesis contributed to clarify the influence of two SODm, MnTnHex-2-PyP and Cu[15]pyN_5, in breast cancer invasiveness and metastasis, thus contributing to novel approaches towards breast cancer treatment. Overall, although differential effects were observed, the alterations induced either by Cu[15]pyN_5 or MnTnHex-2-PyP in dox-treated

cells may be beneficial in breast cancer treatment as it could help to reduce cancer cell migration and invasion, being consistent with a therapeutically favorable outcome. Therefore, these data substantiate the usefulness and safety of SODm-based treatments in breast cancer therapy.

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ANNEX

This Annex contains the theoretical basis of some experimental methodologies used in the work described in Chapter 4, as well as some images or the optimization procedures when they were performed.

1. Cell cycle analysis

Quantitation of DNA content through cell cycle analysis was one of the first applications of flow cytometry. The DNA of several organisms, such as mammalian, plant, yeast or bacterial cells can be stained using a variety of DNA binding dyes, such as propidium iodide (PI), Hoechst 33342, DAPI and 7-aminoactinomycin-D (7-AAD), being the premise of these dyes that they are stoichiometric, which means that they bind proportionally to the amount of DNA in the cell. Therefore, cells that are in S phase will have more DNA than cells in G1. As a consequence, they will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content. Cells in G2, which have twice as much DNA as the cells in G1, will fluoresce twice as bright, than cells in G1 [1]. Therefore, the proportion of cells within each stage of the cell cycle can be determined using a DNA binding dye, like PI (Figure A.1).

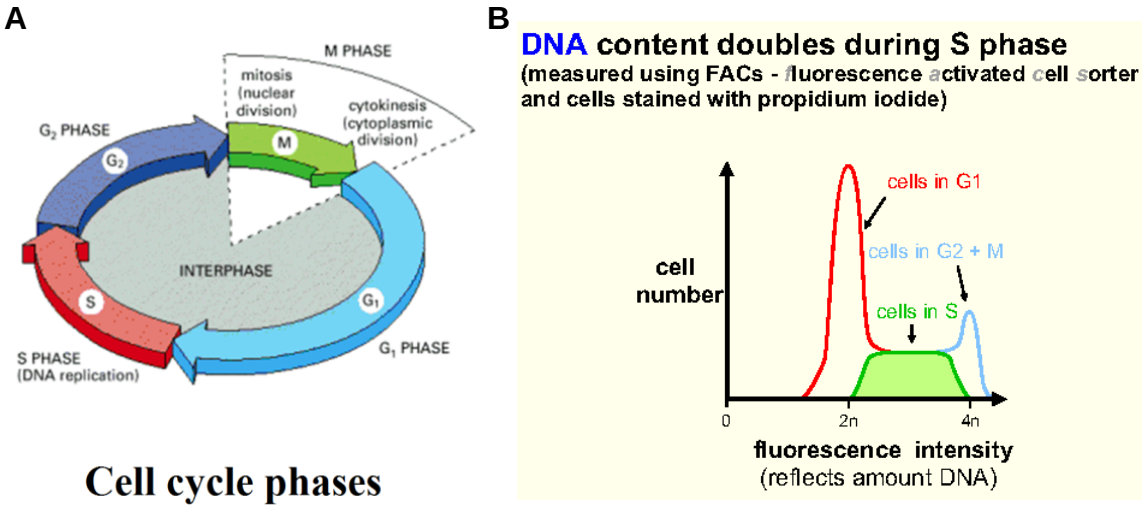

Figure A.1– Cell cycle phases (A). Distribution of DNA levels in a heterogeneous population of cells stained with PI measured using FACS (B) (reproduced from [2]).

In the majority of cases, cells must be first fixed or permeabilized to allow entry of the dye which otherwise is actively pumped out by living cells. For cell fixation, alcohol is commonly used since it is a dehydrating fixative that also permeabilizes cells membrane. For example,

to guarantee good staining, cells should be fixed in cold 70% ethanol, permitting easy access of the dye to DNA and also giving good profiles (low coefficient of variation). After cell fixation, several samples may be stained and analyzed at the end of an experiment since alcohol-fixed cells are stable for several weeks at 4°C.

Cell cycle analysis is usually measured on a linear scale unlike most flow cytometry that uses a logarithmic scale, since the fluorescence differences are usually smaller. Many flow cytometry software programs now offer algorithms which will attempt to fit Gaussian curves to each phase, to estimate with accuracy the cell cycle phases and also to quantitate the percentage of cells in each cell cycle phase [1].

2. *In vitro* wound healing assay

Breast cancer cells migration was initially assessed by the wound healing assay which evaluates collective cell migration. As an example, representative inverted optical microscopy images of MCF-7 cells immediately after the scratch (0 h) and 24 h later (40 x objective) are illustrated in Figure A.2.

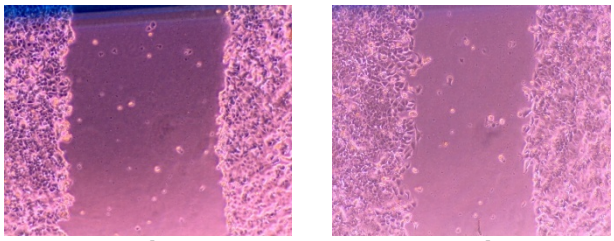

Figure A.2- Representative inverted optical microscopy images of MCF-7 cells immediately after the scratch (0 h) and 24 h later (40 x Objective) (own source).

3. Chemotaxis assay

Chemotaxis was studied by the transwell inserts assay, which evaluated individual directed cell motion towards an external gradient of FBS. As an example, microscopy images in Figure A.3 show representative fields of MDA-MB-231 cells that migrated, after their fixation and staining with CV.

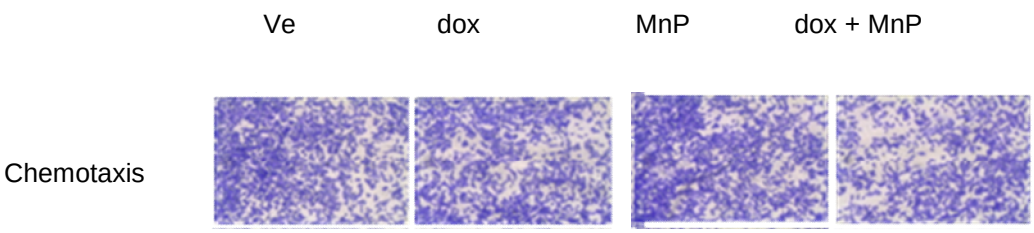

Figure A.3 - Effect of MnTnHex-2-PyP (5 µM), dox (0.1 µM), or both agents (16 h incubation) on the migration of MDA-MB-231 cells, evaluated by chemotaxis using a transwell system with FBS as a chemoattractant. Microscopy images show representative fields of cells that migrated, after their fixation and staining with CV (own source).

4. Random cell migration assay

Random cell migration is related to the intrinsic ability of cells to migrate, frequently called cell motility. Random migration occurs when a cell has low intrinsic directionality, which allows the cells to explore their local environment [3] and differs from directed cell migration, which is characterized by cell migration toward a chemical or physical cue. In fact, migration in the absence of chemoattractant is defined as chemokinesis, or random, background migration [4]. Randomly migrating cells show directional persistence, which means that they are more likely to maintain the movement`s direction that they previously took than to change their direction, even if this direction is randomly chosen in an isotropic environment [5]. There are two main modes of cell motility: one is called mesenchymal which is based on a lamellipodial protrusion that is powered by actin polymerization, and the second mode is called amoeboid that is based on a fast protrusion, termed bleb, which corresponds to a bulge of the cell cortex [5]. The random migration assay describes how to characterize cell motility in the most standard conditions, corresponding to single cells exhibiting random cell migration when plated on a two-dimensional substrate coated with extracellular matrix proteins. The main mode of motility in this situation is mesenchymal migration [5].

One method that allows monitor migrating cells in real time using video is the time lapse microscopy [3]. It sets up time-lapse recording of mammalian cells at low density freely moving on a two-dimensional surface coated with extracellular matrix proteins (Figure A.4) permitting to acquire single cell trajectories from movies and to obtain key parameters that will permit the characterization of cell motility, namely, cell speed, directionality, mean square displacement, and directional persistence [5].

Figure A.4 - Matrigel^{TM}-coated 12-well plates with low density cells (own source).

In order to maintain physiologically adequate conditions for cells, the microscope needs to be equipped with CO_2 supply and also thermostatization. Using a camera attached to the microscope, it is possible to monitor cell movement at regular intervals (Figure A.5). By that way, cell migration can be evaluated by measuring average speed and also average displacement, which is calculated by mathematical software [3].

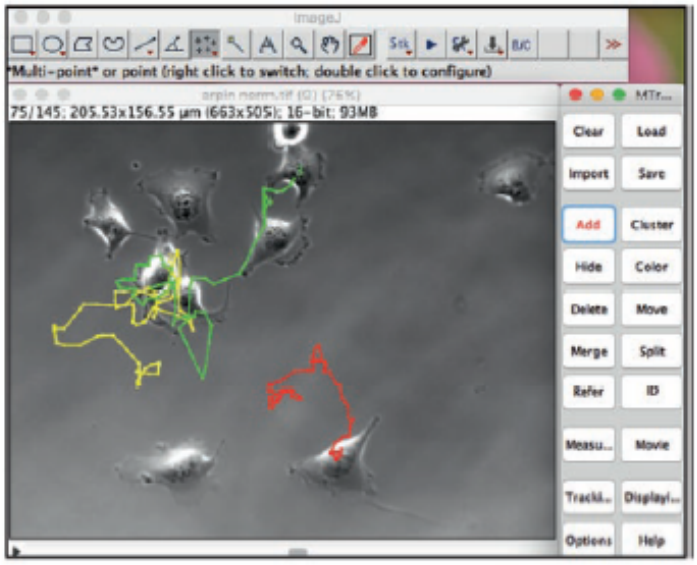

Figure A.5 - Cell tracking and trajectory analysis: single MDA-MB-231 cells were manually tracked using the Manual TrackJ plugin in ImageJ (in red the tracking process was going on) (reproduced from [3]).

An important aspect concerns cell density in the field. If too many cells were in the field, increasing cell-cell interactions would bias random cell motility. In addition, if the tracked cell divided, the tracking must end before cell division, during which cells do not move [5].

5. Gelatin zymography assay

Gelatin zymography is a simple yet powerful *in vitro* assay method based on electrophoretic technique which detects proteolytic enzymes capable of degrading gelatin from various biological sources, separated in polyacrilamide gels under nonreducing conditions [6-9]. After the electrophoresis, the gels were washed, incubated in a reaction buffer and stained with Coomassie blue. This procedure allowed the detection of proteolytic activity at the specific molecular weights of the active MMPs [10], being useful for the assessment of two key MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which have potent gelatin-degrading activity [9].

Before the electrophoresis, the conditioned medium was collected, cleared by centrifugation and concentrated using Vivaspin concentrators (500 µL, 30 kDa cut-off, Sartorius Stedim Biotech, Germany) (Figure A.6).

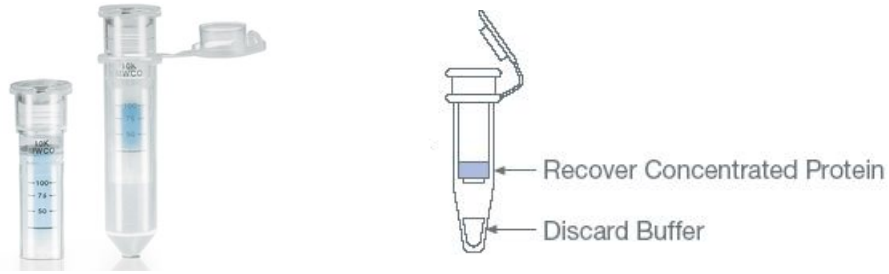

Figure A.6 – Image of a Vivaspin concentrators (500 µL, 30 kDa cut-off, Sartorius Stedim Biotech, Germany) [11].

The concentrated denaturated samples were resolved in a 10% SDS–polyacrylamide gel (PAGE) copolymerized with 0.1% (w/v) gelatine as substrate and prestained molecular weight markers (MW: 10 to 260 kDa; Spectra^{TM} Multicolor Broad Range Protein Ladder, Figure A.7) were also ran with the samples (10 µL).

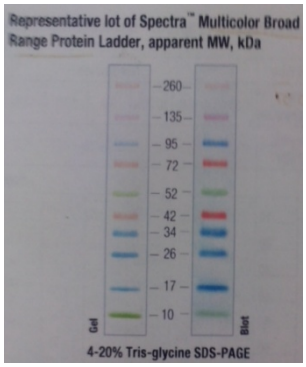

Figure A.7 - Prestained molecular weight markers (MW: 10 to 260 kDa; Spectra^{TM} Multicolor Broad Range Protein Ladder) (own source).

Unstained regions (white bands on the blue background) indicated zones of gelatin digestion corresponding to the presence of different activated MMPs, on the basis of their molecular weight (Figure A.8).

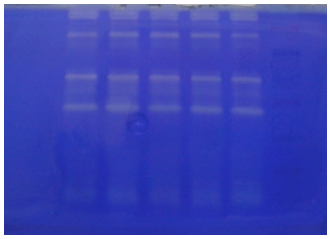

Figure A.8 – Representative image of the stained gel with the white bands on the blue background (zones of gelatin digestion by activated MMP-2 and MMP-9) (own source).

6. Fluorescent-gelatin degradation assay

In the early 1980's, coating fluorescently labeled extracellular proteins on glass coverslips for subsequent microscopic analysis has emerged as the primary technique for evaluating invadopodia function across a wide range of cell types [12]. The protocol followed in this work (section 6.1) refers to the basic methodology used for preparing gelatin-coated coverslips that form a collagenous layer less than 2 μm thick. This methodology allows a rapid production of coated coverslips capable of detecting the initial onset of matrix degradation. This protocol is based on the standard method to generate fluorescently-labeled glass coverslips using a commercially available Oregon Green-488 gelatin conjugate. This method is easily scaled to quickly produce a large number of gelatin-coated coverslips for detecting ECM degradation by cells with fluorescent and confocal microscopes.

The first part of the procedure consisted in the glass coverslips coating (Figure A.9), including the optimization step in order to select the most adequate cover slip coating being this optimization procedure described in section 6.1.

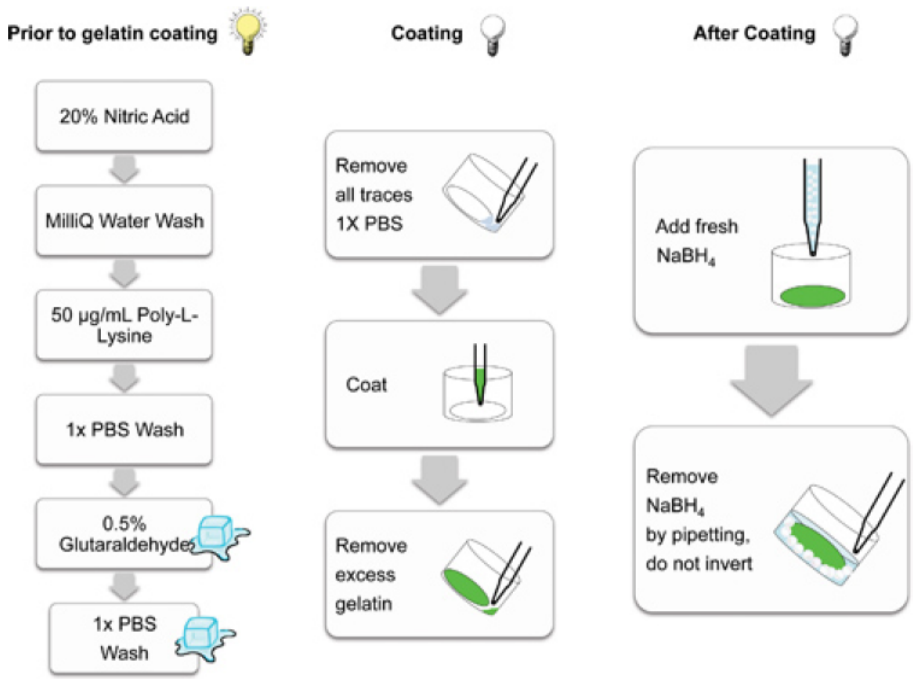

Figure A.9 Schematic demonstration of the individual steps involved in preparing glass coverslips for gelatin matrix coating. Steps conducted in the light (lit bulb), on ice (cubes) and in the dark (non-illuminated bulb) are cartoon indicated (reproduced from [12]).

6.1. Optimization of glass coverslips coating

Glass coverslips (ϕ = 13 mm) were precleaned in a 24-well plate with 20% nitric acid for 30 minutes, followed by nitric acid solution aspiration and extensive washing. Coverslips were coated with 500 µL of Poly-D-lysine solution (50 µg/mL, in dH_2O) for 20 minutes at room temperature (poly-D-lysine coating facilitates even coating and bonding of the overlying labeled gelatin). After that, the poly-D-solution was aspired and the glass coverslips were washed with PBS and cross-linked with 500 µL of ice-cold glutaraldehyde (0.5% in PBS diluted form 25% commercial solution: made fresh before use) for 15 minutes on ice, followed by extensive washing with cold PBS. All traces of PBS were removed prior to gelatin coating and the plate was kept on ice during all washes until gelatin was added. Gelatin from pig skin, Oregon Green 488-conjugate, was ressuspended using the manufacturer`s protocol (final concentration: 1 mg/mL in PBS). Unlabeled 5% (w/w) gelatin/sucrose solution (1:1 ratio) and Oregon Green 488-conjugate gelatin were warm to 37 ºC and Oregon Green gelatin was diluted at a ratio of 8:1 (8 parts of unlabeled 5% gelatin/sucrose solution to 1 part of Oregon Green gelatin), in the dark. After that, 100 µL of Oregon Green gelatin diluted (kept at 37 ºC to prevent premature solidification) was added onto each glass coverslip,

using enough gelatin to coat the coverslip without manual spreading (which can leave to uneven coverslip coating). From this step forward, the glass coverslips were kept in the dark as much as possible to avoid potential photobleaching. Once all glass coverslips were coated, excess of coating was removed (with micropipette), the glass coverslips were incubated for 10 minutes at room temperature, in the dark and then washed three times with PBS. At this point, it was tested the utilization or not of a second glutaraldehyde 0.5% addition to the glass coverslips, 15 minutes at room temperature, followed by washing with PBS. The results of this optimization procedure are presented in Figure A.10.

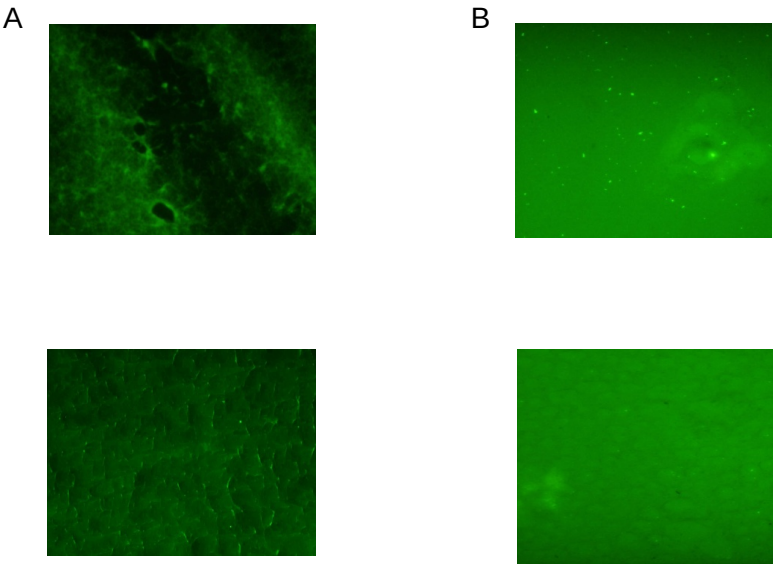

Figure A.10 – Fluorescence microscopy images ("green" channel) of the glass coverslips coated with Oregon Green 488-conjugate gelatin (40x objective) with second glutaraldehyde 0.5% addition (A) or not (B) (own source).

Figures A.10 A and B clear demonstrate that the gelatin coating were much more uniform and homogeneous without the second glutaraldehyde 0.5% addition (Figure A.10 B) being this step eliminated of the glass coverslips coating procedure to perform a gelatin coating as homogeneous as possible (as in Figure A.11).

After washing with PBS, the residual reactive groups in the gelatin matrix were quenched with 500 µL of sodium borohydride $NaBH_4$ (5 mg/mL, in PBS, made fresh before use) for 3 minutes at room temperature in the dark, to reduce and inactivate residual glutaraldehyde ($NaBH_4$ is effervescent and small bubbles were evident on and around each coverslip). The $NaBH_4$ solution around the outside of each well was removed, followed by extensive washing with PBS. At this point, it was important to take care of not picking up any floating glass coverslips that became detached from the bottom of the tissue culture plate during $NaBH_4$ treatment. Detached coverslips that float to the top may be gently pushed back down to the well bottom, but with care in order to avoid damaging the protein coating.

When correctly performed, coverslips are evenly coated with Oregon Green 488-conjugated gelatin, showing homogenous fluorescence when visualized by fluorescence microscopy (Figure A.11).

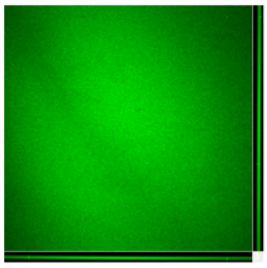

Figure A.11 - Orthogonal view of a confocal z-stack showing the typical color and consistency of an Oregon Green 488-conjugated gelatin coated coverslip produced using this protocol. Coverslips should have a homogenous coating ~1-2 μm thick as shown in the confocal planes (reproduced from [12]).

This protocol utilizes ImageJ to quantify gelatin matrix degradation attributed to individual cells and the matrix degradation area is normalized to either the total area of the cells or the total number of cells in the field. However, if different cell lines having different sized cells are being compared or if the experimental conditions cause cells to change their size, it is more accurate normalizing to cell number. For determining cell numbers in a crowded field, counting nuclei is frequently the method of choice (using DAPI to stain the nuclei).

7. NF-kB gene dual-reporter assay

Actually it is well recognized that genetic reporter systems have contributed a lot to the study of eukaryotic gene expression and regulation, being gene expression evaluation one of the most frequent evaluations performed in molecular and cellular biology. Although reporter genes have played a relevant role in numerous applications, both *in vitro* and *in vivo*, their principal application is as indicators of transcriptional activity in cells through the evaluation of promoter transcriptional activity. This can be achieved evaluating the expression of transiently and/or permanently transfected cloned DNA sequences in an expression vector which contains also the reporter gene attached to a promoter sequence, being this expression vector later transfected into cells [13,14].

Considering the host cell, the reporter gene must not be endogenously expressed in it, to permit developing assays that are quantitative, reproducible, sensitive, rapid, easy and safe. After expression vector`s transfer, cells are assayed for the presence of the reporter protein by detecting inherent characteristics in a direct way, such as enzymatic activity or spectrophotometric characteristics or, in an indirect way, with antibody-based strategies.

Given these various possibilities, in general enzymatic assays are more sensitive since they require only a small quantity of the reporter enzyme to originate reaction products in levels that can be detectable, being frequently necessary to use cell lysates to perform the reaction [13,14].

These reactions give rise to products that emit fluorescence or chemiluminescence where are yielded photons, as a consequence of energy transitions from excited molecular orbitals to lower energy orbitals. However, in both processes the excited orbitals are created in different ways, since in chemiluminescence they result from exothermic chemical reactions, while in fluorescence the excited states result from light absorption [13,14]. Compared to fluorescence, chemiluminescence has an important advantage which is the fact to create the excited states no photons are needed, therefore, they do not constitute an inherent background when measuring photon efflux from a sample. In this way, the low background existent permits measuring very small changes in light with accuracy [15].

Most reporter gene assays involve either one reporter gene or two, in the later case the second reporter is expressed from a "control" vector with the intuit to normalize results of the experimental reporter, *e.g.*, to control transfection efficiency or variation in cell number. Usually, the control reporter gene is trigger by a constitutive promoter being co-transfected with the "experimental" vector. Different reporter genes can be used for control and experimental vectors so that it is possible to assay individually, the relative activities of the two reporter products. Alternatively, it is possible to design dual-reporter bioluminescence assays in which both reporter genes are used as experimental reporters, being this procedure particularly useful for efficiently obtain more information in a single experiment [16].

As luminous organisms were selected through biological evolution by the brightness of their light, the enzymes responsible for this light, the luciferases have evolved to maximize chemical couplings necessary to generate the excited states [13,14]. Bioluminescence is a phenomenon that involves some different chemical processes resulting in light production, which is based on the interaction of luciferase with a luminescent substrate, the luciferin. Bioluminescence is found naturally in several *phyla* including coelenterates, echinoderms, mushrooms and insects. Only a few of these chemical processes have been characterized in detail, two of which, the luciferases from firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, a sea pansy), which represent the majority of the luminescence assays [16] and being both used in the work present in Chapter 4. The luciferase isolated and cloned from the common North American firefly *Photinus pyralis* is one of the most studied enzymes that catalyzes light production in bioluminescent organisms [17], being undoubtedly the most widely used bioluminescent reporter. Firefly luciferase is encoded by the *luc* gene and is a 61

kDa monomeric enzyme that does not require any post-translational modifications, being available as a mature enzyme immediately after release from the ribosome. Firefly luciferase uses luciferin in the presence of O_2, ATP and magnesium to catalyze a two-step reaction, at pH 7.5 to 8.5, in which oxidation of D-luciferin yields light usually in the green to yellow region, typically in the 550–570 nm range with a peak emission at 560 nm [17] (Figure A.12).

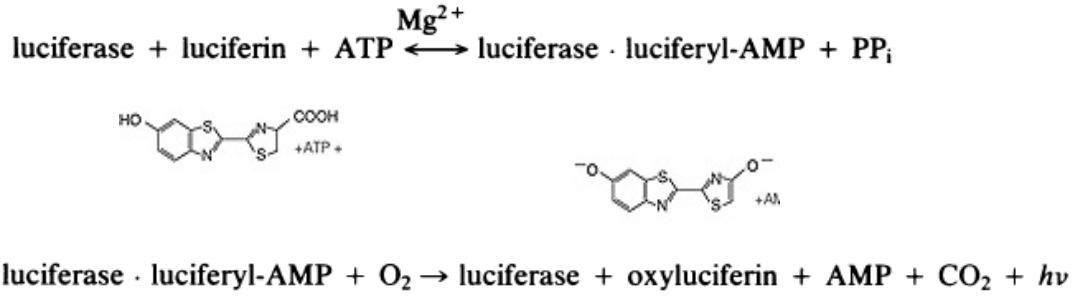

Figure A.12 – Reaction catalized by firefly luciferase in the presence of O_2, ATP and magnesium (adapted from [17]).

The first step consists in luciferin activation with ATP to give an enzyme-bound luciferyl-adenylate and pyrophosphate. Then, in the second step the luciferyl-adenylate reacts with O_2 and undergoes an oxidative decarboxylation resulting in the production of CO_2, AMP and oxyluciferin in an electronically excited state, which then returns to the ground state with simultaneous release of light. When excess of substrates are added to firefly luciferase, the chemical reaction produces a flash of light that is proportional to luciferase quantity in the reaction mixture. After that, light emission decays to 10% of the peak level within 1 min, followed by a long period of low-level light emission, which decays in intensity at a much slower rate [16,17]. In cells and comparing to other commonly used reporters, firefly luciferase has a relatively short half-life [16], however light production triggered by firefly luciferase is a very efficient process, being the quantum yield 0.88 with respect to luciferin [16,17]. Different strategies have been developed to create stable luminescence to make this assay more convenient for routine laboratory use. The most successful ones incorporate coenzyme A (CoA) to yield maximal luminescence intensity, which slowly decays over several minutes. An optimized assay containing CoA generates relatively stable luminescence in less than 0.3 seconds, with linearity over a 100-millionfold range of enzyme concentrations. In addition, the assay sensitivity allows the quantitation of fewer than 10^{-20} moles of enzyme [16]. The popularity of native firefly luciferase as a genetic reporter is due both to sensitivity and commodity of this enzyme assay, as well as due to tight linkage of protein synthesis with enzyme activity. In fact, luciferase assays are rapid, very sensitive, and utilize nonradioactive substrates that are readily available from commercial sources [17].

Renilla luciferase is a monomeric enzyme with 36 kDa that catalyzes coelenterazine oxidation in the presence of O_2, yields coelenteramide and blue light of 480 nm (Figure A.13). The host organism, *Renilla reniformis* (sea pansy) is a bioluminescent marine organisms (a coelenterate), that creates bright green flashes upon tactile stimulation, apparently to ward off potential predators. The interaction between the luciferase and a green fluorescent protein of the coelenterate results in the production of green light [15].

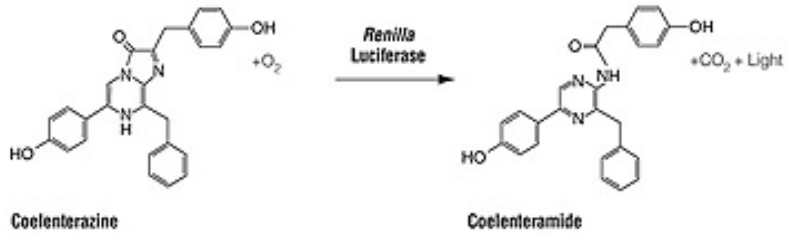

Figure A.13 – Reaction catalized by *Renilla* luciferase in the presence of O_2 (reproduced from [15]).

As a reporter molecule, *Renilla* luciferase which is encoded by the *Rluc* gene, supplies many of the same benefits as firefly luciferase. The simplicity of the *Renilla* luciferase chemistry and, more recently, improvements to the luciferase substrate had allowed quantitation of *Renilla* luciferase from living cells both *in situ* or *in vivo* [15]. *Renilla* luciferase can also be used to generate luminescence, being this technique used in a transient co-transfection system (a reporter construct plus a gene whose effect on certain cell event is intended to be assessed), with the objective to understand how many of the cells expressing the gene were capable to manifest the cellular event under study [18].

The dual-reporter assays most frequently used measure both firefly and *Renilla* luciferase activities. As aforementioned, these luciferases use different substrates and thus can be differentiated by their enzymatic specificities, meaning that they can be used in dual-reporter assays due to their differences in substrate and cofactor requirements and also in light output [15,16]. Most dual-luciferase assays involve adding two reagents to each sample and measuring luminescence after each addition. The addition of the first reagent activates firefly luciferase reaction, while adding the second reagent put out firefly luciferase activity, thus, initiating the second luciferase reaction (*Renilla* luciferase) (Figure A.14). This system demands cell lysis before performing the assay and also requires the use of reagent injectors with multiwell plates [16]. This type of procedure was developed in the work reported in Chapter 4 (section 4.2.12).

In addition to the advantages abovementioned, dual-reporter assays also have the advantage of improving experimental accuracy and efficiency by: i) lowering variability that can obscure meaningful correlations; ii) normalizing interfering events that may be inherent to the experimental system; and iii) normalizing differences in transfection efficiencies

between samples (normalizes well-to-well variations). In addition, dual-reporter assays can reduce the number of non relevant hits, *i.e.*, "false positives" that could result, *e.g.*, from direct interaction between the reporter protein and compounds in high-throughput screenings or due to cell death from compound toxicity [16].

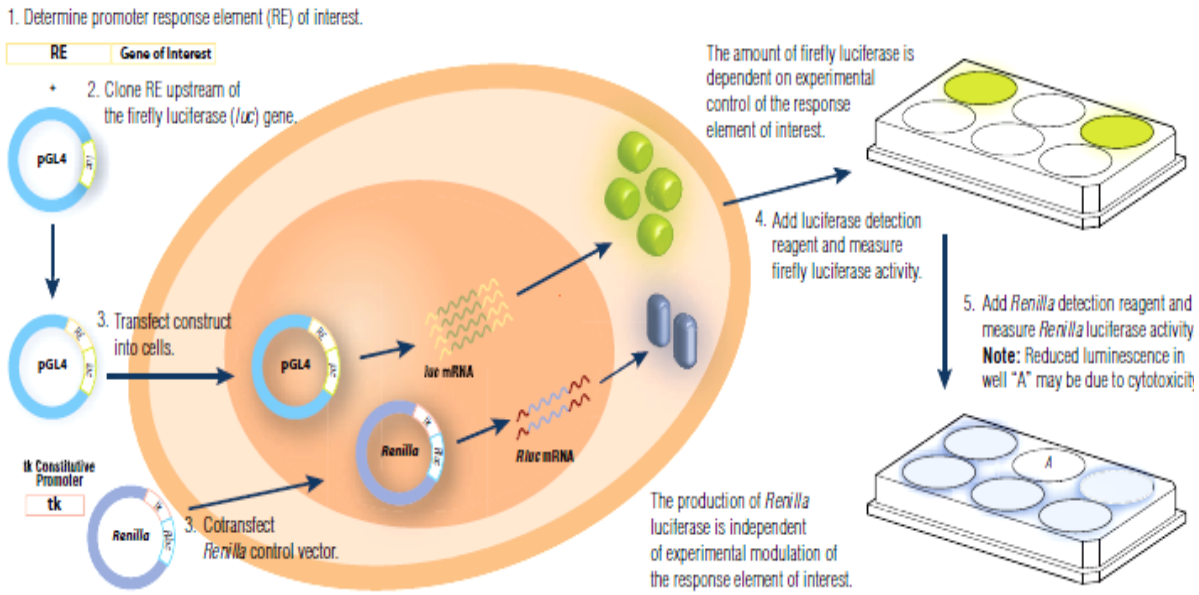

Figure A.14 – Dual luciferase reporter assay measures both firefly and *Renilla* luciferase activities (reproduced from [16]).

7.1. Production of *TK Renilla* and *Firefly* Luciferase-NFkB plasmids

Competent bacteria DH5-α were transformed to produce TK *Renilla* and *Firefly* Luciferase-NFkB plasmids (the plasmid encoding NFkB-firefly luciferase was a gift from R. Hofmeister, University of Regensburg, Germany).

Competent bacteria DH5-α (20 µL) were thawed on ice and 3 µL of each plasmid DNA was added to each eppendorf. The mix was incubated on ice for 30 min and then heat shocked for 30 seconds at 42 ºC. After 5 min incubation on ice, 120 µL of warm LB medium was added and the mix was placed in a shaking bath at 37 ºC for 1 h. Bacterial cells were then spread on LB agar plates containing 100 µg/mL ampicilin and left to incubate overnight (16 h) at 37 ºC. Recombinant colonies were picked and left to grow in liquid medium containing ampicilin, at 37ºC and under constant shaking (200 rpm). Extraction and purification of plasmidic DNA were performed by alkaline lysis with NZY Midiprep (Kit Nzytech Genes & Enzymes) according to the manufacturer's instructions. Plasmidic DNA was quantificated with NanoDrop 2000 Thermo Scientific Spectrophotometer (TK *Renilla*,

214.2 ng/µL, $A_{260}/_{280}$ = 1.91; Luciferase-NFkB, 523.9 ng/µL, $A_{260}/_{280}$ = 1.88), stored at -20 ºC and used to transfect MDA-MB-231 and MCF-7 cells.

7.2. EGFP fluorescence assay – transfection conditions optimization

Optimization of both the technique and appropriate conditions was required for performing the NF-κB gene dual-reporter assay. This optimization procedure can be performed using proteins naturally fluorescents, such as green fluorescent protein (GFP), which can be used to assess gene expression and transfection efficiency. In fact, cells can also readily be transiently or stably transfected with genes encoding fluorescent proteins, such as green or red fluorescent protein (GFP, RFP) from *Renilla* or *Aequoria* or the many improved variants which have brighter fluorescence, or emit wavelengths that broaden both the range of color options and compatibility with fluorescence detection devices [18].

GFP is a 27-kDa monomer protein with 238 amino acids residues that can be obtained from the jellyfish *Aequorea victoria* [19] whose role consists in transducing by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light. The wild type green fluorescent protein (wtGFP) has an absorbance/excitation peak at 395 nm with a minor peak at 475 nm (corresponding to UV to blue light). Regarding the emission wtGFP has a peak at 508 nm and other at 540 nm (corresponding to green light) [20]. Unlike other bioluminescent reporters that require additional proteins, substrates, or cofactors in order to emit light, GFP is inherently fluorescent and its fluorescence is not species-specific. In addition, GFPs do not interfere with cell growth in a wide variety of organisms being, thus, convenient indicators of transformation.

In order to improve one or more characteristics from the wtGFP, various types of mutations have been made. The red-shifted GFPs which have a single excitation peak around 488 nm, rather than the primary excitation and minor peak in the wtGFP are the most widely used GFP variants. However, they have the same emission spectra than wtGFP. Enhanced Green Fluorescent Protein (EGFP) has two mutations in the chromophore region and is reported to have a single, red-shifted excitation peak at 488 nm, presenting fluorescence with higher intensity than wild type GFP, having a peak emission at 509 nm [21]. An important advantage of EGFP application is that it is possible to use the traditional “fluorescein” filter set (485 nm excitation/530 nm emission). Moreover, fluorescence determination using a fluorescence microplate reader also permits to obtain quantitative information, as well as high throughput.

In the work reported in Chapter 4, the optimization of the technique and the determination of appropriate conditions regarding the best cell concentrations and transfection reagent quantities were performed with a DNA plasmid fused to EGFP. In a black 96-well microplate with transparent bottom, four different concentrations of MDA-MB-231 ($1.5x10^4$, $2.0x10^4$, $2.5x10^4$ and $3.0x10^4$ cells/100 µL complete medium) and MCF-7 ($6x10^3$, $8x10^3$, $1x10^4$ and $2x10^4$ cells/100 µL complete medium) were seeded in 10% FBS and antibiotics-free medium so that they would be ~60-70% confluent the next day. 24h after cell seeding, transfection with pEGFP plasmid (100 ng/well) was performed using Lipofectamine® LTX Reagent as transfection reagent, following the manufacturer's recommended instructions. Three different LTX Reagent volumes were tested for both cell lines in the four different cell concentrations: 0,25 µL, 0,35 µL and 0,45 µL. LTX Reagent was diluted in Opti-MEM® medium provided by the manufacturer and pEGFP plasmid was diluted in Opti-MEM® medium, and then was added PLUS™ Reagent. After that the diluted pEGFP plasmid was added to the diluted LTX Reagent (1:1 ratio), the mixture was left to incubate at room temperature for 15 min and then added to the cells drop wise. After that, cells were incubated at 37 ºC. 48h after cell transfection, EGFP fluorescence intensity was measured in cells expressing the gene for EGFP in a fluorescent microplate reader FLUORstar® Omega (BMG Labtech Firmware 1.32, Software 1.20) using an excitation 485 nm/emission 520 nm fluorescent filter. Fluorescent intensity was read using bottom optic readings with orbital averaging of 3 mm.

Measuring the fluorescence emitted by the transfected MCF-7 and MDA-MB-231 cells, the best cells concentrations and transfection LTX reagent quantities were selected. The results obtained are depicted in Tables A.1 and A.2.

Table A.1 – Fluorescence levels for MCF-7 cells transfected with a DNA plasmid fused to EGFP (∅ - cells no transfected)

	LTX 0.25 uL	LTX 0.25 uL	LTX 0.35 uL	LTX 0.35 uL	LTX 0.45 uL	LTX 0.45 uL
	1	2	3	4	5	6
Ø						
($1x10^4$)	145775	145962	152554	141896	143296	141033
($2x10^4$)	139028	141480	158165	151951	162749	164546
($1x10^4$)	142809	144788	164076	160968	164876	158741
($8x10^3$)	128596	132037	155447	156020	157327	151617
($6x10^3$)	138463	136563	152600	149398	156981	151992

Table A.2 – Fluorescence levels for MDA-MB-231 cells transfected with a DNA plasmid fused to EGFP (∅ - cells no transfected)

	LTX 0.25 uL	LTX 0.25 uL	LTX 0.35 uL	LTX 0.35 uL	LTX 0.45 uL	LTX 0.45 uL
	7	8	9	10	11	12
Ø						
($2.5x10^4$)	131774	132401	131997	130478	130307	129788
($3x10^4$)	144106	143576	171789	168686	177172	177735
($2.5x10^4$)	142975	140379	159928	160917	166821	164014
($2x10^4$)	140983	142590	162759	157353	166760	162664
($1.5x10^4$)	138383	141571	150303	155327	152790	156985

The highest fluorescence levels, shown in green, were selected as the best conditions for MCF-7 and MDA-MB-231 cells transfection, which were used in the NF-κB gene dual-reporter assay. For both cell lines, the volume of the LTX transfection reagent selected was 0.45 µL/well and the best cell concentrations were $1.5x10^4$ cells/100 µL complete medium for MCF-7 cell line and $3x10^4$ cells/100 µL complete medium for MDA-MB-231 cell line.

8. References

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