Universidade de Lisboa

Faculdade de Farmácia

Synthesis of Ketone Intermediates for Potential New Antitumor Drugs

Carolina Rego Borges Alves

Trabalho de Campo orientado pelo Professor Doutor Oldrich Farsa, Professor Associado, Universidade de Masaryk e coorientado pelo Professor Doutor Pedro Miguel Pimenta Góis, Professor Auxiliar com Agregação na Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia.

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Abstract

Cancer has been one of the main causes of death in the world since the past century and it is currently one of the major targets when it comes to research in many fields related with science such as Organic Chemistry. Search for new therapies is the main goal of oncology research at the moment, mostly connected with the fact that most of the treatments available have many side effects, although they have become more successful throughout the years.

One group of molecules that has been shown promising in this field are thiosemicarbazones due to their significant biologic activity, this is linked with the fact that they have the ability to chelate essential metal ions. Cancer cells have a particular need of iron which is a key metal for these cells, for their growth and proliferation. Iron chelators like thiosemicarbazones can remove Fe from biological systems and are able to inhibit the activity of Fe-requiring proteins involved in the critical steps of DNA synthesis leading to induced cellular injury. This has dispelled interest in this area of research, that is continuously trying to find more iron chelators that represent a viable option for cancer treatment.

Thiosemicarbazone of the DpT group including di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and its analogue di-2-pyridylketone-4-cyclohexyl-4-methyl-3- thiosemicarbazone (DpC), have been shown to be effective and selective against a wide variety of tumours both in vitro and in vivo.

The aim of this study was to try to synthesise analogues of the di-2-pyridylketone'4,4 dimethyl 3 thiosemicarbazone (Dp44mT) or di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) using 2-aminophenol as a starting material. During this project we focused on synthesising the analogues in the most optimised way possible and in order to potentially synthesise new molecules that would overcome some problems that the original molecules showed. With the aim of these analogues being studied in the future in terms of efficacy and activity.

Keywords: Cancer; Treatment; Iron; Thiosemicarbazones; Analogues;

Resumo

O cancro tem sido uma das principais causas de morte no mundo desde o século passado e é atualmente um dos principais alvos no que concerne a investigação em muitos campos relacionados com a ciência, nomeadamente a Química Orgânica. A procura por novas opções terapêuticas é o principal objetivo da investigação oncológica neste momento, principalmente devido ao facto de a maioria dos tratamentos disponíveis terem muitos efeitos secundários, embora se tenham tornado mais bem sucedidos ao longo dos anos.

Um grupo de moléculas que se tem revelado promissor neste domínio é o das tiossemicarbazonas, devido à sua importante atividade biológica, que está relacionada com o facto de terem a capacidade de quelar iões metálicos essenciais. As células cancerígenas têm uma necessidade particular de ferro, que é um metal essencial para estas células, para o seu crescimento e proliferação. Os agentes quelantes de ferro, como as tiossemicarbazonas, conseguem remover o Fe dos sistemas biológicos e são capazes de inibir a atividade de proteínas que necessitam de Fe e que estão envolvidas nas etapas críticas da síntese de ADN, induzindo lesões celulares. Este facto tem despertado o interesse por esta área de investigação, estando continuamente à procura de encontrar novos agentes quelantes de ferro que representem uma opção viável para o tratamento do cancro.

As tiossemicarbazonas do grupo DpT, incluindo a di-2-piridilcetona-4,4-dimetil-3-tiossemicarbazona (Dp44mT) e o seu análogo di-2-piridilcetona-4-ciclohexil-4-metil-3-tiossemicarbazona (DpC), demonstraram ser eficazes e seletivas contra uma grande variedade de tumores, tanto in vitro como in vivo.

O objetivo deste trabalho foi tentar sintetizar análogos da Dp44mT ou da DpC utilizando o 2-aminofenol como material de partida. Durante este projeto, concentramo-nos em sintetizar os análogos da forma mais otimizada possível e de modo a sintetizar novas potenciais moléculas que ultrapassassem alguns dos problemas já levantados pelas moléculas originais. Sempre com o objetivo de estes análogos serem estudados futuramente em termos de eficácia e atividade.

Palavras-chave: Cancro; Tratamento; Ferro; Tiossemicarbazonas; Análogos;

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First of all, I would like to thank Professor Oldrich Farsa. I would like to thank you for seeing the necessary potential in me and for giving me the opportunity to work under his guidance for 3 months in this area that is so dear to me. Your guidance and mentorship have been fundamental in shaping my understanding of research and have left an indelible mark on my academic and personal growth.

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Carolina Rego Borges Alves

Abbreviation

DNA - Deoxyribonucleic acid

ATP - Adenosine triphosphate

Tfa - transferrin

TfR1- transferrin receptor 1

LCN2 - lipocalin2

LIP - labile iron pool

ROS - Reactive oxygen species

NDRG1 - N-myc down-regulated gene 1

DFO - Deferoxamine

Dp44mT - Di-2-pyridylketone-4,4, -dimethyl-3-thiosemicarbazone

DPC - Di-2- pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone

LMP - lysosomal membrane permeabilization

TNF-α - Tumour necrosis factor alpha

Pgp - P-glycoprotein

MDR - Multidrug resistance

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Index:

Figures Index:

1 Introduction

Cancer is a major cause of death globally, accounting for nearly 10 million deaths in 2020, or nearly one in six deaths. It is a leading cause of death in both less or more economically developed countries. It is a disease that can affect any part of the body and it is caused by the uncontrolled growth of cells with abnormalities that are not eliminated by our immune system. These cells that are not eliminated when they should become tumours which are tissue masses that can be cancerous or not and, in this case, they are referred to as benign. Cancerous tumours can metastasize, that is the process through they migrate to distant parts of the body and invade neighbouring tissues to produce new tumours. Malignant tumours are another name for cancerous tumours. Malignancies of the blood, including leukaemia, rarely become solid tumours although many other types of tumours do. Non-cancerous tumours do not penetrate or spread to neighbouring tissues. Benign tumours often don't come back after removal, however malignant ones can (1,2).

There is no particular cause of cancer. Cancer is a genetic disease that can start in anyone but there are some factors that can make the risk of developing cancer increase such as smoking, exposure to radiation, and a family history of cancer. The best way to prevent it is to eliminate the risk factors that can lead to its development. Nowadays, there are many options for cancer treatment. The treatment you will receive will depend on how advanced is the disease, the type of cancer and where it is located. The most common treatments are radiotherapy and chemotherapy, these can be used together or in separated depending on the type of cancer (3). These treatments destroy the damaged cells by limiting their growth but also damage normal tissues and organs. There are some side effects that appear when healthy cells are destroyed like nausea and hair loss but after chemotherapy is over, they frequently improve or even disappear (4). Despite the development of novel chemotherapeutic agents and technologies for radiotherapy, the side effects of these treatments remain. The scientific community continues to search for new therapeutic options that address the shortcomings of current therapeutics and works towards being more target specific, minimising the damage provoked in healthy cells. The path to do it is to gain a better understanding of the mechanism that cancerous cells use to maintain their activity and to multiply. One group of compounds that have been shown promising in this field are Thiosemicarbazones.

Hamre et al. reported thiosemicarbazone as compounds with anticancer action in 1950. Numerous research has been conducted since 1950 to look into the potential and antitumor effects of these compounds (5).

1.1 The role of Iron in Cancer cells

Iron is an essential element for almost all living organisms especially due to his role in cellular metabolism. Iron is required for the synthesis and repair of DNA, ATP production and oxygen transport. All of these processes mentioned before are very important for cancer cells because of their ability to proliferate and to grow. Iron from the serum Fe-transport protein, transferrin (Tfa) enters the cells using the transferrin receptor 1 (TfR1) that is present on the outside of the cell membrane and that allows iron to be internalised by endocytosis (6). Usually, the intake of iron is a very well controlled process by normal cells, but when it comes to cancer cells it was observed an uncontrolled metabolism because they demonstrated a higher dependence on iron. Cancer cells can obtain more iron because they have an increased expression of TfR1 and a downregulation of ferroportin that exports iron to the outside of the cell. Besides these two receptors, more recently it was found that lipocalin2 (LCN2), a member of the lipocalin protein family, is a secreted glycoprotein that was identified as a novel transport system of iron into cells and that high levels of LCN2 could lead to an increased cell proliferation, angiogenesis, cell invasion, and metastasis (7). There is an iron storage protein called ferritin that is decrease in some cancers, which allowed more iron to be stored inside the cell in the labile iron pool (LIP) that is used for cellular metabolism (8).

After realising the importance that Fe represented in the proliferation of cancer cells, the research for a way to remove iron from these cells became a very important topic.

Iron chelators are molecules that have the ability to remove iron from the biological systems and with this to inhibit the Fe-requiring proteins and activity. They were initially developed to treat Fe overload diseases but due to the iron role in cancer cells more recently they have revealed potential in cancer chemotherapy and it represents a novel avenue of investigation. The anticancer potential of these chelators its due to iron depletion they provoke that leads to inhibition of proliferation through the activity of ribonucleotide reductase, Fe-requiring enzyme, and through formation of redox active

metal complexes with iron that create reactive oxygen species and oxidative stress. The generation of reactive oxygen species (ROS) such as hydroxyl radicals (OH•), induce cellular injury, including DNA Oxidation and mitochondrial damage. In this process iron ions catalyse Fenton reaction of H2O2 and induce excessive ROS in cells, leading to accumulation of phospholipid peroxides, which eventually result in damage of intracellular proteins, lipids and DNA (9,10). The other important aspect is connected with ribonucleotide reductase which is an enzyme that is rate-limiting for the synthesis of all four 2′-deoxyribonucleotides from their 5′-ribonucleotide counterparts that are required as precursors for DNA synthesis and repair. Without iron this enzyme stops the production of 2′-deoxyribonucleotides and DNA synthesis is interrupted leading to cell cycle arrest and apoptosis (11). This process that leads to cellular death trough iron it's called Ferroptosis and it is a non-apoptotic form of programmed cell death which is important in cancer treatment because most of the cancers can already bypass the normal apoptosis process induced by chemotherapy, they are becoming apoptotic resistant (10).

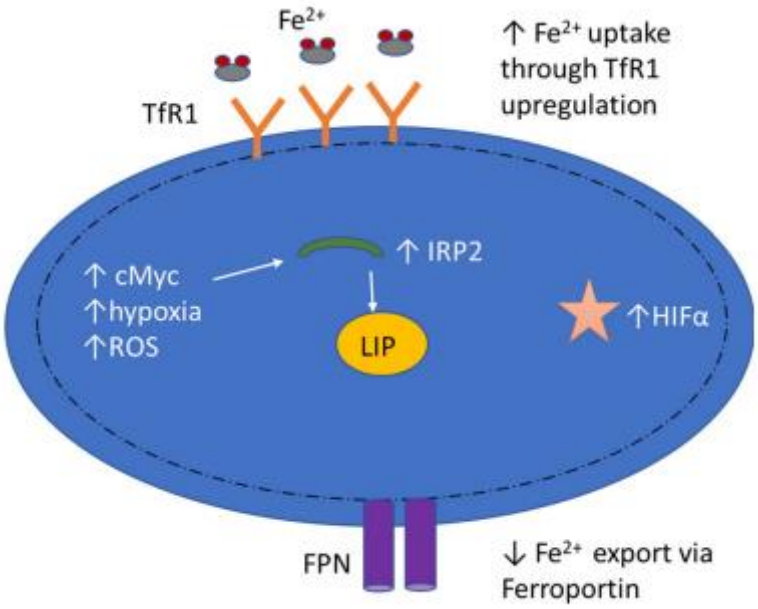


Figure 1. Iron metabolism. Adapted from (9).

1.2 Iron chelators

Iron Chelators were initially designed for the treatment of iron overload diseases, but more recently they have proved to have very interesting antiproliferative properties. This interesting group of molecules have many molecular targets resulting in their impact in terms of mechanisms of action, which consist in iron deprivation, the inhibition of ribonucleotide reductase (Fe-requiring enzyme), cell cycle arrest mediated by decreased expression of cyclin D1 and CDK2, generation of ROS and induction of the expression of the iron regulated metastasis suppressor, N-myc downstream regulated gene-1 (NDRG1), and the tumour suppressor protein, p53 (Fig.4)(11). The iron chelator will be more efficient if it has high selectivity for iron and forms more stable complexes and to achieve this, we need to design an efficient molecule (12). Iron can coordinate six ligands in an octahedral arrangement although chelators with the highest affinity for iron will normally be hexadentate, binding iron in a 1:1 ratio (chelator: iron) (Fig.2)(13).

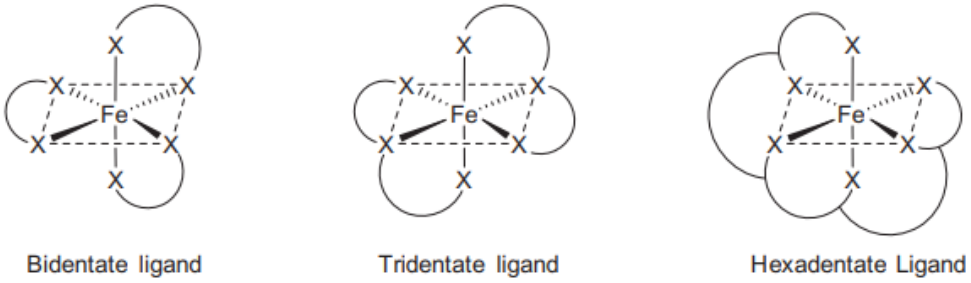


Figure 2. Schematic representation of possible iron chelators. Adapted from (12).

The first iron chelator to be studied was deferoxamine (DFO), which was initially used to treat iron overload diseases such as β-thalassemia (14). As chelators showed some potential the scientific community carried out studies in vivo and in vitro using DFO as the chelator to test the efficacy of these treatments in cancer cells (15,16). DFO proved to be very effective when it came to cancer treatment with the best well-studied tumours being neuroblastoma, in vitro. It was shown that it was effectively used in monotherapy or with other agents (17). Unfortunately, DFO have a rapid metabolism and poor lipophilicity which makes their efficacy compromised because it limits its ability to enter cells and also makes it difficult to be taken orally (12).

After realising that DFO had potential but its structure needed to be improved the search for other iron chelators with other properties capable of having a better antitumor activity began. Several alternative chelating agents were studied using DFO as a base structure, this led to the di-2-pyridylketone thiosemicarbazone the base of the (DpT) family. This group of very interesting molecules are substituted thiosemicarbazones. Two thiosemicarbazones that stood out for their potential activity, it was DpC and Dpm44T.

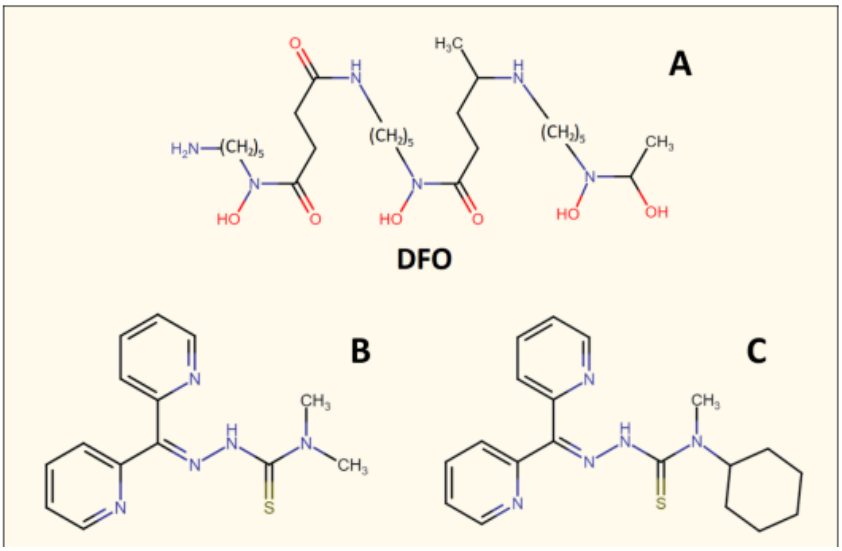


Figure 3. Line drawings of the chemical structures of the metal-binding ligands described herein: (A) deferoxamine, (D.F.O.); (B) di-2-pyridyl ketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), and (C) di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC). Adapted from (8).

It was found that unlike their predecessor DFO, thiosemicarbazone chelators were capable of inducing reactive oxygen species (ROS)(18). When it comes to the chemical properties of iron chelators, they always contain oxygen, nitrogen and sulphur donor atoms that form coordinate bonds with iron. The donor atoms of the ligand affect the preference of the chelator for either the Fe (II) or Fe (III) oxidation states. Chelators that prefer Fe (II) contain ‘soft’ donor atoms, such as nitrogen and sulphur, and, consequently, retain a relatively high affinity for other biologically important divalent metals such as Cu2+ and Zn2+ (13). Both DpC and Dp44mT have chemical properties that make them more lipophilic than DFO, which promotes their membrane

permeability and ability to bind intracellular overcoming the problems associated with DFO.

1.3 DpT Group

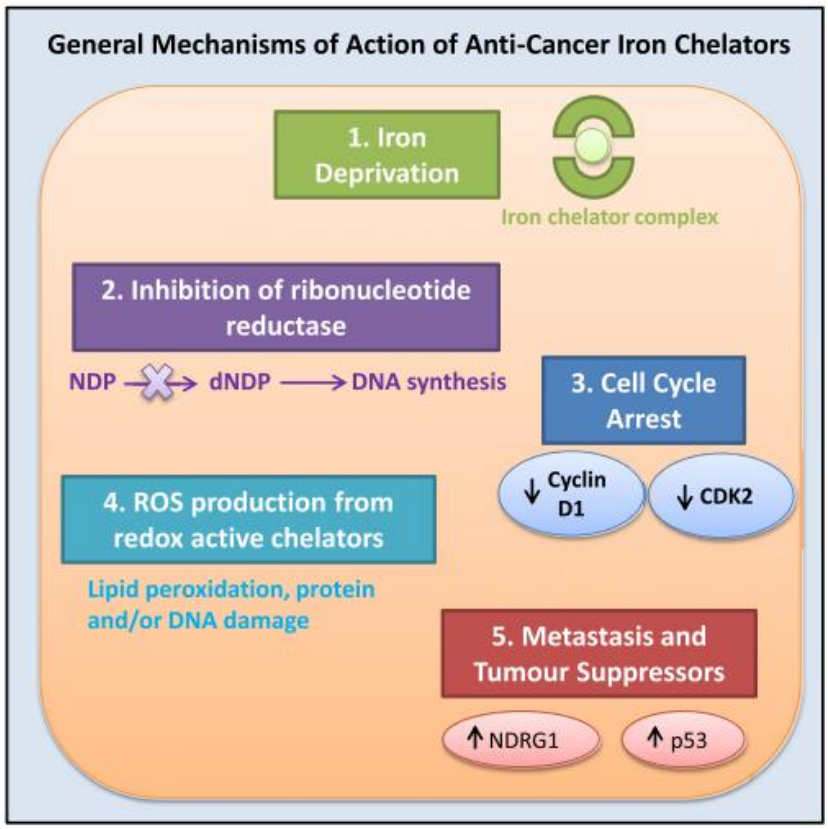


Figure 4. General mechanisms of action of iron chelators that are responsible for their anti-tumour activity. Adapted from (10).

The DpT group of compounds, which includes Dp44mT and DpC, were derived from assessment of the pyridoxal isonicotinoyl hydrazone analogue group of agents. Since the derivation of DpT compounds, numerous types of biological activity, including antiviral, antibacterial, antitumor, anti-leprosy, anti-tubercular and anti-malarial activities, have been reported in multiple studies. In this study we will focus on the antitumor activity (5).

The DpT family is a group of thiosemicarbazones. Thiosemicarbazones are a class of Schiff bases usually obtained by the condensation of thiosemicarbazide with a suitable aldehyde or ketone (18). The N1NH(CS)N4H structure is the key structure, and it is necessary for their biological activity. Due to the presence of N, S, the C=N group and other elements, thiosemicarbazones readily form stable complexes with a variety of metal ions, including iron. The biological activity of thiosemicarbazones was markedly

enhanced following the formation of complexes, particularly in terms of anticancer activity (19).

The first ligand to be developed from the first generation of DpT analogue was Di-2-pyridylketone-4,4, -dimethyl-3-thiosemicarbazone (Dp44mT), this molecule was tested and showed marked up-regulation of the Fe-responsive tumour growth and metastasis suppressor Ndrg1 (N-myc downstream regulated gene-1) (Fig.5)(15). Unfortunately, Dp44mT showed that it is capable of inducing cardiac fibrosis when given at high non optimal doses, it also induced oxyhemoglobin oxidation in vivo and like DFO was not well tolerated orally (20). When the scientific community realised that Dp44mT presented some problems they decided to synthetise a second generation of agents that resulted in the compound, di-2- pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) (Fig.5). DpC exhibits a synergistic effect with chemotherapy on mouse lung tumours and a potential independent antitumor activity (5). DpC overcomes the problems lifted by Dp44mT by being very well tolerated orally, having greater tolerability in vivo, and unlike the last one does not induce cardiac fibrosis or oxyhemoglobin oxidation. Although they have a similar structure and both present a good activity towards cancer cells, DpC demonstrated a higher activity against some tumours and despite the fact that both exhibit adequate pharmacokinetics, DpC showed a significantly longer half-life (t1/2 = 10.7 h for DpC vs. 1.7 h for Dp44mT) emphasising its potential even further (20,21).

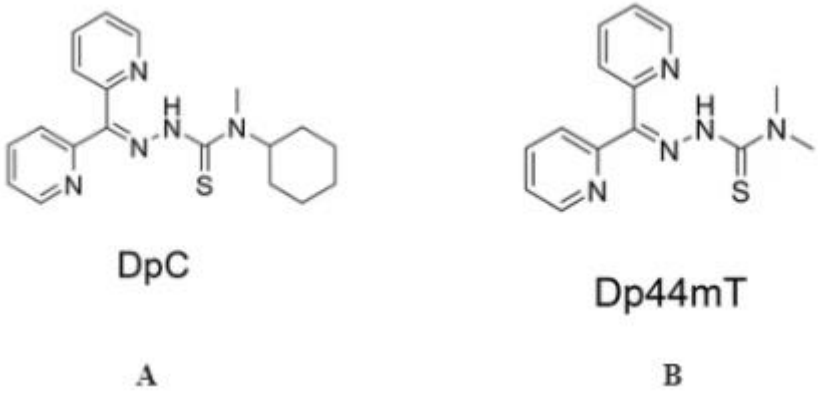


Figure 5. (A) Di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC). (B) Di-2-pyridyl ketone-4,4, -dimethyl-3-thiosemicarbazone (Dp44mT).

These results highlight the potential and comprehensive antitumor activity of the novel Fe chelators. Due to their unique method of function, these chelators also show the ability to overcome resistance to traditional chemotherapeutic drugs.

1.4 Mechanisms of action of DpT family

1.4.1 Chelation of iron and copper within cancer cells, which is critical for proliferation

Iron is an important element in the DNA synthesis and because neoplastic cells are continuously multiplying and growing the demand for iron in these cells is higher, so if there is an intracellular depletion of iron the tumour activity also decreases (22). Cancer cells have, for this need, developed a higher expression of the TfR1 on their membranes allowing them to be able to remove more iron from the bloodstream to use for their proliferation and growth. Besides this, these cells also present a lower quantity of ferritin, an iron storage protein, which allows more iron to reside in the LIP and be used for cellular metabolism. The ability to bind intracellular iron results in inhibitory activity of various molecular targets. But the thiosemicarbazones activity is not just connected to their ability to chelate iron, they also bind to copper depriving cancer cells of this metal that is also essential to angiogenesis (8). Angiogenesis is a process that, in healthy conditions, is controlled by negative and positive angiogenic modulators being strictly regulated. For the cancer cells to grow they need to get proper nutrients and have an effective elimination of metabolic waste that is produced at a very fast rate while cancer cells multiply (23). The deprivation of iron and copper through chelation leads to the activation of various cytotoxic mechanisms inside a cell, for example by generating reactive species (ROS) that induce apoptosis (Fig.6). The key about the thiosemicarbazones from the DpT family is that they have a marked potency and selectivity against tumour cells compared to other iron chelators.

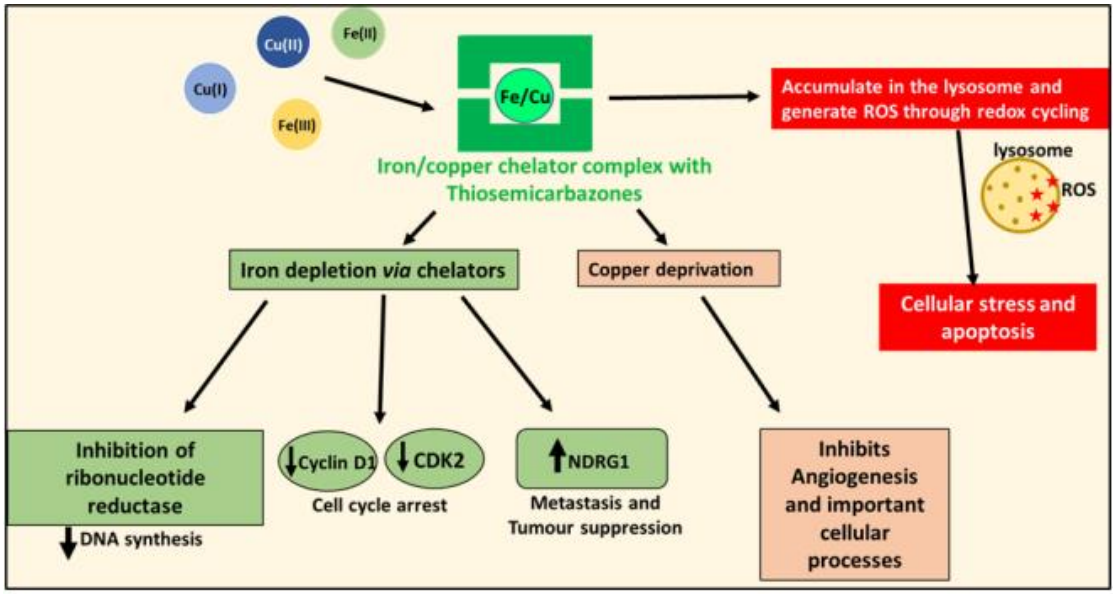


Figure 6. Anti-oncogenic effects of iron and copper-binding ligands. Adapted from (8).

1.4.2 Induction of the expression of the potent metastasis suppressor N-myc downstream regulated gene-1 (NDRG1)

Metastasis is a process in which cancer cells spread from a primary tumour tissue to another being able to grow and multiply in the whole body, becoming the lead cause of death among cancer patients (24). Metastasis is controlled by various factors, one of the most interesting ones is N-myc down-regulated gene 1 (NDRG1), which revealed in several studies that it may have a role in tumour and metastasis suppression. This makes neoplastic cells and tissues to express low levels of NDRG1 in contrast to normal cells. The NDRG1 receptor has various functions in our organism (Fig.7) such as cell growth and differentiation, stress response, which can be up-regulated in situations of elevated homocysteine levels or hypoxia, among others. When it comes to cancer it plays an important role in the promotion or inhibition of carcinogenesis depending on the cell type affected. The tumour suppressor protein, p53, has been reported to induce NDRG1 expression by binding to the NDRG1 promoter but it cannot be the only promoter once it is still expressed even when p53 is not present (25,26).

Iron chelators are related to this receptor because it was proved that NDRG1 is up-regulated in cancer cells after being treated with these molecules (25). Thiosemicarbazones, by chelating iron, have the capacity to mimic hypoxia which leads to the induction of the NDRG1 as a stress response leading to decreased proliferation

and induced apoptosis in both neoplastic and normal cells. It's also curious how the depletion of iron by itself causes cellular stress which once again leads to an up-regulation of NDRG1, via an eIF3a-dependent mechanism. This mechanism is important because the elF3a functions as a translational regulator for several key nucleotide excision repair proteins, and that's why this molecule overexpression in cancer is an important key factor. Because elF3a plays a role in proliferation it has been proved that the depletion of this molecule leads to decreased malignant activity (27).

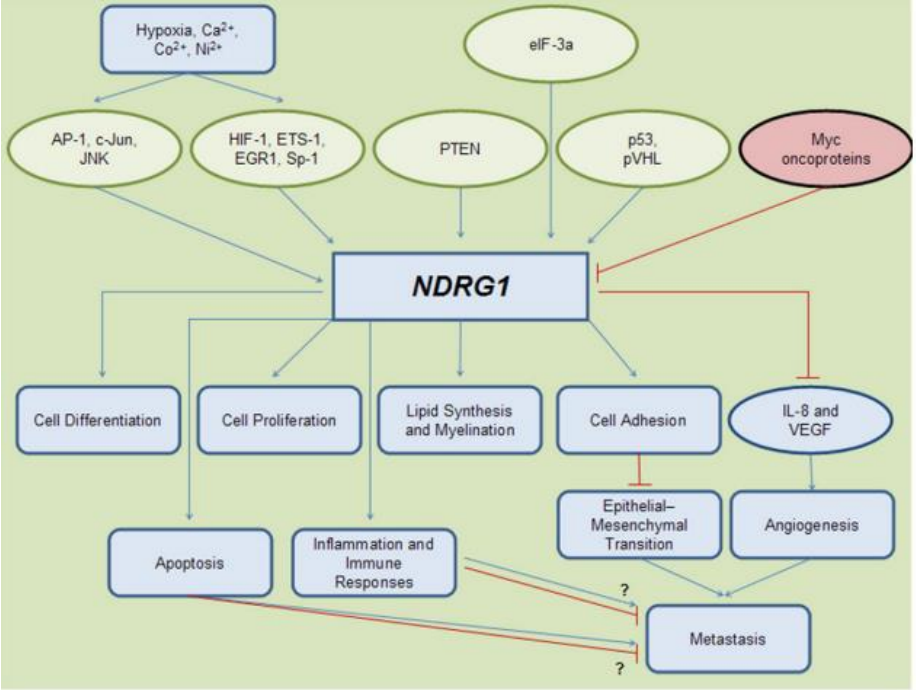


Figure 7. Biological functions and regulation of NDRG1. Adapted from (25).

1.4.3 TNFα expression

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with many functions that are connected with apoptosis, cell survival, inflammation and immunity acting (28). In cancer cells its expression is very low because it leads to cell death. TNF-α has two receptors the TNFR-1 and the TNFR-2 but the only that is considered a death receptor is TNFR-1 which has the ability to induce apoptosis (29). The ability of DpC to increase TNF-α expression in tumours in vivo may contribute to these pro-apoptotic signalling effects shown in vitro, as TNF-α binds to the TNF-α receptor (TNFR) to activate the MAPK/p38/JNK and NF-κB signalling cascades, which lead to nuclear transcription of genes that induce apoptosis (Fig.8). Besides this, DpC and Dp44mT

can also promote the release of caspase 9 from the mitochondria, by forming ROS, which again leads to apoptosis (20).

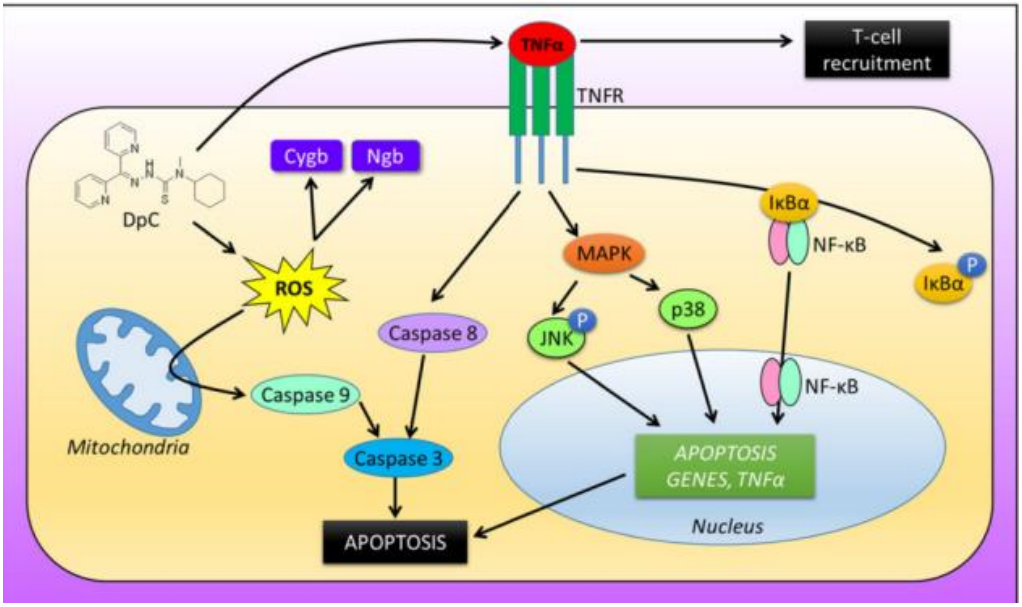


Figure 8. Overview of the potential mechanisms involved in the DpC-mediated effects on neuroblastoma. Adapted from (20).

1.4.4 Inhibition of Autophagy

Apoptosis is the form of programmed cell death in multicellular organisms and it's the main process that leads to cell death in the majority of the organisms. When it comes to cancer cells, they have been developing mechanisms to overcome the cytotoxic stress provoked by anticancer agents and one of them is the up-regulation of autophagy which allows them to gain more resistance to these agents. Autophagy is a process that involves the degradation of cytoplasmic content like proteins and organelle, this gives cancer cells an advantage because they use autophagy as a recycling mechanism of the damaged organelles by the chemotherapeutic agents.

This makes us believe that by inhibiting the autophagy process we would be able to cause cancer cell death. That's when the DpT series enters, especially Dp44mT, their activity consists in their ability to induce lysosomal membrane permeabilization (LMP), which stops the lysosome from fusing with the autophagosome to produce an autolysosome that completes the autophagy (30).

Dp44mt due to its pH and ionization characteristics can enter the lysosomes easily and then be retained there (31). Inside the lysosome, Dp44mT will form cytotoxic iron and copper complexes that will create reactive oxygen species (ROS) that will lead to death of the lysosome by increasing the permeabilization of the membrane and leading to apoptosis (31). This is only possible due to the N and S donors present in the coordination sphere in Dp44mT and DpC, that leads to generation of redox-active metals complexes (20).

After its disruption the lysosome cannot be merged with autophagosome so the autolysosome it's never formed. Studies also revealed that Dp44mT was not only capable of inducing autophagy but also reducing the degradation of autophagosomes that were continuously being formed, being able to overcome the pro-survival process of cancer cells converting autophagy in a cytotoxic mechanism (31).

1.5 The role of thiosemicarbazones in Multidrug resistance

Multidrug resistance (MDR) is a major obstacle in cancer treatment, it decreases the effect of chemotherapy and the scientific community continues to try to solve this issue but it has not been easy. One family of compounds that is under investigation as a future hypothesis for the solution of this problem are thiosemicarbazones from the DpT family.

One of the most well studied resistance mechanisms in cancer cells is the cellular efflux of chemotherapeutic agents, including vinblastine or doxorubicin, using "drug pumps" such as P-glycoprotein (Pgp)(32). When chemotherapeutic agents enter the lysosome due to Pgp they become trapped inside this organelle and cannot distribute to the most important targets like the nucleus leading to resistance to treatments (33).

Dp44mT ability to overcome MDR is also connected with Pgp, present in the lysosome membrane, that allows Dp44mT to be transported inside of the lysosome and also being trapped. When inside of the lysosome, in contrast to vinblastine and doxorubicin, it will form ROS that will lead to LMP and apoptosis (Fig.9). This characteristic is distinct, not present in the existing Pgp substrate chemotherapeutics, and depends on the redox activity of the Dp44mT-Cu complex in Pgp expressing cells.

The fact that cancerous cells have enhanced metabolism of metals, including copper, explain why thiosemicarbazones have a higher selectivity for cancer cells when compared to normal cells (15).

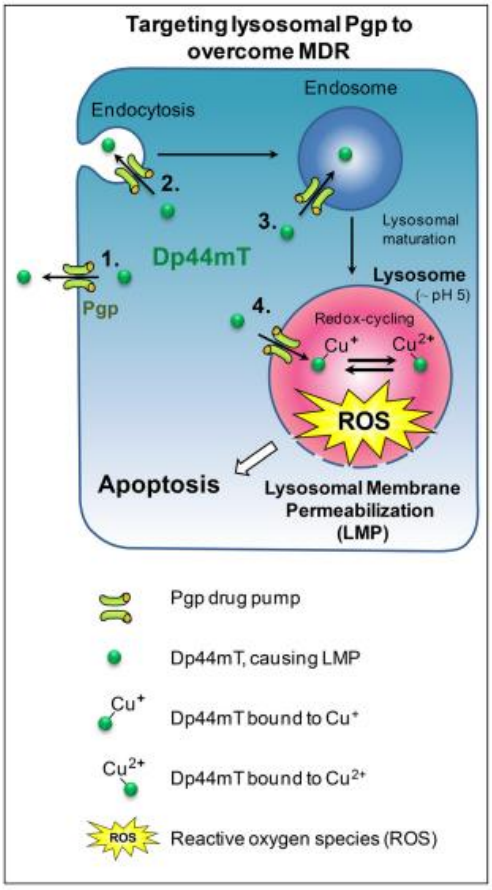


Figure 9. Schematic diagram of the mechanism of Pgp-mediated cytotoxicity by Dp44mT. Adapted from (32).

1.6 Dp44mt and DpC

Both Dp44mt and DpC stood out inside the DpT family and both of them showed a pronounced activity against cancer cells. These two thiosemicarbazones were both found to have a higher selectivity to cancer cells, this happens because of 3 main reasons. One is the amount of iron and copper that these cells need compared to normal ones which makes these cells more sensitive to chelation by these molecules. Secondly, the significant changes observed in lysosomes and autophagy in cancer cells create a dependency on lysosomal function and make them vulnerable to lysosomal targeting. Thirdly, it has been demonstrated that Pgp is located not only in the plasma membrane

for efflux of cytotoxic agents, but also in lysosomes. All these factors make these molecules good targets to study more deeply (32).

The first analogue to be formed was Dp44mT and the second-generation analogues resulted in DpC which turned out to be more orally effective than Dp44mT and having better tolerability in vivo (15). Because almost all anti-cancer therapies are given in combination, it was studied the possibility of Dp44mT and DpC being given along other standard drugs like Tamoxifen and they resulted in synergism. It was also studied the possibility of using both Dp44mT and DpC at the same time which demonstrated a strong antagonism (21).

Considering that the importance of these molecules has been well established. Further research was conducted into enhancing these molecules by adding unique substituents in specific locations to produce the compound with the greatest efficacy and potency against cancerous cells possible. Since then, various analogues have been studied as a mechanism to achieve this goal.

2 Objectives

Cancer treatment is an area that continues to undergo development and improvement due to various issues associated with the available cancer treatments. One of the key concerns is addressing the side effects that patients experience during treatment which can significantly impact a patient's quality of life. Efforts are being made to find ways to minimise these side effects and improve the overall well-being of individuals undergoing cancer treatment. Another important aspect is the effectiveness of current treatments. Researchers are constantly exploring new approaches, such as targeted therapies and immunotherapies, to enhance the efficacy of treatments and increase the chances of successful outcomes.

The use of thiosemicarbazones has been shown to have a powerful anti-tumour activity and an ability to overcome resistance to cancer treatment, two molecules that have a major role in the thiosemicarbazones family are Dp44mT and DpC.

In our research, we aimed to contribute to the ongoing development of cancer treatment by focusing on the synthesis of an analogue of these molecules that have shown promise in this area, with this research we were trying to synthesise analogues that can be more effective and have even better tolerability. The primary objective was to successfully synthesise the analogue of Dp44mT and DpC, both of which are thiosemicarbazones, starting from 2-aminophenol. However, synthesising these molecules proved to be challenging, especially due to the complicated Fries rearrangement step. Therefore, our research also involved finding and studying new viable pathways to overcome this difficult step.

In addition to our primary objective of synthesising analogues of Dp44mT and DpC, our research also encompassed the exploration and study of the intermediates generated during the synthesis process. By analysing these intermediates, we aimed to gain a deeper understanding of the underlying chemical reactions and mechanisms involved in the synthesis pathway.

For this the following research question will be addressed:

“Is N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl] -2 - hydroxyphenyl}-2- -(diethylamino)acetamide possible to synthesise in an ortho position and still have therapeutic value?”

3 Results and Discussion

Increased resistance among cancers to standard treatment has led to the investigation of new therapeutic strategies and to our examination of the activity of novel DpT analogues. With the importance of thiosemicarbazones increasing throughout the years we have decided to design a new molecule, analogue to DpC and Dp44mt, starting in the di-2-pyridylketone thiosemicarbazone the base of the DpT family of compounds but aiming to enhance their activity and effectiveness, by changing its structure.

3.1 Changing of Molecule Structure

The N1NH(CS)N4H structure is the key active structure, and it is necessary for the biological activity of the compound. Due to the presence of N, S and the C=N group, thiosemicarbazones readily form stable complexes with a variety of metal ions which is essential for their activity against cancerous cells. After realising the key elements that needed to be present in the molecule, to maintain an anti-tumoral activity, we have focused our attention on the elements that we could add or change to improve its effectiveness.

We decided to synthetise a molecule that would have a different position compared to previous ones. For testing this, our starting compound begins in an -ortho position instead of -para versus the N-acetyl side chain attached to the aromatic ring – **Step 1**. Having the DpC and Dp44mT as a model for our design process we also decided to remove the second aromatic ring common in both structures – **Step 2.**

In addition, after step 2 we have decided to add 2-(Diethylamino)acetamide to our investigational compound to test the eventual benefit in adding more N and O atoms to our molecule – **Step 3.**

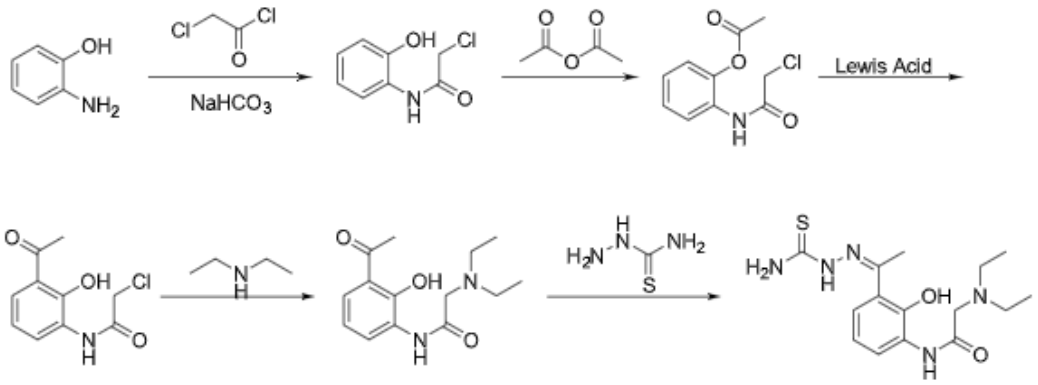


Figure 10. Synthetic pathway to obtain the N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl]-2-hydroxyphenyl}-2-(diethylamino)acetamide.

3.2 Chemical Process Selection – Fries Rearrangement

One of the goals of our work was to obtain **compound 6**, in the most stable and effective way possible, using the fries rearrangement.

We needed to prepare our molecule to be able to receive the semicarbazide group that is essential for this synthesis. The fries rearrangement is a chemical process that converts phenol esters to hydroxyaryl ketones. This process proved to be very important in our synthesis because it allowed us to have the control over the positions of substituents on the aromatic ring, which is crucial in designing and synthetizing molecules with specific structures and properties as ours. Furthermore, this process allowed us to modify the functional group within our molecule.

3.3 Limiting Factors of the Synthesis

One of the limiting factors appeared in the Fries rearrangement, the process we decided to follow in order to obtain **compound 4** proved to be more difficult than we initially thought. The control over the temperature was a difficult factor since we used melting as the process to perform the fries rearrangement, we heated our mixture up to 160ºC during 2h. This revealed one of the hardest steps in our synthesis because if we heated our mixture too much, we could lead to decomposition of our compound but if we did not heat our mixture enough nothing would happen and the rearrangement would not occur.

Other limiting factor we had was when it came to the separation phase that succeeded the fries rearrangement since there were always solid particles that did not allow the separation of phases to go fluid. From the NMR of **compound 4** we were able to realise that the Fries Rearrangement didn't work as well as we thought since we obtained a very low yield in all our attempts never being able to overcome these difficulties.

From this step on we were only working with a very low quantity of our desired compounds which made it harder to proceed. In the posterior steps the challenge was to purify the molecules that we were obtaining.

4 Materials and Methods

All the reactions were performed in oven dried glass. All the water used as solvent in the reactions was previously distilled. The reactants were purchased from commercial sources and used without further purification.

Reaction mixtures were analysed by thin layer chromatography TLC using Silica Merck F254, detection: UV lamp Benda, Germany, wavelength 254 nm. NMR spectra were recorded in Jeol EZCR-400, 400 MHz, Jeol, Japan, using DMSO-d6 as a solvent (possible to recognize after a quintet at 2.49 ppm) or CDCl3 (singlet at 7.26 ppm); All coupling constants (J values) are expressed in Hz and chemical shifts (δ) in ppm.

4.1 Synthesis of 2-Chloro-N-(2-hydroxyphenyl)acetamide

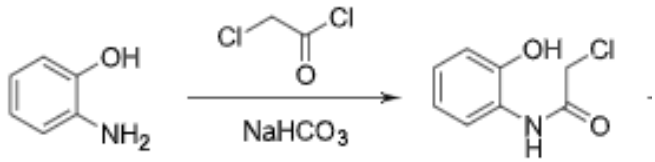


Figure 11. Synthesis of 2-Chloro-N-(2-hydroxyphenyl)acetamide

The starting material, 2- aminophenol (6.15g) was mixed in a suspension with $NaHCO_3$, in acetone (90 ml) under an argon atmosphere. We used an argon atmosphere because this reaction is sensitive to the humidity in the air. At this suspension, was added dropwise chloroacetyl chloride, this needs to be done with extreme caution as if it drops too fast it can cause an accumulation of CO_2 leading to tension. After 2h of stirring we separated the precipitate by suction and washed it with acetone. The combined filtrate was concentrated under vacuum and the residue was recrystallized from ethyl acetate to obtain **compound 2** as a colourless crystal (8.05g, 0.043 mol, 76%).

1H NMR: (400 MHz, Chloroform-d) δ 11.57 (s, 1H), 10.68 (s, 1H), 10.26 – 10.17 (m, 1H), 8.86 – 8.75 (m, 2H), 8.63 – 8.54 (m, 1H), 5.24 (s, 2H).

4.2 Synthesis of 2-[(Chloroacetyl)amino]-phenyl acetate

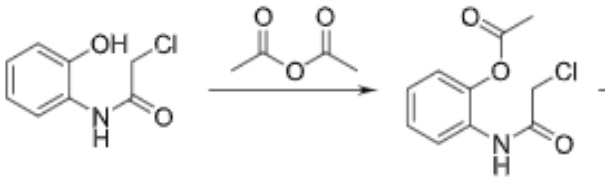


Figure 12. Synthesis of 2-[(Chloroacetyl)amino]-phenyl acetate

We added to Chloro-N-(2-hydroxyphenyl)-acetamide (43 mmol), sodium acetate (7.95 g) in acetic anhydride (39.75 ml) and heated the mixture up to 110ºC for 2h. The reaction mixture was concentrated in vacuo, and washed successively with an aqueous solution saturated with Na_2CO_3 and was left to dry in Na_2SO_4. After drying we evaporate the excess of acetic anhydride and acetic acid that was formed during the reaction. After the evaporation we added water (160 ml) and dichloromethane (50ml) and proceeded with the phase separation. To decompose the remaining acetic anhydride and neutralise the rest of the acetic acid we prepared a solution with 11g of Na_2CO_3 and 50 ml of water and added to our organic phase, although this needed to be done carefully because it can hydrolyse our compound. We also decided to add brine (40ml) to our organic phase to remove the remaining Na_2CO_3 that could have stayed and that way we minimise the possibility of our phenolic compound being hydrolysed. After drying we obtained a solid with 10.42mg.

1H NMR: (400 MHz, Chloroform-d) δ 11.48 (s, 1H), 10.46 (dd, J = 7.2, 1.7 Hz, 1H), 9.12 (dd, J = 7.3, 1.7 Hz, 1H), 8.99 (dtd, J = 23.2, 7.5, 1.7 Hz, 2H), 5.27 (s, 2H), 2.91 (s, 3H).

4.3 Synthesis of N-(3-acetyl-2-hydroxyphenyl)-2-chloroacetamide

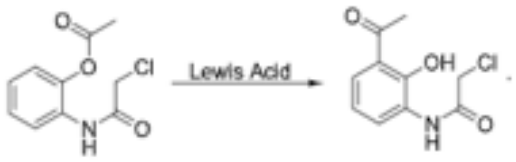


Figure 13. Synthesis of N-(3-acetyl-2-hydroxyphenyl)-2-chloroacetamide

In this next step we made a Fries rearrangement using Aluminium Chloride as the Lewis Acid. We started by adding Toluene (50 ml) and petroleum ether (45 ml) to **compound 3** and then we heated our reaction carefully until it dissolved. When it dissolved, we leave our mixture crystallise at room temperature. When the crystals (2.52g) formed we filtered them and added to them aluminium chloride (4.44g). We mixed everything using a mortar and heated our mixture for 2h at 160ºC. In the final steps of this stage, we washed the mixture with ethyl acetate (45 ml) and acidified it with HCl until PH=1 was obtained. After evaporation of ethyl acetate, we let it dry with Sodium Chloride. We obtained a solid. (0.45g, 1.98 mmol, 17%).

1H NMR: (400 MHz, Chloroform-d) δ 9.40 (s, 1H), 8.07 (dd, J = 7.5, 1.5 Hz, 1H), 7.70 (dd, J = 7.5, 1.5 Hz, 1H), 7.18 (t, J = 7.5 Hz, 1H), 4.21 (s, 2H), 2.61 (s, 3H).

4.4 Synthesis of N-(3-acetyl-2- hydroxyphenyl) -2- (diethylamino) acetamide

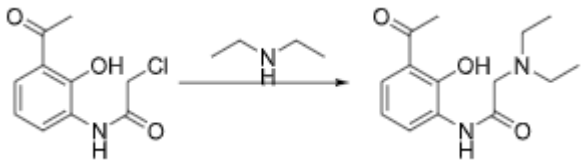


Figure 14. Synthesis of N-(3-acetyl-2- hydroxyphenyl) -2- (diethylamino) acetamide

The N-(3-acetyl-2-hydroxyphenyl)-2-chloroacetamide is suspended in 10 ml of toluene in a round bottom flask equipped with a condenser. Diethylamine (0.63 ml) is added. The reaction mixture is refluxed for 4 hours at 200ºC. After cooling, precipitated diethyl ammonium chloride is filtered off. The filtrate is extracted three times with 2 ml of water in a test tube because we had a very low volume and after separation of the third

portion of water, we dried our filtrate with anhydrous sodium sulphate in a stoppered flat-bottom flask for 45 min. After evaporating the toluene, we ended up with a small dark solid (0.20g, 0.87 mmol, 44%).

1H NMR (400 MHz, Chloroform-d) δ 9.42 (s, 1H), 8.23 (dd, J = 7.5, 1.5 Hz, 1H), 7.65 (dd, J = 7.5, 1.5 Hz, 1H), 7.23 (t, J = 7.5 Hz, 1H), 3.28 (s, 2H), 2.74 – 2.59 (m, 7H), 1.09 (t, J = 8.0 Hz, 6H).

4.5 Synthesis of N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl]-2-hydroxyphenyl} -2-(diethylamino)acetamide

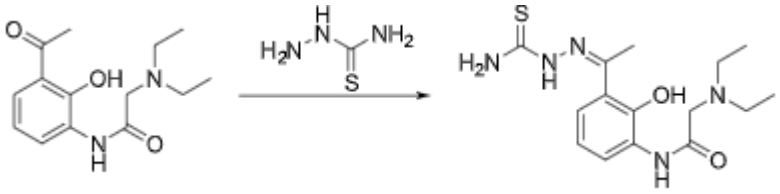


Figure 15. Synthesis of N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl]-2- hydroxyphenyl} -2-(diethylamino)acetamide

We added to **compound 5,** 3 ml of ethanol, 98 mg of thiosemicarbazide and 3 drops of acetic acid. The reaction mixture was refluxed for 2 hours at 100ºC. We filtrated the mixture and let it to dry. We synthesise 30 mg of our desired compound.

1H NMR (400 MHz, Chloroform-d) δ 9.54 (s, 1H), 9.46 (s, 1H), 8.01 (s, 2H), 7.59 (dd, J = 7.5, 1.6 Hz, 1H), 7.38 (dd, J = 7.5, 1.5 Hz, 1H), 7.28 (t, J = 7.4 Hz, 1H), 3.29 (s, 2H), 2.68 (q, J = 8.0 Hz, 4H), 2.37 (s, 3H), 1.09 (t, J = 8.0 Hz, 6H).

5 Conclusions

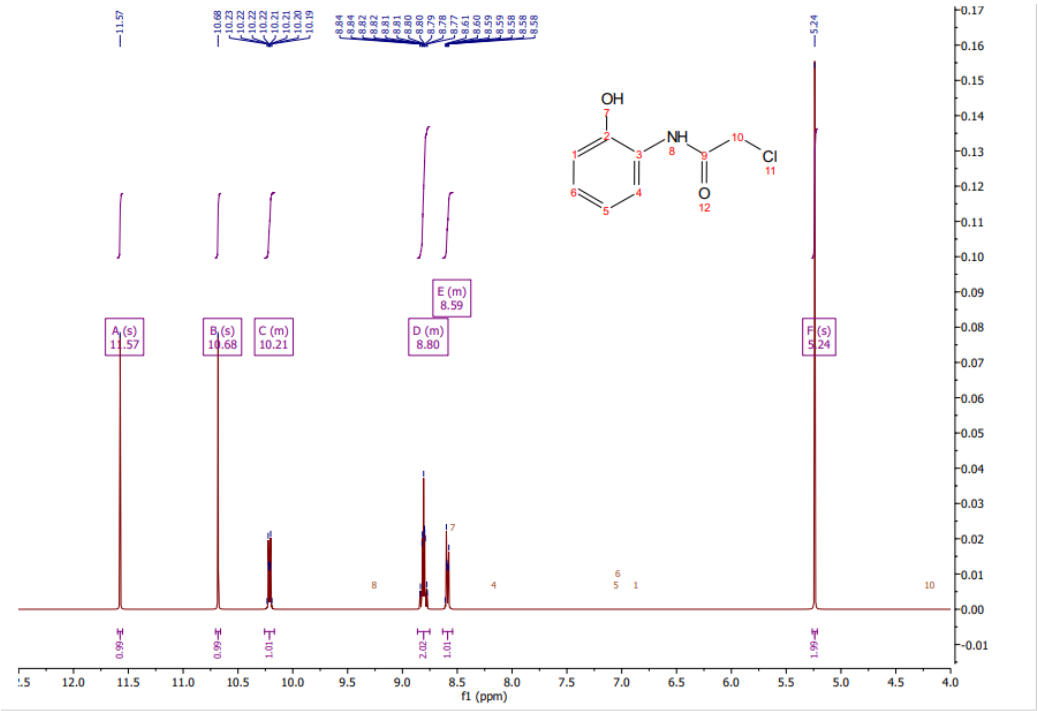
In the course of this project, we successfully synthesized an analogue of thiosemicarbazones DpC and Dp44mT, namely N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl]-2-hydroxyphenyl}-2-(diethylamino)acetamide. Despite achieving the synthesis of our desired compound, the yield was significantly low, highlighting the imperative need for process optimization, especially in the challenging Fries rearrangement step, which proved to be more challenging than initially anticipated.

Further studies are essential to uncover the full extent of thiosemicarbazones capabilities. In particular, ongoing investigations may reveal more efficient and well-established synthetic routes. The exploration of more effective substituents is a crucial facet of future research. Improving the molecular structure of thiosemicarbazones, can strive for greater selectivity against tumour cells, minimizing potential off-target effects and enhancing their therapeutic potential.

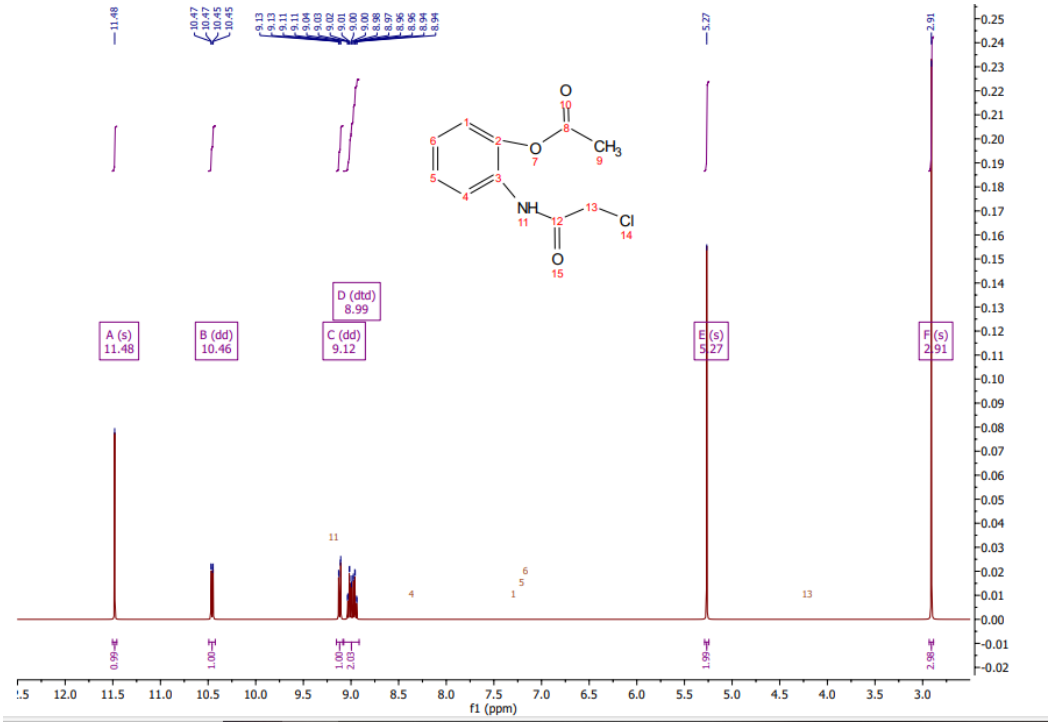
This work has made a significant contribution to the ongoing path of developing a viable process for synthesizing thiosemicarbazones with anti-tumoral properties. It is worth noting that the process of discovery often involves learning from our mistakes, and each misstep brings us one step closer to achieving success.

Appendix

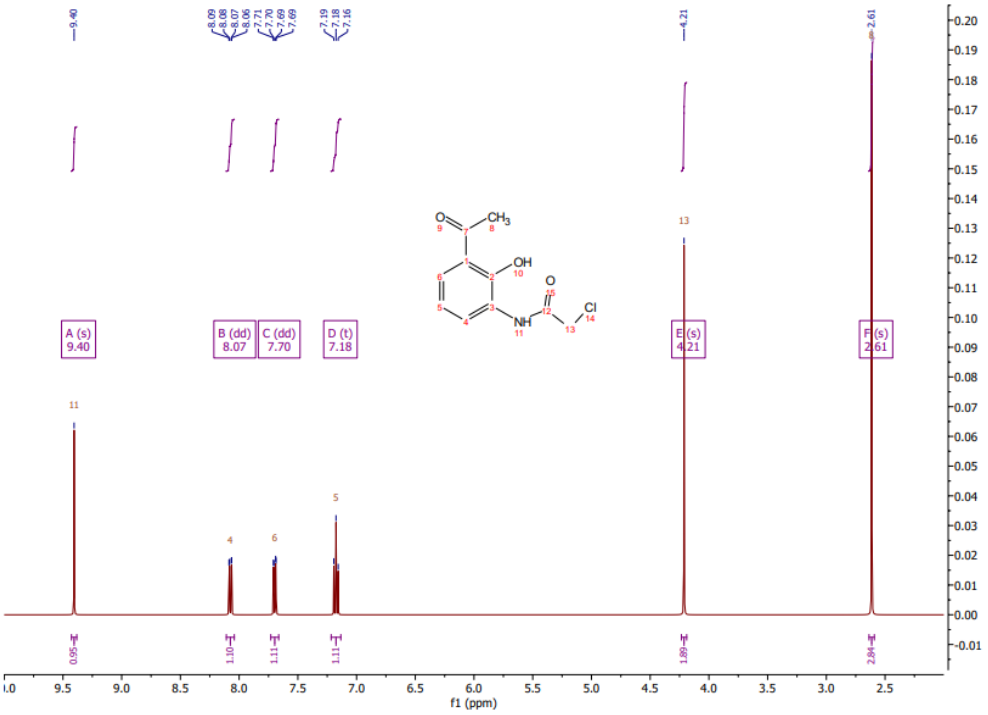
A1. 1H NMR Synthesis of 2-Chloro-N-(2-hydroxyphenyl)acetamide



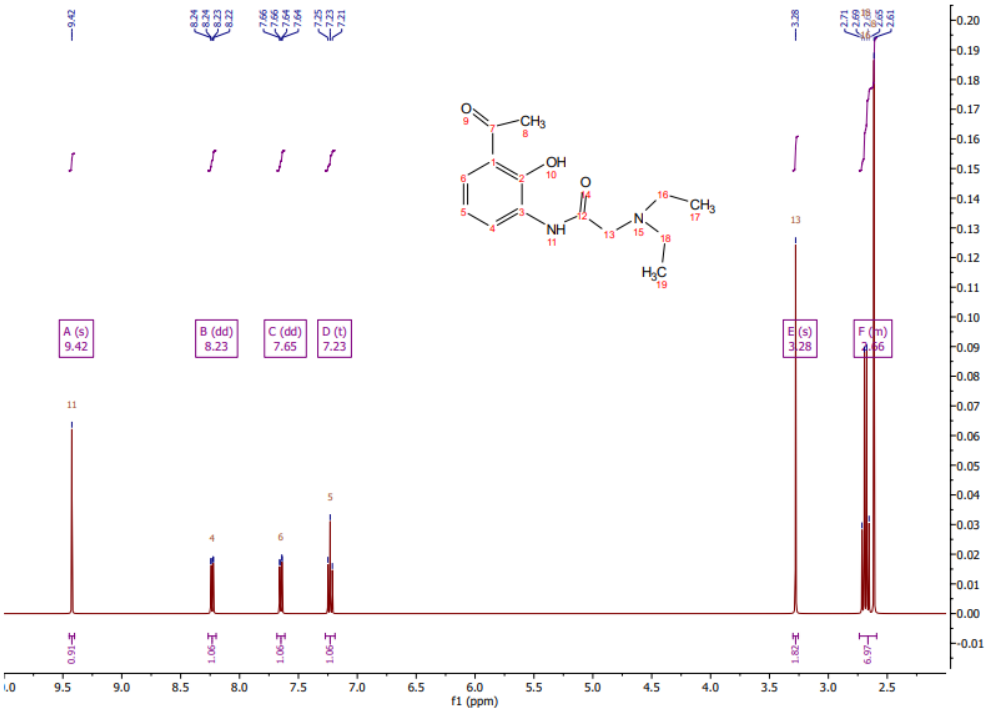
A2. 1H NMR Synthesis of 2-[(Chloroacetyl)amino]phenyl acetate



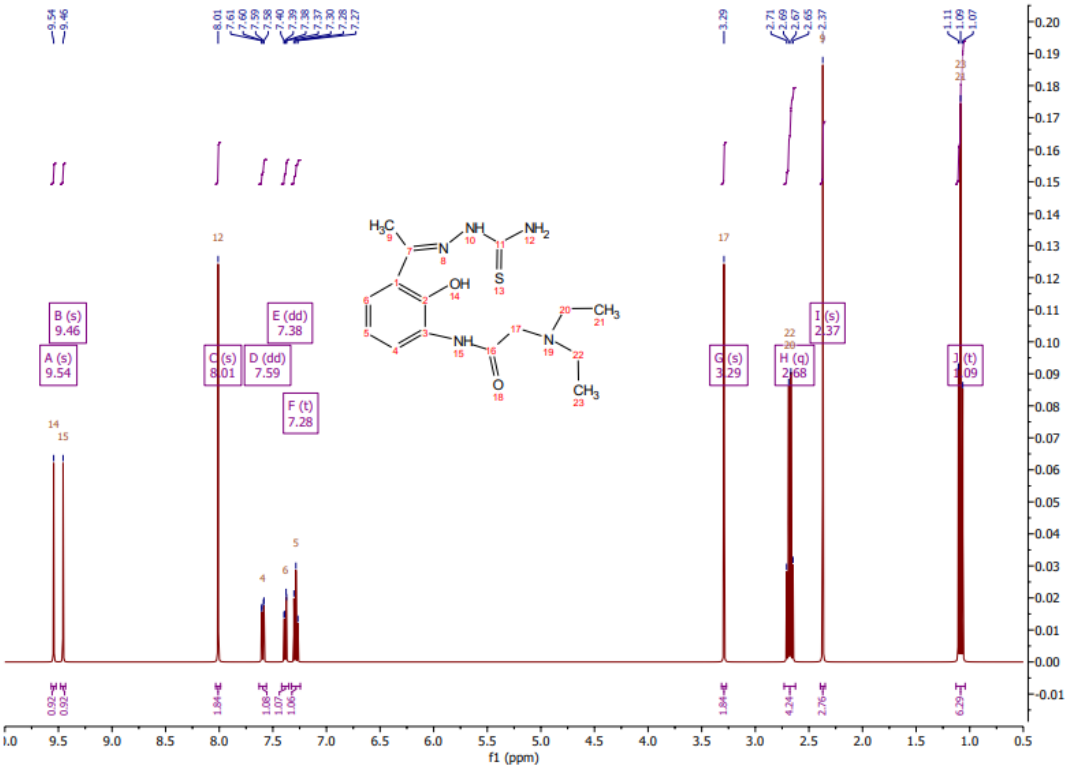
A3. 1H NMR Synthesis N-(3-acetyl-2-hydroxyphenyl)- 2 chloroacetamide



A4. 1H NMR Synthesis N-(3-acetyl-2- hydroxyphenyl) -2- (diethylamino) acetamide



A5. 1H NMR Synthesis N-{3-[1-(2-carbamothioylhydrazinylidene)ethyl]-2 - hydroxyphenyl}-2-(diethylamino)acetamide



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