Universidade de Lisboa

Faculdade de Farmácia

MANAGEMENT OF SKIN CANCER BY AGONISTS OF 5-HT1A AND ANTAGONISTS OF 5-HT2A RECEPTORS

Ana Catarina da Silva Fernandes Saraiva de Menezes

Dissertação

Mestrado em Ciências Biofarmacêuticas

2015

Universidade de Lisboa

Faculdade de Farmácia

MANAGEMENT OF SKIN CANCER BY AGONISTS OF 5-HT1A AND ANTAGONISTS OF 5-HT2A RECEPTORS

Ana Catarina da Silva Fernandes Saraiva de Menezes

Dissertação orientada por:

Prof.ª Doutora Andreia Ascenso e Doutora Helena Oliveira

Mestrado em Ciências Biofarmacêuticas

2015

À minha Avó

Agradecimentos

A concretização desta tese de mestrado fica a dever-se a um conjunto de pessoas que sempre me acompanharam ao longo deste percurso emocionante, quer a nível profissional, quer a nível pessoal, e às quais não poderei deixar de verdadeiramente agradecer.

Antes de mais, um primeiro agradecimento merecidamente dirigido à minha orientadora, Professora Doutora Andreia Ascenso, por ter acreditado neste projeto desde o início e ter batalhado por ele, mesmo com tantas adversidades que nos foram surgindo. Agradeço-lhe profundamente todo o apoio que me deu, toda a dedicação e esforço que demonstrou dia após dia e toda a disponibilidade e orientação prestada que contribuíram fortemente para o meu crescimento académico. Acima de tudo, agradeço-lhe por ter acreditado e confiado em mim para a concretização deste projeto. Espero sinceramente ter conseguido corresponder às elevadas expectativas em mim depositadas!

À minha co-orientadora, Doutora Helena Oliveira, agradeço-lhe profundamente toda a orientação prestada durante o meu percurso em Aveiro. Obrigada por todo o apoio, disponibilidade, paciência e calma que sempre me transmitiu, mesmo quando eu me preocupava demais e provavelmente nem devia! Foi sempre uma batalha difícil, ter de manter o ânimo em bons níveis e o trabalhar no “escuro” que envolve toda e qualquer investigação, sem saber o que esperar deste fármaco. Ainda assim conseguimos levantar uma “pontinha do véu” sobre o seu mecanismo e com essa vitória, poderei hoje afirmar que valeu, sem dúvida, todo o esforço.

Queria ainda agradecer ao Doutor Miguel Oliveira pela ajuda fundamental e enorme apoio que me deu durante o meu trabalho em Aveiro. Sempre que uma dúvida surgisse sabia que podia contar com um incondicional apoio quer no seu esclarecimento, como no entusiasmo e perseverança que me conseguiu transmitir! Obrigada por todas as conversas, todas as explicações e boa disposição que fazia questão de oferecer sempre.

Agradeço a todo o Laboratório de Biotecnologia e Citómica da Universidade de Aveiro por tão bem me receberem, pelo bom ambiente e o à vontade com que me puseram desde o início. Obrigada Verónica e Fernanda! Agradeço-vos a amizade e o apoio que me deram ao longo do meu trabalho, dada a vossa maior experiência. Sempre curiosa e cheia de perguntas e vocês sempre disponíveis para me responder!

À minha Ritinha, obrigada por tudo! A melhor amiga que podia imaginar ter conhecido em Aveiro, que sempre me ajudou quando precisei e sempre esteve lá para mim. Somos tão parecidas, que connosco é só alegria! Quer nos momentos bons, quer nos de maior pressão, estivemos sempre juntas. E mesmo cheias de trabalho lá arranjávamos um tempinho para nos irmos divertir. Afinal, só vivemos uma vez e temos que aproveitar. Foi sempre o nosso pensamento. Tenho a certeza que esta amizade é para durar, e não é a distância que nos vai impedir de continuarmos a estar juntas!

À Márcia também, obrigada por todos os momentos que passámos juntas em Aveiro. Agradeço-te imenso naquele dia teres ido ter comigo ao laboratório e me teres ajudado até tarde quando já só me apetecia desistir. Obrigada pelas conversas, pelos desabafos e pela nossa amizade! Mesmo quando estávamos mais em baixo lá íamos nós aquela nossa terceira casa comer qualquer coisinha e simplesmente conversar. Espero igualmente que continuemos sempre amigas e que, juntamente com a Rita, partilhemos mais momentos destes ao longo da nossa vida.

Queria agradecer ainda às Doutoras Sandra Simões, Manuela Colla e Manuela Gaspar por toda a preciosa ajuda prestada no meu trabalho em Lisboa. Obrigada pelos prontos esclarecimentos sempre que uma ou outra dúvida surgisse. Agradeço ainda a todos os que trabalharam comigo no grupo NanoBB do iMed.UL e que, estando eu fora da minha área de conhecimentos, me apoiaram sempre, ajudarando a aprofundar novas ideias e conceitos. Curiosa por natureza, gostei imenso de trabalhar e ganhar experiência na área de tecnologia farmacêutica!

Aos meus amigos que sempre me acompanharam, obrigada por tudo! Maria Inês e Cristiana, vocês que sempre estiveram ao meu lado para tudo e me apoiaram quando mais precisei, agradeço do fundo do coração. Vocês sabem a importância que têm para mim! Ana e Nuno, obrigada por todos os momentos que passámos juntos! Pipa, Natasha, Sofia, Susana e Mafalda, colegas de mestrado e sobretudo amigas, agradeço-vos todos os nossos momentos, todos os trabalhos que enfrentámos juntas e acima de tudo a amizade que daí resultou. Maria João, obrigada por me acompanhares ao longo dos anos e por todos os momentos que já partilhámos. Sem querer esquecer ninguém, agradeço a todos os meus colegas de licenciatura e mestrado que comigo partilharam gargalhadas, histórias e momentos bem passados.

Por último, aos que serão sempre os primeiros, agradeço do fundo do coração à minha família e namorado. Mãe e Pai, obrigada pelo vosso constante apoio mesmo a mais de 6,000 kms de distância. Obrigada por me incentivarem, acreditarem em mim e por me proporcionarem a realização de todos os meus estudos e dos meus sonhos. Espero que se sintam orgulhosos de mim! Aos meus irmãos, agradeço a companhia, a cumplicidade e todos os momentos que partilhámos ao longo da vida e, se também aqui cheguei, foi com a vossa ajuda. E ao meu namorado, de quem tenho recebido um apoio e confiança incondicionais, sabes que sem a tua presença neste momento tão importante da minha vida nada disto seria possível. Sempre tiveste lá para mim, para me ouvires, para me dares força e nunca duvidaste da importância desta vitória. Obrigada por tudo!

Dedico esta tese à minha Avó Helena que, apesar de não estar aqui hoje para me ver concluir mais esta etapa, foi de longe a pessoa que mais acreditou em mim, mais força me deu e mais orgulhosa estava por todo o meu percurso até hoje. Só pedias a Deus para me veres concluir os estudos e felizmente viste isso acontecer. Com todas as adversidades dos últimos meses consegui terminar o meu trabalho e a escrita da tese contigo a olhares por mim, tenho a certeza. Só tu sabias o quão cansada andava, o quão trabalhava até tarde e todo o sacrifício que as duas fizemos para conseguir finalizar esta etapa da minha vida. Espero somente que estejas feliz e orgulhosa de mim, obrigada vó!

Publications & Communications

All studies included in this thesis are present in the following publications and communications:

- Publications in international peer-reviewed journals:

Menezes AC, Raposo S, Simões S, Ribeiro H, Oliveira H, Ascenso A (2015) *Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors*. Molecular neurobiology. doi: 10.1007/s12035-014-9068-z.

- Poster communications:

Menezes AC, Ferreira de Oliveira JM, Carvalheiro M, Oliveira H, Ascenso A. *Cytotoxicity of the Serotonergic Drug 1-(1-Naphthyl)Piperazine (1-NPZ) in Human MNT-1 Melanoma Cells*. 51st Congress of the European Societies of Toxicology. Porto, 2015 (accepted);

Menezes AC, Ferreira de Oliveira JM, Carvalheiro M, Oliveira H, Ascenso A. *Management of Skin Cancer by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors*. IV Encontro Nacional de Pós-graduação em Ciências Biológicas. Aveiro, 2015;

Menezes AC, Oliveira H, Ascenso A. *Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors*. iMed Conference 6.0 – Nova Medical School. Lisboa, 2014.

- Competitions & Awards:

Participation in the Fundação AstraZeneca Innovate Competition, having won **3rd place** in the poster presentation contest with an original Translational Research project entitled *Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors*. This competition was held during the iMed Conference 6.0 – Nova Medical School, Lisboa, 2014.

"Pleasure in the job puts perfection in the work"

Aristotle

Greek critic, philosopher, physicist & zoologist (384 BC - 322 BC)

Resumo

A pele é o maior órgão humano e apresenta funções importantes quer a nível neuroendócrino, quer imunológico. A presença de um análogo do eixo hipotalâmico-hipofisário-adrenal na pele permite reagir a fatores externos de *stress* e modular as funções da mesma, tais como a melanogénese. A serotonina (5-hidroxitriptamina, 5-HT) é um neuromodelador importante que atua como fator de crescimento no cancro da pele, uma vez que os seus recetores na pele poderão estar envolvidos na imunossupressão induzida pela radiação UV, danos no ADN, *stress* oxidativo e proliferação celular. O *1-(1-Naftil)piperazina* (1-NPZ), agonista do recetor 5-HT1A e antagonista do recetor 5-HT2A, tem vindo a revelar efeitos promissores ao inibir a imunossupressão induzida pela radiação UV e, consequentemente, a fotocarcinogénese. Para tal, a presença do fotorecetor ácido *trans*-urocânico (ácido 3-(1H-imidazol-4-li)-2-propenóico, AUC) na epiderme é fundamental. Com a exposição aos raios UV, o *trans*-AUC isomeriza na sua configuração *cis* e esta, por sua vez, leva à imunossupressão. Contudo, o mecanismo de ação do *cis*-AUC ainda não foi devidamente elucidado. Desta forma, nós tentámos investigar se o 1-NPZ seria igualmente capaz de tratar a forma maligna e mais letal do cancro da pele. O melanoma tem origem nos melanócitos da epiderme que, por sua vez, são os principais produtores de serotonina ao nível da pele e possuem ambos os recetores 5-HT1/2A. O melanoma é ainda uma das doenças mais desafiantes, o que enfatiza a importância do nosso trabalho ao nível da comunidade científica.

A administração tópica de fármacos anticancerígenos representa uma abordagem terapêutica promissora para o tratamento eficaz do cancro da pele. As vesículas ultradeformáveis (VUD) são nanosistemas recentes que permitem melhorar o transporte dérmico e/ou transdérmico de vários compostos. Os benefícios destes sistemas vão desde o aumento da absorção do fármaco através da pele com uma libertação continuada a uma menor degradação do fármaco encapsulado ou incorporado. Os transetossomas (TE) são um tipo de VUD que deriva de duas vesículas lipídicas distintas, transferssomas e etossomas, abrangendo as vantagens de ambas. Os TE apresentam assim novas

propriedades de penetração mais eficaz das vesículas ao nível das camadas mais profundas da pele o que, por sua vez, se deve à presença de um tensioativo e de um álcool na sua composição. De facto, a presença sinérgica destes dois componentes contribui para a elevada deformabilidade dos TE, permitindo a sua fácil penetração através dos poros da pele mais pequenos do que o seu próprio tamanho. Os dmsossomas (DM), por outro lado, são um novo tipo de vesícula lipídica que contém dimetilsulfóxido (DMSO) na sua composição, tendo sido recentemente desenvolvidos pelo nosso grupo de investigação. O DMSO atua diretamente na pele enquanto promotor de penetração ao invés de aumentar a deformabilidade das vesículas. Deste modo, o mecanismo de ação do DMSO envolve a sua interação com os domínios lipídicos do estrato córneo, interferindo com essa mesma organização estrutural. Adicionalmente, o DMSO é considerado um "solvente universal" e possui ainda atividades biológicas relevantes.

O objetivo deste estudo prendeu-se com a investigação do efeito terapêutico do 1-NPZ em células humanas de melanoma MNT-1 e com o subsequente desenvolvimento de formulações de transetossomas (NPZ-TE) e dmsossomas (NPZ-DM) com 1-NPZ enquanto novos nanotransportadores de administração tópica para o tratamento do cancro da pele.

Assim sendo, este trabalho procurou explorar uma perspetiva importante e original da ação dos neurotransmissores ao nível da pele e pretendeu ainda ultrapassar alguns obstáculos dos tratamentos atuais para o cancro da pele, tais como: inacessibilidade do tumor, eficácia reduzida, efeitos secundários graves, elevados custos e disponibilidade limitada. Ambas as formulações tópicas desenvolvidas neste trabalho, NPZ-TE e NPZ-DM, deverão ser mais seguras e benéficas, com um grande potencial para gerar avanços no tratamento do cancro da pele. Uma melhor evolução clínica e maior qualidade de vida para as pessoas que padecem desta doença seria o mais desejável. O verdadeiro impacto deste trabalho, quer a nível científico, quer ao nível da sociedade, conta desde logo para o seu sucesso.

Inicialmente, tanto as condições de exposição ao 1-NPZ como a viabilidade celular foram analisadas com recurso ao método do MTT. De seguida, avaliou-

se a dinâmica do ciclo celular, produção de espécies reativas de oxigénio (ERO) e eventos apoptóticos por citometria de fluxo. A técnica da reação em cadeia da polimerase com transcrição reversa em tempo real foi ainda utilizada com o intuito de quantificar os níveis de expressão de genes envolvidos no processo de imunossupressão e na progressão do cancro.

O tratamento com 1-NPZ durante 24 h levou à redução da viabilidade celular, bem como à indução da apoptose nas células MNT-1 de uma forma dependente da concentração. Simultaneamente, o 1-NPZ conduziu a um atraso na fase S do ciclo celular e aumentou a produção de ERRO nestas células. Para além disso, a expressão da ciclooxigenase-2 (COX-2) aumentou significativamente após o tratamento com 1-NPZ. O conjunto destes resultados sugere que o 1-NPZ foi capaz de induzir *stress* oxidativo e a paragem do ciclo celular o que, por sua vez, levou à morte celular por apoptose das células de melanoma.

Por outro lado, a caracterização físico-química dos NPZ-TE e NPZ-DM baseou-se na avaliação de vários parâmetros, incluindo o tamanho médio das vesículas e o potencial zeta por dispersão dinâmica e eletroforética da luz, respetivamente. Adicionalmente, a deformabilidade das vesículas foi avaliada pela filtração sob pressão e os estudos de reologia foram realizados por viscosimetria. A espetroscopia e a cromatografia líquida de alta eficiência foram ainda usadas para determinar ambos os rendimentos lipídico e de encapsulação do fármaco, respetivamente. Por último, os estudos de administração tópica *in vitro* foram realizados utilizando células de difusão de Franz e pele de leitão com o objetivo de avaliar os perfis de penetração e permeação das formulações vesiculares.

Em ambos os sistemas, NPZ-TE e NPZ-DM, obtiveram-se resultados positivos ao nível do tamanho médio, potencial zeta, deformabilidade e reologia. O rendimento de encapsulação do 1-NPZ foi de 90,6% para os NPZ-TE e de 95,8% para os NPZ-DM, revelando que ambas as formulações são excelentes candidatos para fins terapêuticos. Os resultados *in vitro* também demonstraram uma penetração aumentada do 1-NPZ na pele de leitão, especialmente por NPZ-TE (0,31 μg/cm^2).

Em suma, este estudo demonstrou o potencial dos recetores da serotonina, nomeadamente os 5-HT1/2A, enquanto alvos terapêuticos para o tratamento do cancro da pele do tipo melanoma, identificando pela primeira vez o 1-NPZ como um agente quimioterapêutico promissor. Os resultados obtidos encorajam-nos a continuar os estudos com ambas as formulações para o tratamento tópico do melanoma.

Palavras-chave: Cancro da pele, Recetores 5-HT1/2A, 1-NPZ, Apoptose, Administração tópica

Abstract

The skin is the largest organ in the body with important neuroendocrine and immune functions. In fact, skin has its own hypothalamic–pituitary–adrenal axis, which in turn reacts to external stress factors and regulates skin functions, including melanogenesis. Serotonin (5-hydroxytryptamine, 5-HT) is an essential neuromodulator that can act as a growth factor for skin cancer, since its skin receptors may be involved in UV-induced immunosuppression, DNA damage, oxidative stress and cells proliferation. 1-(1-Naphthyl)piperazine (1-NPZ) is both an agonist of 5-HT1A and antagonist of 5-HT2A receptors that has shown promising effects by inhibiting UV-induced immunosuppression and, consequently, photocarcinogenesis. Therefore, we attempted to investigate whether 1-NPZ was also capable of treating the malignant and most deadly form of skin cancer. Melanoma arises from epidermal melanocytes, which are the main producers of serotonin in the skin and possess both 5-HT1/2A receptors. It is also one of the most challenging malignancies to address therapeutically, emphasizing the importance of our work in the scientific community.

Topical administration of anticancer drugs represents a potential therapeutic approach for the effective treatment of skin cancer. Ultradeformable vesicles (UDV) are novel advantageous nanosystems capable of improving the dermal and/or transdermal delivery of several drugs. Transethosomes (TE) descend from both transfersomes and ethosomes, thereby having pioneering permeation-enhancing properties due to the existence of an edge activator and an alcohol in their composition. The synergistic presence of these two components contributes to the high deformability of TE, allowing their easy penetration across skin pores much smaller than the vesicles size. Dmsosomes (DM), on the other hand, are a new type of lipid vesicles containing dimethyl sulfoxide (DMSO), and were recently developed by our research group. DMSO acts directly on the skin as a penetration enhancer, rather than increasing vesicles deformability. Moreover, DMSO is regarded as a “universal solvent” and has important biological activities.

The aim of the present study was to investigate the therapeutic effect of 1-NPZ on human MNT-1 melanoma cells, and posteriorly develop 1-NPZ-loaded transethosomes (NPZ-TE) and dmsosomes (NPZ-DM) as novel topical delivery nanocarriers for the treatment of skin cancer. Therefore, this work aimed to explore an important and original perspective of neurotransmitters action at skin level, and intended to overcome some drawbacks of the current treatments for skin cancer, such as tumor inaccessibility; poor effectiveness; strong side effects; high costs and limited availability. Both topical formulations developed in this work should be more secure and beneficial with a high potential to generate advances in skin cancer management. An improved clinical outcome and higher quality of life for people diagnosed with this malignancy would be desirable. The real impact of this work, from scientific to societal, counts to its success.

Firstly, the exposure conditions of 1-NPZ as well as cell viability were evaluated by MTT assay. Cell-cycle dynamics, reactive oxygen species (ROS) production and apoptosis were all evaluated by flow cytometry. Reverse transcription RT-PCR was also performed to quantify the expression levels of genes involved in immunosuppression events and cancer progression.

Treatment with 1-NPZ for 24 h reduced cell viability and induced apoptosis on MNT-1 cells, in a dose-dependent manner. Simultaneously, 1-NPZ mediated S-phase delay in cell-cycle dynamics and increased ROS production. Moreover, the expression of cyclooxygenase-2 (COX-2) increased significantly following treatment with 1-NPZ. All these findings suggest that 1-NPZ was able to induce oxidative stress and a cell cycle delay, which in turn led to apoptotic cell death in melanoma cells.

The physicochemical characterization of both NPZ-TE and NPZ-DM was based on the evaluation of several parameters, including the mean vesicles size and zeta potential by dynamic and electrophoretic light scattering, respectively. Vesicle deformability was assessed by pressure driven transport, whereas rheology studies were performed by viscometry. Spectrophotometry and high performance liquid chromatography were both used to determine lipid and drug entrapment yields, respectively. Finally, *in vitro* topical delivery studies were also achieved using Franz diffusion cells and newborn pig skin in order to evaluate the penetration and permeation profiles of the vesicle formulations.

Either NPZ-TE or NPZ-DM showed positive results in terms of mean size, zeta potential, deformability and rheology. 1-NPZ entrapment yield was 90.6% for NPZ-TE and 95.8% for NPZ-DM, which indicated that both formulations are excellent candidates for therapeutic purposes. *In vitro* data also exhibited an improved penetration of 1-NPZ into newborn pig skin, especially by NPZ-TE (0.31 $\mu g/cm^2$).

In summary, this study revealed the potential of serotonin receptors, namely 5-HT1/2A, as therapeutic targets for the treatment of melanoma skin cancer, identifying for the first time 1-NPZ as a promising chemotherapeutic agent. Additionally, the positive results here obtained encourage us to carry on the studies on both formulations for the topical treatment of such malignancy.

Keywords: Skin cancer, 5-HT1/2A Receptors, 1-NPZ, Apoptosis, Topical delivery

Aims & Thesis Organization

The main focus of this thesis was to assess the potential of a novel therapeutic strategy based on the topical delivery of 1-(1-Naphthyl)piperazine (1-NPZ), both an agonist and antagonist of serotonin receptors (i.e. 5-HT1/2A), posteriorly encapsulated into ultradeformable vesicles (UDV) formulations towards the treatment of melanoma skin cancer.

The thesis is divided into three main chapters written in a scientific paper format. The first chapter comprises the introduction of the work by means of a review article already published containing the state-of-the-art. The second and third chapters correspond to the research work, including *in vitro* evaluation of 1-NPZ effects on melanoma cells, and further formulation and characterization of 1-NPZ-loaded lipid UDV for dermal delivery, respectively. Certain information might be repeated between chapters, as they are independent from each other.

Chapter 1, entitled *Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors*, provides an overview of the role of serotonin receptors as therapeutic targets for the prevention and/or treatment of skin cancers. This literature review helped us select the drug that best suited our purpose. The dual mechanism exhibited by 1-NPZ was considered an important advantage for counteracting the negative effects of skin cancer through serotonin receptors present in skin cells.

Chapter 2, entitled *Effect of 1-(1-Naphthyl)Piperazine on Human MNT-1 Melanoma Cells*, covers the *in vitro* evaluation of 1-NPZ effects on MNT-1 cells by cytotoxicity, genotoxicity, apoptosis, cell cycle dynamics and oxidative stress assays.

Chapter 3, entitled *Development and Characterization of Novel 1-(1-Naphthyl)Piperazine-loaded Lipid Vesicles for Dermal Delivery*, reports 1-NPZ entrapment into two innovative UDV, and subsequent comparative study between their physicochemical characteristics. These UDV comprised either a combination of ethanol and sodium cholate (surfactant) or DMSO alone in order to improve the penetration of vesicles into deeper skin layers.

The thesis concludes with **Final Remarks**.

Abbreviations

1-NPZ	1-(1-Naphthyl)piperazine
5-HIAL	5-Hydroxy-3-indolacetaldehyde
5-HIAA	5-Hydroxy-3-indolacetic acid
5-HT	5-Hydroxytryptamine or serotonin
5-HTR	5-Hydroxytryptamine receptors
5-HTPOL	5-Hydroxytryptophol
5-MT	5-Methoxytryptamine
5-HTP	5-OH-tryptophan
8-OH-dG	8-Hydroxy-2-deoxyguanosine
8-oxo-dG	8-Oxo-7,8-dihydroguanine
AIF	Apoptosis inducing factor
Ag	Antigens
ADH	Alcohol dehydrogenase
ALDH2	Aldehyde dehydrogenase
ALDR	Aldehyde reductase
APC	Antigen-presenting cells
BCCs	Basal cell carcinomas
BER	Base excision repair
BH4	Cofactor 6-tetrahydrobiopterin
Cdc42	Cell division control protein 42 homolog
CHS	Contact hypersensitivity
COX	Cyclooxygenase
CPD	Cyclobutane-type pyrimidine dimers
DAG	Diacylglycerol
DAT	Dopamine transporter
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DI	Deformability index
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTH	Delayed-type hypersensitivity
FAD	Flavin adenine dinucleotide
FapydG	2,6-Diamino-4-hydroxy-5-formamidopyrimidine
FasL	Fas ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High Performance Liquid chromatography

HSV	Herpes simplex virus
IC	Inhibitory concentration
IFN-γ	Interferon gamma
IL	Interleukins
IP3	Inositol 1,4,5-triphosphate
L-aromatic AAD	L-aromatic amino acid decarboxylase
LC	Langerhans cells
MAO	Monoamine oxidase
MHC	Major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCo	Sodium cholate
NAS	N-acetylserotonin
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NMSC	Nonmelanoma skin cancers
NO	Nitric oxide
NPZ-DM	1-NPZ-loaded Dmsosomes
NPZ-TE	1-NPZ-loaded Transethosomes
OCT	Polyspecific organic cation transporters
OGG1	8-Oxoguanine DNA glycosylase-1
OS	Oxidative stress
PAF	Platelet-activating factor
PAK1	p21-activated kinase
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
PDI	Polydispersity index
PDT	Photodynamic therapy
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PG	Prostaglandin
PKA/C	Protein kinase A/C
PLC	Phospholipase C
PPAR-γ	Peroxisome proliferator-activated receptor gamma
Rac1	Ras-related C3 botulinum toxin substrate 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
qRT-PCR	Quantitative real-time reverse-transcriptase polymerase chain Reaction
SC	*Stratum corneum*
SCCs	Squamous cell carcinomas

SERT or 5-HTT	5-Hydroxytryptamine transporter
SPC	Soybean phosphatidylcholine
SSRIs	Selective serotonin reuptake inhibitors or serotonin-specific reuptake inhibitors
T4N5	T4 endonuclease V
TGF-β	Transforming growth factor
Th	Helper T cells
TNF-α	Tumor necrosis factor-α
TPH	Tryptophan hydroxylase
Treg	Regulatory T cells
UCA	Urocanic acid
UDV	Ultradeformable vesicles
UVR	Ultraviolet radiation
VMAT	Vesicular monoamine transporter

Table of contents

Chapter 1
INTRODUCTION

Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors

Ana Catarina Menezes[1], Sara Raposo[2], Sandra Simões[2], Helena Ribeiro[2], Helena Oliveira[3], Andreia Ascenso[2]

[1]Faculdade de Farmácia da Universidade de Lisboa
Lisbon, Portugal

[2]NanoBB Research Group of iMed.UL
Lisbon, Portugal

[3]Departamento de Biologia, Laboratório de Biotecnologia e Citómica,
CESAM, Universidade de Aveiro
Aveiro, Portugal

Published in *Molecular Neurobiology* 2015 Jan 15 [Epub ahead of print]

Abstract

Exposure to UV radiation is the principal cause of nonmelanoma skin cancer, a process in which serotonin (5-HT) is intimately involved. This review focuses on the potential of serotonin receptors, namely 5-HT1/2A, as therapeutic targets for the prevention of photocarcinogenesis.

UV-induced immunosuppression is triggered by a cascade of events initiated when *cis*-urocanic acid (UCA), a UV photoreceptor present in the skin, binds to the serotonin receptor. Serotonin receptor antagonists will therefore attempt to block this association, and in turn, prevent skin cancer induction. In addition, 5-HT2A receptor antagonists are also capable of regulating DNA repair, including the acceleration of nucleotide excision repair. At the same time, these agents also reduce UV-induced formation of reactive oxygen species (ROS).

Since the involvement of serotonin in photocarcinogenesis process is somewhat underexplored as a pertinent therapeutic effect, this review intends to reveal the use of serotonergic drugs as an important strategy to prevent and/or inhibit photocarcinogenesis. Considering the emergency of developing novel therapeutic strategies for skin cancer management, the use of these agents, whose benefits have partially been studied, may be crucial especially if topically applied. Topical nanoformulations containing serotonin receptor agonists and/or antagonists also represent a pioneer concept in this area.

Keywords: Serotonin, Serotonin Receptors Agonists and Antagonists, Immunomodulation, DNA Repair and Cells Proliferation, Photocarcinogenesis

Introduction

The *skin* is the largest organ in the body with a vast structural and functional diversity. It represents a metabolically active barrier separating the body's internal from external environment [1]. Therefore, the skin is continuously exposed to environment stimuli such as UV radiation, and the need to maintain its integrity at all levels becomes evident. There are numerous mechanisms to restore the barrier properties of the skin through its ability to recognize and integrate different signals and to trigger appropriate responses [2]. It is the precise coordination of these responses, locally and systemically, that defines the *neuroendocrine system*. The crosstalk between the skin and the neuroendocrine system can be described as a multidirectional exchange of signals involving the brain, endocrine and immune systems and peripheral organs [1], leading ultimately to rapid (neural) or slow (humoral) responses [2].

The skin not only responds to signals, but also actively synthesizes *regulatory molecules* as neurotransmitters, hormones, neuropeptides, and corresponding functional receptors [3,4]. These molecules can be locally produced in epidermal and dermal layers or secreted from cutaneous nerve endings [3]. Their presence in the skin can also be due to active transport from blood and migrating immune cells [4]. Although the novel synthesized molecules act at a local level in paracrine or autocrine fashions, there is still the possibility of acting at a greater distance and influence central organs including the brain [4]. Besides having endocrine functions, the skin also exerts exocrine activities important for the maintenance of the epidermal barrier, thermoregulation, defense against microorganisms, and social communication [1,3].

The *hypothalamic-pituitary-adrenal* (HPA) axis is a crucial central system of regulation of stress response, which triggers a cascade of reactions with the production and release of neurotransmitters, regulatory peptides, and hormones [4]. The idea that skin may possess an equivalent HPA axis with similar mediators was formulated nearly 14 years ago [4]. In fact, skin and brain organs share a common embryologic origin as both derive from ectoderm, one of the three primary germ layers of the embryo. Besides these anatomic links, there are also functional links between brain and skin established by neuromediators that participate in the regulation of skin functions.

Serotonin, also known as 5-hydroxytryptamine (5-HT), is one important neuromediator among others involved in the interactions between skin and the neuroendocrine system [5]. There are seven general families of 5-HT receptors (R) with at least 21 subtypes. Skin cells express membrane-bound receptors for 5-HT (mainly 5-HT1R and 5-HT2R), as well as transporter G-proteins, and can stimulate (5-HT7R) or attenuate (5-HT1R) adenylate cyclase activity or enhance phosphoinositol hydrolases activity (5-HT2AR) [5]. Synthesized from L-tryptophan, 5-HT is a crucial signaling molecule that mediates neurocutaneous interactions at all levels [5]. It is involved in a variety of physiological processes throughout the body acting as a neurotransmitter, hormone, cytokine, growth factor, morphogen, and antioxidant [2]. These functions affect several behaviors including mood, stress response, sleep, appetite, pain sensation, tissue regeneration, platelet aggregation, and gastrointestinal function, among others [6,7]. Additionally, 5-HT is fundamental for basic cell functions such as proliferation, differentiation, migration, and synaptogenesis [8], as well as for immune responses through the regulation of leukocyte chemotaxis, cytokine production and dendritic cell activation of T cells [9]. The production and secretion of 5-HT can be affected by other molecules sharing its regulatory character and lead to changes in the maturation, migration, and mitosis of its target cells, including skin cells [5]. 5-HT is widely distributed throughout the body, which emphasizes its central role in maintaining homeostasis [5]. However, 95 % of the total 5-HT content in the body is localized essentially in platelets and enterochromaffin cells of gastric mucosa [10].

In previous studies, 5-HT was reported to act as a growth factor for numerous types of human cancer [8] including *skin cancer*, as its receptors may be involved in UV-induced immunosuppression [11].

Exposure to UV radiation is the principal cause of nonmelanoma skin cancer, the most prevalent human cancer, and is also associated with the induction of malignant melanoma [11-13]. The global incidence of both types of skin cancer has been increasing over the past few years. Currently, approximately 2.5

million and 132,000 of nonmelanoma and melanoma skin cancers, respectively, occur worldwide each year [14]. This perpetual incidence is associated with the depletion of ozone layer, and inevitably with cumulative sun exposure.

Currently, there is a wide variety of *treatment* approaches for patients with skin cancer either surgical, such as simple excision or Mohs surgery, or non-surgical, including radiotherapy, cryotherapy, topical immunomodulators (imiquimod), 5-fluorouracil (5-FU), and photodynamic therapy (PDT) [15]. The option for the most adequate treatment of skin cancer depends on many factors associated with age, anatomic location, number of lesions and type of tumor, among others [15]. Although the surgery is the usual procedure in most cases, it is not always possible due to the location of many skin cancers on the head or face [16]. Other disadvantages of the current treatments include poor effectiveness of chemotherapy for more advanced melanomas, strong side effects, high costs, and limited availability (e.g., imiquimod, 5-FU, and PDT) [15]. In addition, PDT has not yet contributed with consistent results and been clinically accepted for the treatment of skin cancer [17]. Recently, researchers have suggested that scoring systems like ABCDE (Asymmetry, Border, Color, Diameter, Evolving) may have a low specificity for melanoma detection since seborrheic keratosis and atypical nevi sometimes do not meet the ABCDE criteria [18]. Epiluminescence microscopy (ELM) is a noninvasive examination technique, which facilitates diagnosis and improves reliability. The Quiz of Dermatoscopy is a postgraduate medical training tool [18]. Therefore, in order to overcome some drawbacks of the current treatments of skin cancer, an urgent search for new therapeutic agents should be considered.

This review will mainly focus on the potential of a novel therapeutic strategy based on the use of agonists of 5-HT1 and antagonists of 5-HT2 receptors towards the prevention and/or inhibition of photocarcinogenesis.

Photocarcinogenesis

Skin cancer is the most predominant form of human cancer [13], and its incidence is rapidly increasing around the world in the past few decades [19]. Numerous factors can lead to the development of skin cancer, including ionizing radiations, viruses (such as the human papilloma virus, HPV), inflammation, and genetic factors, among others. However, the most significant environment factor is *UV radiation* (UVR) which may induce the photocarcinogenesis process [19]. The most prevalent forms of skin cancer are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), which arise from keratinocytes and are collectively known as *nonmelanoma skin cancers* (NMSC) [19]. UVR is also implicated in the induction of *malignant melanomas*, the most common fatal form of skin cancer that derive from melanocytes [19]. Although quite different at first sight, these three types of skin cancer share some common aspects, such as a higher incidence with increased patients age and exposure to UVR [19].

Several studies have been made in order to investigate the role of UVR in carcinogenesis, which revealed that it can induce irreversible alterations in cell genetic material by mutation, amplification or deletion of some genes, leading to final modifications of signaling pathways [19]. Besides its carcinogenic character, UVR is also immunosuppressive [13]. Immunosuppression is a major risk factor for the induction of skin cancer, as evidenced by studies with biopsy-proven skin cancer patients and immunosuppressed transplant patients [12,13]. Thus, it is crucial to understand the mechanism behind UV-induced immunosuppression and its connection with carcinogenesis.

Wavelength and Time-Dose Relationships

The development of a tumor is not a one-dimensional process, depending mainly on the nature of the carcinogenic challenge to the target organ, the dosages and their distribution, and time interval [20].

The process of carcinogenesis can be defined as the clonal expansion of a cell with critical alterations in genes that are important for the regulation of proliferation and differentiation mechanisms [20]. To achieve oncogenic transformation, these genes have to be activated in the case of proto-oncogenes, and deactivated in the case of tumor suppressor genes [20]. Therefore, the central cause of photocarcinogenesis is the induction of mutations in those genes by UVR.

UVR electromagnetic spectrum can be subdivided into three *wavelength ranges*: shortwave UVC (200–290 nm), midwave UVB (290–320 nm) and longwave UVA (320–400 nm) [19,21]. There is an inverse relationship between the energy of each part of the radiation and its wavelength and therefore, *UVC* is the most energetic one [19]. This type of radiation is extremely harmful and it can penetrate skin to a depth of approximately 60–80 μm [21]. Nevertheless, UVC is almost completely filtered by the ozone layer and thus, its impact on human health may be negligible [19,21]. *UVB* rays represent about 5 % of UVR that reaches the earth surface, being the most active component of UVR responsible for nonmelanoma and melanoma skin cancers (**Fig. 1**) [19,21]. This type of radiation acts primarily on the epidermal basal layer of the skin, penetrating the skin to a depth of approximately 160–180 μm [21]. It can cause inflammation, apoptosis, DNA damage, oxidative stress (OS), immunosuppression, and premature aging of the skin [19,21]. Despite the fact that UVB radiation is more mutagenic than UVA, the extent of UVA reaching the earth surface is much higher [22]. *UVA* rays represent approximately 95 % of solar radiation, reaching the earth surface and penetrating deeper into the skin to around 1000 μm [19,21]. The exposure to UVA radiation can produce a variety of reactive oxygen species (ROS), including hydrogen peroxide, superoxide radical, singlet oxygen and hydroxyl radical, which indirectly cause damage to cellular proteins, lipids, and DNA [19,21].

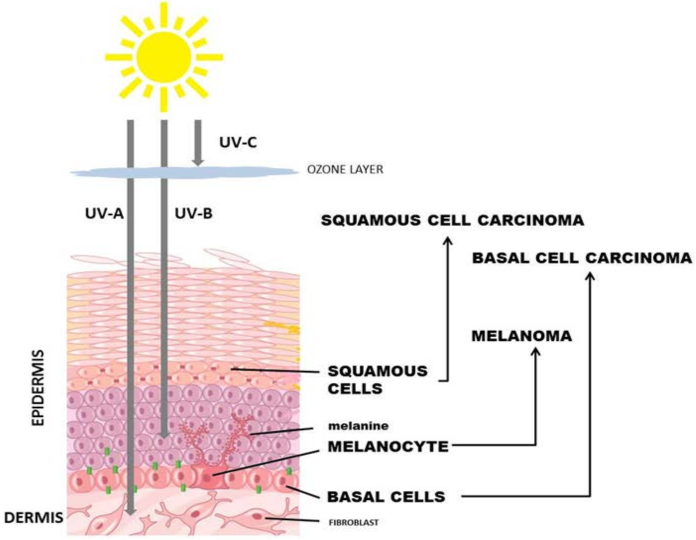


Fig. 1. Exposure to UV radiation and development of melanoma and nonmelanoma skin cancers. UVA radiation reaches the dermis and to some extent the subcutis, whereas UVB does not pass beyond the epidermal layer.

The target cells are likely to be the proliferating cells at the epidermis basal layer in *in vivo* UV-induced carcinogenesis [20]. Either UVC or UVB radiation are powerfully absorbed in the upper epidermis, with a lesser extent for the latter [20]. For wavelengths over 300 nm, the induction of DNA damage falls deeply, and becomes almost negligible in the UVA region above 340 nm [20].

The time of carcinogenic UV exposure is another essential aspect to be considered. Thus, if this exposure is increased in a group of mice, that group will eventually develop more tumors earlier [20]. However, there is no straight reciprocity between daily dose and induction time, since the kinetics of tumor induction is a process with several rate-limiting steps [20], which may or may not depend on UVR. Therefore, the probability of occurrence of a UV-independent step will increase over time, making the process of tumor induction less UV dose-dependent [20]. This may also involve adaptive mechanisms, such as epidermal thickening [20].

Stages of Photocarcinogenesis

Photocarcinogenesis is a complex multistage process that can be divided into three different stages: initiation, promotion, and progression. Each of these stages involves biochemical and molecular alterations in the cell. The tumor *initiation* stage is an irreversible phenomenon in which genetic alterations occur upon UVR exposure, leading to DNA mutation in normal cells [21,23]. Within these mutations, C to T and CC to TT transitions have been found in tumor suppressor gene p53 in human BCC, SCC, and actinic keratosis [21]. Tumor *promotion* is defined as a slow and reversible process consisting of clonal expansion of initiated cells and giving rise to a benign tumor with high proliferative capacity [21,23]. This stage is the most suitable for anticarcinogenic agents to prevent, reverse or slow down the process of photocarcinogenesis [24]. Finally, tumor *progression* comprises the conversion of the benign tumor into an aggressive, and eventually, metastatic malignant tumor [21,23]. Among the types of UVR, UVA is the weakest initiating agent but it is a fairly potent tumor-promoting agent [23].

UV-induced Damage

The direct effects of UVR include the generation of DNA photoproducts, whereas the indirect effects comprise the cell cycle arrest and melanogenesis, whose processes are regulated by several UV-induced cytokines and growth factors that mediate the survival, proliferation, and function of epidermal and dermal cells [22].

DNA Damage, Gene Mutations and Repair Mechanisms

The main target of carcinogenesis is the DNA, which absorbs the UVR in the form of photons, resulting in DNA damage. UVR induces several DNA lesions when it is absorbed by a double bond of pyrimidine bases, such as thymine and cytosine, which leads to the opening of this bond, thus becoming more reactive [25]. Two major types of damaging crosslinks between adjacent pyrimidine bases are: (1) *cyclobutane-type pyrimidine dimers* (CPD), which result from the formation of two new bonds between these neighboring bases, creating a four-membered cyclobutane ring; (2) *6-4 photoproducts* (pyrimidine (6-4) pyrimidone), which arise from the formation of a single bond between two carbons on the adjacent rings (**Fig. 2**) [25,26]. The latter is formed three- to five-fold less frequently than the CPDs, but it is considered more mutagenic [27]. In addition, the predominant resulting mutations are *C to T transitions* and CC to TT double base mutations, representing a specific marker of UVR contribution to photocarcinogenesis [19,28].

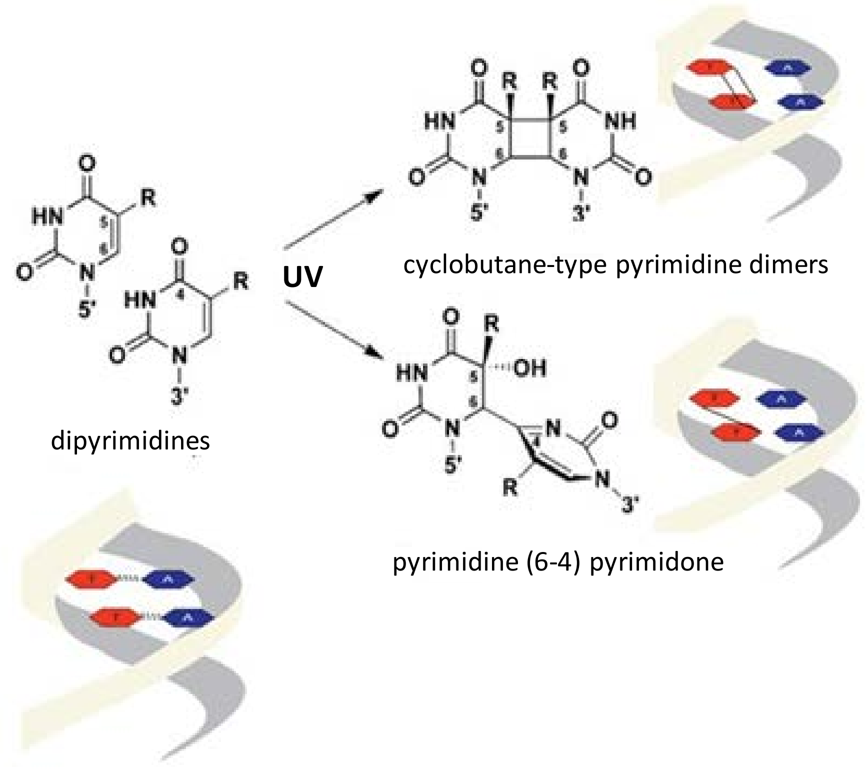


Fig. 2. Chemical structures of the main DNA photoproducts (adapted from [29,30]).

Supporting the role for DNA damage as an instigating event in UV-induced immunosuppression is the fact that immune function can be reestablished when liposomes containing the bacteriophage T4 endonuclease V (T4N5) are applied to the skin of UV-irradiated mice [31]. T4N5 is particular for UV-induced pyrimidine dimers [31]. UV-induced dimer formation specifically causes the release of immunosuppressive cytokines from keratinocytes, including interleukin (IL)-10 [32]. Abnormal *DNA methylation* is a hallmark of most cancers. It is a marker of epigenetic events, i.e., susceptible to change with environmental factors, and a fundamental process that modulates gene expression and regulates the chromosomal stability. Hyper or hypomethylation in G-C rich regions can contribute to carcinogenesis by silencing tumor suppressor genes (e.g., p53), upregulating oncogenes at dipyrimidine sites and by decreasing genomic stability. Chemopreventive agents may be used to correct aberrant methylation patterns and restore the control of tumor cells growth and/or against UVR side effects [33].

Other types of UV-induced damage, including protein DNA crosslinks, single-strand breaks, oxidative damage and purine photoproducts may also occur, but unlike the previous DNA damage, these are not specific and can be produced by other carcinogens [19,20]. If these lesions are not repaired before DNA replication, it will cause mutations in *cancer-related genes*, such as suppressor genes (involved in the regulation of DNA damage, apoptosis, and cell cycle control), proto-oncogenes (involved in organ growth and tissue repair), and other genes associated with the regulation of cell cycle [19]. The genes involved in skin pigmentation are also imperative to the development of skin cancer [19]. Thus, photocarcinogenesis involves the inactivation of tumor suppressor genes by altering the amino acid sequences of the encoded protein products, and the mutation or overactivation of some proto-oncogenes becoming oncogenes, whose encoded proteins allow cells to grow without restrictions [19]. In the first case, the products of suppressor genes can be inactivated by binding to other proteins or by phosphorylation in normal cells, or can be mutated or deleted in tumor cells [19]. However, most mutations that lead to loss of tumor suppressor genes function are recessive, and so it is required the inactivation or deletion of both alleles for a cell to become cancerous [34]. This idea was firstly proposed by the geneticist Alfred Knudson in 1971, and it is known as the “two-hit” hypothesis [34]. The most frequently altered gene in human cancers is *p53* which is activated by several forms of cell stress, such as irradiation, hypoxia, chemicals, among others [19]. Its protein product is an important transcriptional activator for cell regulation, being expressed at low levels in normal cells [19]. UV-induced mutations in this gene occur usually on pyrimidine sites [19]. In fact, in most UV-induced human skin cancers, CPDs can be found within the cell cycle regulatory gene p53, which also acts as a tumor suppressor gene, indicating an important role of UV-induced DNA damage in photocarcinogenesis [32]. Human double minute2 (Hdm2) regulates p53 gene by binding to it and inhibiting its transcriptional activity, as well as stimulating p53 degradation [19]. Following DNA damage, p53 induces the transcription of p21 (cyclin kinase inhibitor), which in turn arrests the cell cycle at the G1 phase, facilitating the DNA repair. In addition, p53 increases the apoptosis of mutated cells by upregulating the expression of proapoptotic genes, and prevents angiogenesis [19]. These p53 cellular functions have already been demonstrated in UV-irradiated transgenic mice (p53 −/−), which presented a reduction of apoptosis, and subsequent development of tumors [19].

On the contrary, mutations in proto-oncogenes are generally dominant in nature, and therefore alteration in one allele is sufficient to stimulate the cells growth or proliferation [19,34]. The mutated version of a proto-oncogene is an oncogene and, in humans, these genes include the Ras family [19,34]. In previous studies, Ras genes were detected by PCR in BCC, SCC, and melanoma [19]. Proto-oncogenes encode proteins involved in the stimulation of cell division, inhibition of cell differentiation and cell death arrest, which are all crucial processes for normal human development and maintenance of tissues and organs [34]. Therefore, oncogenes contribute to increased cell division, decreased cell differentiation, and inhibition of cell death [34].

Generally, DNA photolesions are rapidly repaired by the *nucleotide excision repair* (NER) mechanism described by the following steps: recognition of the lesion, unwinding of the DNA helix, demarcation of the lesion, dual incision and excision of the damaged fragment, resynthesis of the gap, and ligation of the new strand by DNA polymerase and ligase [19,20]. Mismatch repair and double-strand break repair are examples of other types of DNA repair processes [19].

The repair mechanisms for UV-induced DNA damage in humans have been primarily elucidated from studies of *xeroderma pigmentosum*, an inherited autosomal recessive disorder characterized by a deficiency in the NER process that leads to high sensitivity to UVR [19,20]. This occurs when DNA photolesions are not recognized or incorrectly repaired, and the bases continue permanently mutated, which lead to a very high incidence of skin cancer [19,20].

In other studies [35], after measuring the rate of removal of thymine dimers and 6-4 photoproducts from the DNA of UV-irradiated human dermal fibroblasts derived from donors of different ages, it was found that the NER mechanism of photoproducts substantially decreases with age. An age-associated decline of mRNA and protein levels for selected NER proteins [35] was also demonstrated.

Oxidative Stress

In addition to DNA photoproducts, there is the possibility that certain mutations arise from UV-induced oxidative DNA damage. The most important source of OS in human skin is UVA irradiation [36], which leads to an increase of ROS, damaging lipids, proteins, and nucleic acids in both epidermal and dermal cells [37]. This type of indirect damage is potentially mutagenic since it has already been demonstrated that the amount of oxidative damage induced by sunlight in skin fibroblasts is, at least, equivalent to the extent of CPDs [22]. UVA can produce similar DNA lesions associated with ROS, including usually apurinic/apyrimidinic (abasic) DNA sites, oxidized purines and purimidines, single- and double-strand DNA breaks and DNA-protein crosslinks, among others [20,36,38]. It has also been demonstrated that UVA radiation is capable of inducing photoaging and SCC in mice, as well as malignant transformation and apoptosis resistance in human keratinocytes HaCaT cells under chronic UVA exposure [36].

The generation of *ROS*, such as superoxide radical ($O2^{\bullet -}$) and singlet oxygen ($^1O_2$), results primarily from the light-driven redox cycling of non-DNA skin chromophores (e.g., porphyrins, riboflavin, and collagen crosslinks) behaving as endogenous photosensitizers [39]. In addition, UVR leads to an enhanced activity of xanthine oxidase in human keratinocytes, increasing the production of $O2^{\bullet -}$ [40]. Usually, most ROS have a short half-life, causing damage only at a local level [38]. However, in the case of hydrogen peroxide (H_2O_2) and others oxidants, they exhibit a relatively long half-life and are able to travel long distances, which in turn leads to DNA damage at distant locations [38]. In addition, hydroxyl radical ($^{\bullet}OH$), and to a lesser extent $^1O_2$, may react with DNA and other important biomolecules [38]. Unlike these, both $O2^{\bullet-}$ and H_2O_2 are relatively unreactive towards biomolecules, as H_2O_2 requires the presence of reduced transition metals (e.g., Fe2+) to promote the Fenton-type reaction [38].

A typical UV-induced DNA lesion emerges from the oxidation of the guanine base by ROS to form 8-oxo-7,8- dihydroguanine (*8-oxo-dG*) and 8-hydroxy-2-deoxyguanosine (*8-OH-dG*) [37,41]. 2,6-Diamino-4-hydroxy-5-formamidopyrimidine (*FapydG*) is another common endogenous DNA base alteration, namely guanine [38]. In fact, under low oxygen conditions such as hypoxia, FapydG is the most predominant guanine-derived modification in DNA [38]. On the other hand, interaction of $^{\bullet}OH$ with pyrimidines (thymine and cytosine) can produce numerous base alterations, including 5,6-dihydroxy-5,6-dihydrothymine (*thymine glycol*) and 5,6-dihydroxy-5,6-dihydrocytosine (*cytosine glycol*) [38]. Thymine glycol and 8-oxo-dG are considered reliable markers of OS in cancer patients [38].

The photoproducts are repaired via a very efficient *base excision repair* (BER) mechanism initiated by the 8-oxoguanine DNA glycosylase-1 (OGG1) [41,42]. In previous studies [43], it has been shown that OGG1 knockout mice are compromised regarding the removal of 8-oxo-dG from DNA in epidermal cells after UV exposure. Therefore, after chronic UV irradiation, OGG1 knockout mice develop an expressively higher number of skin tumors when compared to the wildtype or the heterozygous models [43]. Accumulation of unrepaired 8-oxo-dG and 8-OH-dG photoproducts can eventually cause mutations, and thus lead to carcinogenesis. Huang and colleagues recently found low OGG1 protein expression in human BCC compared to overlying or normal epidermis [44]. They have also demonstrated the presence of higher levels of 8-OH-dG within the BCC when compared to the basal layers of epidermis overlying the BCC lesions [44]. These experiments highlight the importance of OS in the process of carcinogenesis, leading to new perspectives of targeted therapies for cancer management.

Reactive nitrogen species (RNS) represent another group of photoproducts that can lead to DNA lesions [41]. They are formed as a consequence of UV-induced upregulation of nitric oxide (NO) synthases, which leads to excess levels of NO, as well as through the release of NO by UVA decomposition of endogenous NO supplies [41]. The combination of excess NO and ROS will generate more genotoxic NO derivatives, such as peroxynitrite ($OONO^-$) [41]. In turn, these derivatives will cause base modifications to DNA by oxidation or nitration, and ultimately lead to mutagenesis and carcinogenesis [41]. Nitrotyrosine and 8-nitroguanosine are formed through nitration of tyrosine in proteins and guanine in DNA, respectively, and are considered the markers of inflammation and carcinogenesis [41]. Indeed, the most sensitive DNA base to oxidation is guanine, since it has the lowest oxidation potential [40].

Cells can be protected against UV-induced oxidative damage by numerous enzymatic and nonenzymatic antioxidants. The skin comprises a network of endogenous antioxidant defenses, including superoxide dismutase, glutathione peroxidase, and catalase, which are enzymes that help removing ROS before they can cause severe cell damage [40,45]. Free radical scavengers, such as vitamin E, carotenoids, glutathione, ubiquinone, and ascorbic acid are also present in the skin [40]. However, UV-induced ROS may deplete these endogenous antioxidants, leaving the skin quite vulnerable [40,45]. Therefore, systemic or topical application of certain antioxidants, such as polyphenols, helps to protect the skin against UV-induced oxidative damage.

Immunosuppression

Experimental studies performed by Kripke et al. [46] in UV-irradiated mice showed the importance of the immune response in the process of carcinogenesis, and led to the hypothesis that *UVR suppresses the immune system* both locally and systemically [19]. In other studies, it was shown that most UV-induced skin tumors in mice are immunogenic, and consequently are rejected upon transplantation into immunocompetent hosts [47,48]. On the other hand, if the animal is immunosuppressed by UVR or other methods, these tumors undergo unrestricted growth, further showing that the rejection is immunologic [47,48]. In other words, the immunosuppression induced by UVR is capable of disturbing the immunological mechanism of tumor immunosurveillance [19].

UV-induced DNA photoproducts may cause immunosuppression by: (a) modifying antigen presentation [49]; (b) depleting Langerhans cells from the epidermal layer; (c) inducting T cells [19]; (d) recruiting immunosuppressive CD11b$^+$ macrophages into the skin; and (e) by changing the cytokine environment, which is important for the cell-mediated immune response (**Fig. 3**) [48].

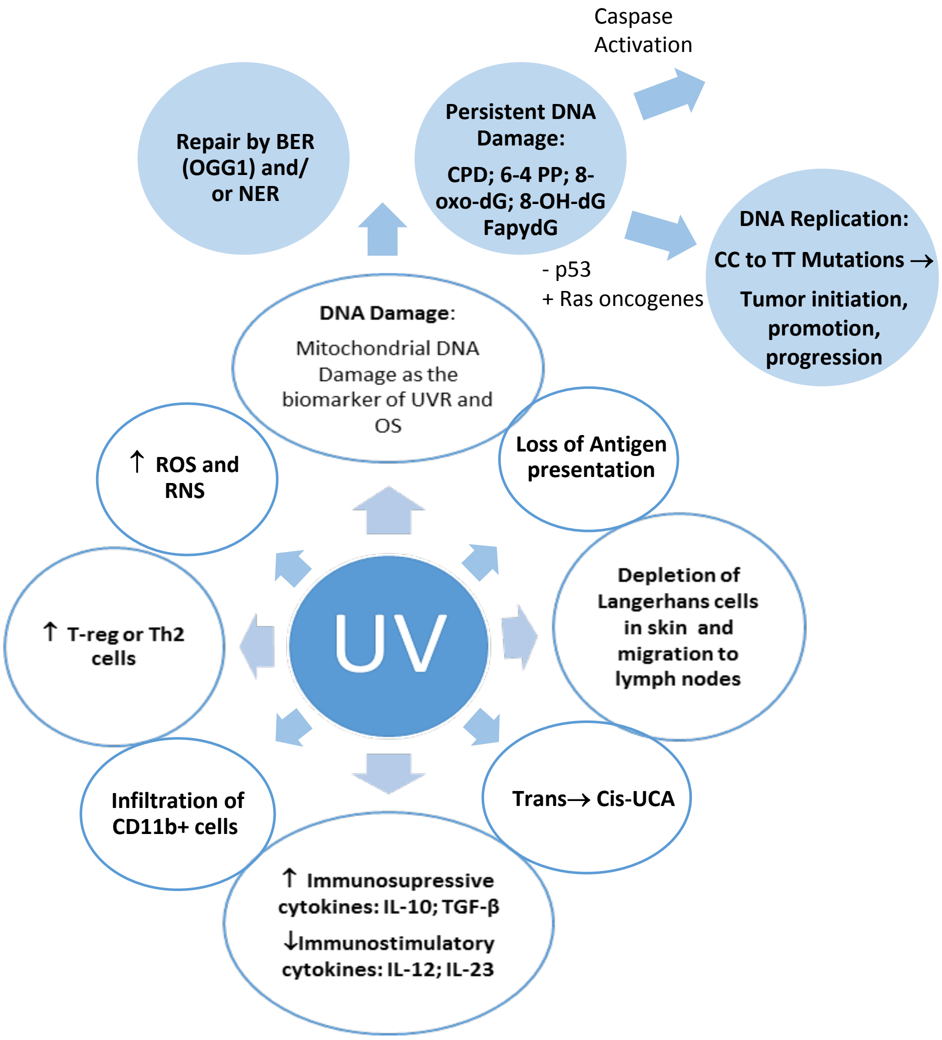


Fig. 3. Factors involved in photoimmunosupression and photocarcinogenesis processes.

Langerhans cells (LC) are a type of dendritic cells present in the skin, whose role has been reevaluated over the past few years. Being firstly known as sensory nerve endings, it is currently recognized that LC are also responsible for inducing most of the immune responses in the skin, particularly in the epidermis layer [50]. In fact, in healthy epidermis, LC are the only type of antigen-presenting cells (APC), whereas in the dermis, it is possible to perceive different hematopoietic cells, such as dendritic cells and macrophages [50]. These cells can migrate directly from the epidermis to the draining lymph nodes and promote antigen-specific T cell activation, as it happens after UVR [51]. In fact, LC and dendritic cells of lymphatic system are the major cellular chromophores absorbing UVB radiation [52].

The notion that LC are universally immunogenic has been challenged over the years. In previous studies [53,54], it has been demonstrated an immune-dampening function of LC, which can be due to the expansion of *regulatory T cells* (Treg) prompted by them. Indeed, Schwarz et al. have already suggested that the presence of UV-irradiated damaged but still viable LC in the regional lymph nodes is necessary to induce Treg cells [55]. However, further evidence reveals that immune responses can be produced in the skin without the presence of LC and that dermal dendritic cells are more significant at an immunogenic level [55]. Therefore, it has been proposed that LC are more involved in downregulating immune responses in the skin than inducing them [55].

Regarding epidermal biology, there is an intense and bidirectional communication between LC and keratinocytes [50]. Keratinocytes are capable of producing cytokines and growth factors that will have an impact on LC phenotype and functions [50]. In turn, LC are able to modulate epidermal homeostasis via helper T cells, inducing them to produce IL-22, which in turn will promote the proliferation of keratinocytes [50].

When UVB-irradiated skin is treated with haptens, higher numbers of Treg and smaller numbers of *effector T cells* are observed, which causes a shift in the equilibrium from T cell-mediated immunity to immunosuppression [48]. It has been demonstrated that hapten-induced effector T cell-mediated immunity is responsible for the secretion of interferon gamma (IFN-γ), whereas Treg express CD4 and CD25 and secrete IL-10 [48]. In fact, T cells that mediate UV-induced immunosuppression and tumor development exhibit complementary characteristics. $CD8^+$ T cells are effector cells in contact hypersensitivity (CHS) and release IFN-γ, inhibiting tumor development, whereas $CD4^+$ T cells have the opposite effect and secrete IL-4, IL-10, and IL-17 [48].

Regarding the role of *cytokines*, UV exposure can lead to an increase in the levels of immunosuppressive mediators, IL- 10 and transforming growth factor (TGF-ß), as well as a reduction in the production of immune-stimulatory molecules, such as IL-12 and IL-23 [48]. *IL-10* activates the synthesis of other proinflammatory cytokines, appearing to be a key mediator of UV-induced immunosuppression [56,57]. In fact, intraperitoneal injection of IL-10 in mice prevents delayed-type hypersensitivity (DTH) responses, whereas injection of anti-IL-10 antibody repressed UV-induced tolerance stimulation [58,59]. *IL-12* was found to accelerate the removal of UV-induced DNA lesions in keratinocytes by inducing NER. These findings strongly suggest that IL-12 plays a protective role in photocarcinogenesis [56,60]. In fact, IL-12 regulates the growth and functions of T cells, and enhances helper T (Th1-type) cells development by promoting the secretion of IFN-γ [59]. It has already been demonstrated that intraperitoneal injection of IL-12 in mice leads to the inhibition of UV-induced immunosuppression, which suggests that a cytokine discrepancy between Th1 and Th2 may be responsible for the development of immunosuppression caused by UVR [59]. Moreover, when UV-exposed mice were treated with IL-12, Treg were not induced and lost their suppressive activity [49]. IL-12 still has the ability to reduce UV-induced DNA damage, as it decreased both *in vitro* and *in vivo* UV-induced apoptotic cell death [49]. The process through which IL-12 causes the reduction of UV-induced DNA damage implies the activation of NER mechanism, since this effect was not demonstrated in *Xpa* knockout mice lacking NER [49]. Besides IL-12, IL-18 and IL-23 can also reduce UV-induced damage, ultimately preventing immunosuppression [49]. Nevertheless, only IL-23 shares with IL-12 the ability to restore immune responses due to its influence on Treg [49].

Once the electromagnetic energy of UVR is absorbed by an epidermal photoreceptor and transformed into a biological perceptible signal, that signal must be transferred to the immune system in order to induce immunosuppression [61]. Two epidermal photoreceptors have been established, *DNA* and *trans-urocanic acid* (3-(1H-imidazol-4-ly)-2-propenoic acid, UCA) [12]. While some authors [62] consider DNA damage as the main cause for immunosuppression, others [63] believe that it is due to the presence of the naturally occurring *trans*-UCA in the *stratum corneum* (SC), which upon UVR, isomerizes to the *cis* configuration with immunosuppressive effects [12,19]. Indeed, it has already been suggested that topical or systemic application of *cis-UCA* is able to inhibit cell-mediated immunity and mimic several immunomodulatory effects of UVR in mice [64]. The mechanism of action of *cis*-UCA is still unclear [64]. *Trans*-UCA is produced in the mammalian SC by L-histidine ammonia lyase (HAL, histidase) from L-histidine [65]. Urocanase is absent in the skin (unlike the liver), and therefore, the catabolism of UCA does not occur, leading to its accumulation in the epidermis layer [12,65]. Upon UV exposure, *trans*-UCA is isomerized to *cis*-UCA in a dose-dependent manner, until a photostationary phase is reached with equivalent amounts of both isomers present in the skin [12]. As the photoisomerization of *trans*-UCA to its *cis*-isomer occurs in the epidermis, keratinocytes may be a feasible target for *cis*-UCA. In previous studies [66] with primary human keratinocytes, it was revealed that *cis*-UCA upregulates UV-inducible genes (e.g., COX-2), leading to an enhanced production of immunomodulatory mediators, including prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF-α), and IL-6. The release of PGE_2 from keratinocytes has been proposed to induce the IL-4 and IL-10 production in serum, and thus inducing systemic immunosuppression [64]. However, Kaneko and colleagues [64] were unable to identify either IL-4 or IL-10 in keratinocytes upon UCA or UVR exposure.

Some authors [67] also consider that UV exposure leads to the generation of free radicals and membrane lipid peroxidation, which in turn causes immunosuppression [19]. There is also the probability that upon UVR exposure, keratinocytes secrete neuropeptides and the alpha melanocyte-stimulating hormone (α-MSH) which have immunoregulatory effects [19]. The synthesis of the lipid modulator of inflammation named platelet-activating factor (PAF) represents an alternative early UV-induced event leading to immunosuppression [13]. Commonly, UVR induces *cis*-UCA binding to skin cells and stimulates the PAF release by epidermal cells, which in turn induces the COX-2 upregulation and PGE_2 release. All these events lead to immunosuppression and photocarcinogenesis [13].

Taken together, these events result in the *downregulation of cellular immune response*, including induction and promotion of CHS, and also in the inhibition of natural killer (NK) cell activity [19]. In addition, the suppression of T-dependent antibody formation is likewise a result from UV-exposure, with mast cells-derived IL-10-producing T cell being involved in the process [68]. These immunosuppressive effects cause the development of skin cancer, not only because the cellular immune processes are capable of destroying tumor cells, but also because under UV irradiation, keratinocytes acquire a substantial cytotoxicity [19].

For a long time, it was thought that the signal transduction pathway associated with UV-induced DNA damage was unidirectional, however, the fact that various cytokines are able to control DNA repair, and consequently, UV-induced DNA damage, suggests the existence of a biofeedback process [49]. Indeed, this might represent a new defense mechanism of the host against UV-induced immunosuppression (**Fig. 4**), and potentially carcinogenesis [49].

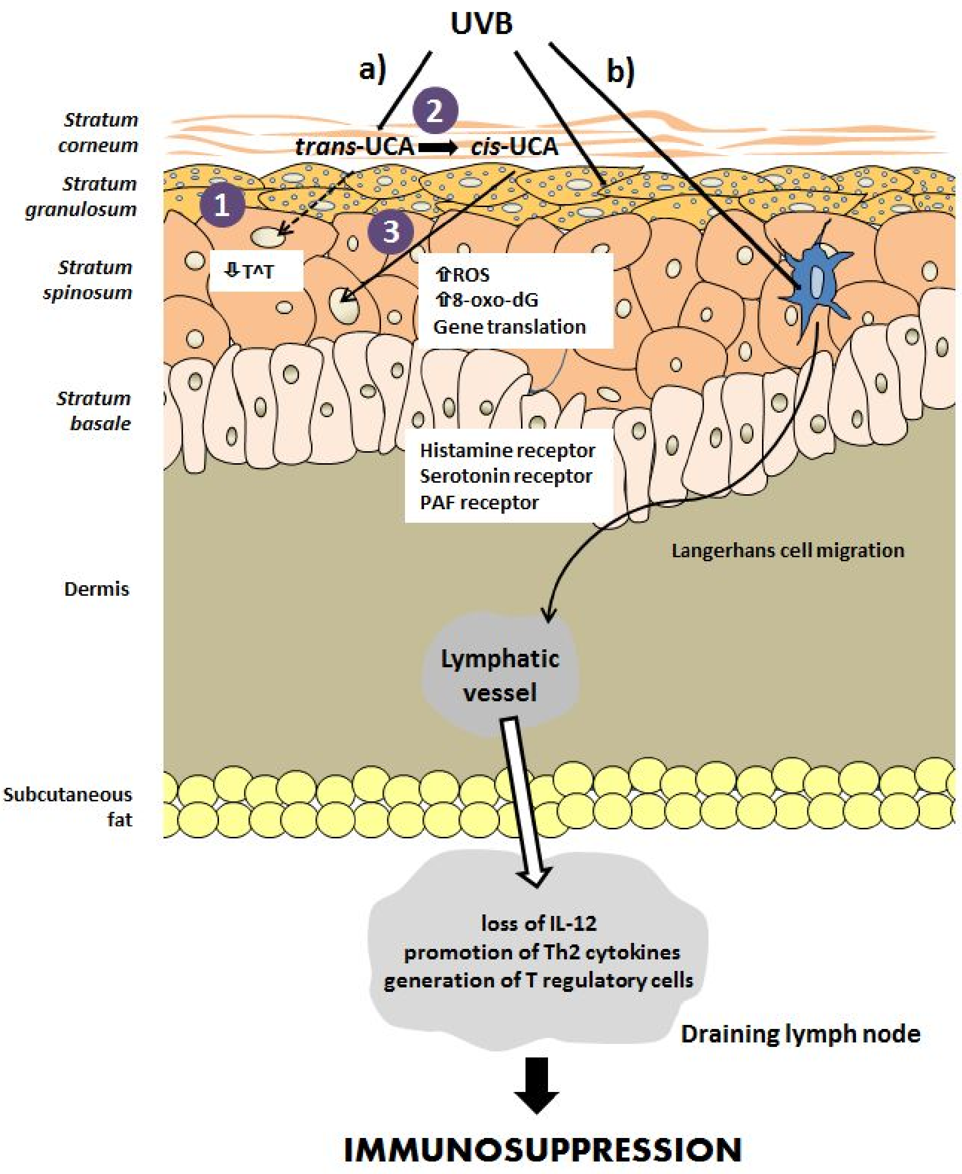


Fig. 4. Immunosuppression resulting from exposure to UVB. (a) UVB is absorbed by *trans*-UCA (in the SC) (b) and by DNA, tryptophan and membrane lipids of epidermal cells (keratinocytes and Langerhans cells). (1) *Trans*-UCA absorbs UVB and protects against thymine dimer (T^T) formation in keratinocytes; (2) *Trans*-UCA is photoisomerized in *cis*-UCA. (3) *Cis*-UCA induces intracellular ROS formation, and consequently, oxidative DNA damage (8-oxo-dG), and initiates translation of genes associated with apoptosis and immunosuppression. Acute signaling pathways are stimulated, cellular trafficking to draining lymph nodes via lymphatic vessels increases. The production of regulatory cells and soluble mediators is ultimately responsible for the downregulation of T cell responses (adapted [65,69,70]).

Keratinocytes Apoptosis

Keratinocytes, the primary cellular component of epidermis, are capable of releasing into circulation a number of soluble mediators, which in turn can affect the outcome of immune response [51].

Recent studies have focused the investigation on the regulation of *apoptosis* in the skin and its application for understanding skin carcinogenesis [71]. Acute UV irradiation causes apoptosis involving p53 and Fas-FasL pathways (Fas ligand or FasL, a member of TNF superfamily involved in the elimination of apoptotic cells and prevention of cell transformation). Chronic UV irradiation results in deregulation of apoptosis, leading to abnormal proliferation of photodamaged keratinocytes, acquisition of p53 mutations, loss of Fas-FasL interaction besides decreased death receptors, Stat3 activation and variation of survivin, Bcl-2 and Bcl-XL expression. All of these events contribute to the onset of skin cancer [52,71]. In fact, gene silencing by RNA interference may become a new approach to reverse apoptosis resistance [72], as it has been successful in tissue cultures and tumor tissues, as well as, in a mouse model [73].

As already mentioned, phosphorylated p53-induced transcription of p21 causes G/S cell cycle arrest, allowing the cellular repair pathway to remove DNA lesions before DNA synthesis and mitosis [52]. However, if the damage persists into the S-phase, other repair mechanisms might lead to mutagenesis resulting mainly in the characteristic C to T substitutions. When such mutations occur in the p53 gene, keratinocytes lose their ability to undergo apoptosis following UVR exposure. The *degenerative changes in keratinocytes* include mitochondrial swelling and rupture, condensation of the cytoplasm and the appearance of pyknotic nuclei. Macrophages bind and phagocytize apoptotic keratinocytes and their number increase significantly in the skin after UVB exposure [33,56,74-76].

Excessive UVR leads to calcium increase in keratinocytes, resulting in the activation of the inflammasome (a multiprotein of the innate immune complex) and in the synthesis and release of IL-1 [56,77]. In addition, UVR induces the synthesis of granzyme B and perforin in keratinocytes (proteins only present in cytotoxic lymphocytes and NK cells), transforming them. This cellular alteration strongly suggests that UV-irradiated keratinocytes participate in skin cancer surveillance [78].

Keratinocytes apoptosis contributes to the regulation of epidermal development and carcinogenesis restrain. The extrinsic pathway (via cytoplasm) is stimulated by UVB or binding of FasL, TNF, or other cytokines to death receptors that results in the activation of a caspase cascade. The intrinsic pathway (via mitochondria) is also stimulated by UVB and involves mitochondrial depolarization and higher membrane permeability, leading to the release of multiple proapoptotic factors including cytochrome c, Smac, and the apoptosis inducing factor (AIF) [79]. Bid protein links both extrinsic and intrinsic pathways, and the activated Bid fragment will cause the mitochondrial content release. Activation of upstream caspases (caspase-8 or -9) leads to the activation of downstream caspases (caspase-3 or -7) resulting in the cleavage of intracellular substrates, cellular condensation and nuclear fragmentation. Apoptosis inhibitors include caspase inhibitors and the big complex Bcl-2 family proteins, most of which prevent mitochondrial membrane permeability [71].

Caspase-14 is a keratinocyte-specific caspase only found in skin. It is only expressed in HaCaT cells and normal human keratinocytes under culture conditions mimicking terminal differentiation. In addition, death receptor signaling or UVB irradiation could not induce caspase-14. Although the targets of activated caspase-14 have not been defined yet, it seems to correspond to focal epidermal hyperplasia [71].

Serotonin: A Key Mediator between the Skin and the Neuroendocrine System

Serotonin or 5-hydroxytryptamine (5-HT) is a key mediator between the skin and the neuroendocrine system [5], as it acts in both central and peripheral nervous system [80]. Biosynthesized from L-tryptophan, 5-HT is widely distributed throughout the body. Nevertheless, the greatest concentration of this signaling molecule is found in enterochromaffin cells and platelets. There are many different sources of 5-HT in the human skin, such as keratinocytes, melanocytes, dermal fibroblasts, Merkel cells, Langerhans cells, mast cells, T cells, NK cells, sensory nerve endings, and mainly platelets (upon aggregation) [5,80].

There are seven general families of *5-HT receptors* with at least 21 subtypes. Skin cells express membrane-bound receptors for 5-HT (mainly 5-HT1R and 5-HT2R), as well as transporter G-proteins, and can stimulate (5-HT7R) or attenuate (5-HT1R) adenylate cyclase activity or enhance phosphoinositol hydrolases activity (5-HT2AR) [5].

The effects of 5-HT are associated with many *neurological disorders*, such as Alzheimer’s and Parkinson’s diseases [7]. In the gastrointestinal tract, it is involved in pain perception, fluid secretion, regulation of ion transport and intestinal motility [7]. Furthermore, 5-HT is implicated in the regulation of bone formation and resorption [7]. Liver regeneration, platelets mechanisms, and regulation of blood pressure are other processes in which 5-HT is involved as a mediator [7]. In fact, this mediator has additional effects on cardiovascular function and breath control [7]. Proinflammatory mediators can also be produced upon 5-HT activation of immune cells [7]. Particularly in the skin, 5-HT is involved in vasodilatation, inflammation (associated with mast cells secretion), immunomodulation, and pruritogenic effects [80]. In fact, 5-HT1AR modulates the skin barrier and, at the same time, might promote inflammation by prolonging the lifespan of inflammatory cells [5]. However, a complete understanding of signaling cascade for the different 5-HTR subtypes in various cells, including skin cells, is still needed.

Synthesis and Metabolism of Serotonin in the Skin

The *biosynthesis of 5-HT* starts from L-tryptophan which is converted to 5-OH-tryptophan (5-HTP) by tryptophan hydroxylase (TPH) in the presence of Fe^{2+}, the reducing cofactor 6-tetrahydrobiopterin (BH4) and molecular oxygen [5] [81]. This conversion is the initial and rate limiting step of 5-HT synthesis. The TPH enzyme exists in two forms: TPH1, the main enzyme expressed in peripheral tissues and TPH2, which is mainly found in central nervous system [3,5]. The final reaction is catalyzed by the ubiquitously expressed L-aromatic amino acid decarboxylase (L-aromatic AAD), and implicates the pyridoxal phosphate cofactor [5]. The initial substrate (*tryptophan*) is not synthesized by the body, and therefore it must be obtained through the diet [5]. This essential amino acid exists in plasma at steady state in a free form, possibly taken-up by the cells and reversibly associated with serum albumin [5]. Tryptophan levels show some oscillations through time, which can arise from stress, diet, age, and gender [5]. The physiological levels of *BH4 cofactor* are associated with a regulatory mechanism of 5-HT synthesis, in which BH4 potentiates the nitric oxide attack on free protein thiol groups, and consequently, the inactivation of TPH [3]. It has already been shown the capacity of skin in synthesizing this important cofactor [5]. Another essential condition for the synthesis of 5-HT is the presence of *Fe^{2+}*, which leads to the inherent inhibition of TPH by the oxidized form of iron (Fe^{3+}), and to the intimate relationship between TPH activity and the redox state of the cell [3]. Additionally, the regulation of TPH activity can be performed by means of phosphorylation or by stress hormones [3]. Findings from previous studies [82] indicated that phosphorylation by protein kinase A (PKA) increases the V_{max} of TPH, maintaining the K_m value constant. In another study [83], it was also found that UVR is capable of inhibiting TPH synthesis in SCC C1-4 cells and human melanoma cells, which may suggest a possible regulatory mechanism of the TPH following external environment stimulation. Considering that THP is the first key regulatory point of both 5-HT and melanin synthesis, it could be an interesting therapeutic target in melanoma and/or pigmented disorders [84].

On the contrary, *monoamine oxidase* (MAO) expressed in the skin deaminates 5-HT by converting it to 5-hydroxy-3-indolacetaldehyde (5-HIAL), which is then transformed to 5-hydroxy-3-indolacetic acid (5-HIAA) by an isoform of aldehyde dehydrogenase (ALDH2) and 5-hydroxytryptophol (5-HTPOL) by aldehyde reductase (ALDR) or alcohol dehydrogenase (ADH) [5,81]. The contribution of MAO for the production of 5-HIAA and 5-HTPOL was further confirmed in previous studies [85] using pargyline (specific MAO inhibitor) in rodent skin [3,5]. The levels of these metabolites were analyzed in both mouse and rat skin, and the results obtained revealed the production of 5-HIAA and 5-HTPOL in mouse skin [86] and almost only 5-HIAA in rat skin [85], with 5-HIAA being the major degradation product (**Fig. 5**).

MAO is located in the outer mitochondrial membrane of the cells and has two isoforms, MAO A and MAO B, being the first one more selective for 5-HT oxidation with a much lower K_m value, and consequently, with a higher affinity for the substrate compared to MAO B [81]. As a redox cofactor, flavin adenine dinucleotide (FAD) participates in the reaction catalyzed by MAO and is reduced to $FADH_2$ when the amine group of 5-HT is converted to imine [81]. Afterwards, $FADH_2$ is oxidized back to FAD converting oxygen into hydrogen peroxide and, in the final step, the imine group is hydrolyzed forming aldehyde and ammonia [81].

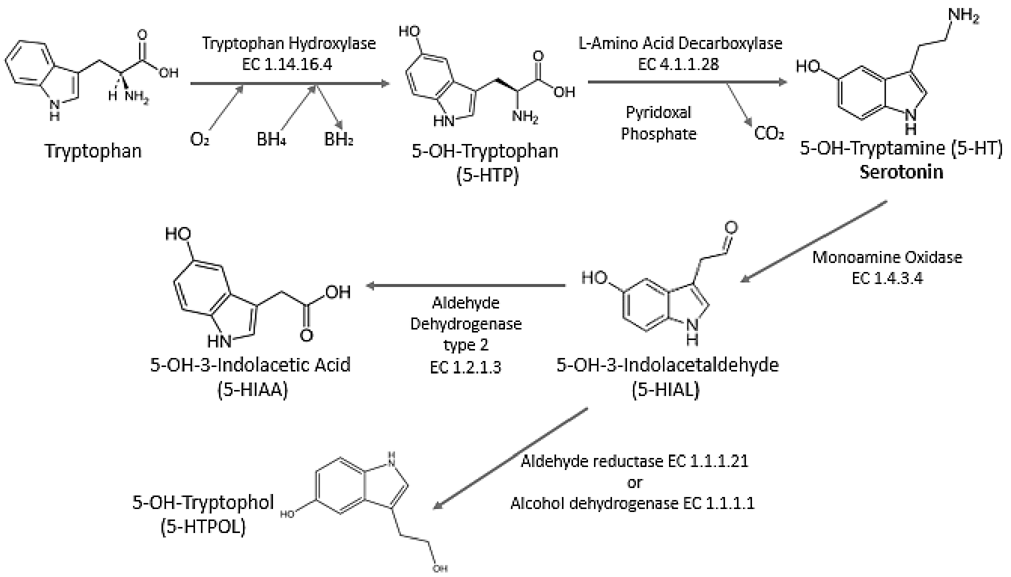


Fig. 5. Schematic illustration of 5-HT metabolism in the skin.

Location and Transport of Serotonin in the Skin

The skin is a dynamic and complex organ organized in three structural layers: epidermis, dermis, and subcutis [87]. The epidermis is the outer layer whose main cells are keratinocytes, responsible for the production of keratin. It is the presence of different stages regarding keratin maturation that subdivides epidermis into four separate layers: *stratum basale*, *stratum spinosum*, *stratum granulosum*, and *stratum corneum* [87], ordered from the inner to the outermost layer. The skin dynamic character is due to the synthesis of new cells and its continuous movement towards the outer layer of this organ. *Stratum basale* contains essentially keratinocytes, melanocytes and Merkel cells, which are generally associated with cutaneous nerves [87]. Langerhans cells are located in the *stratum spinosum,* and are specialized in presenting antigens that may have a role in the modulation of skin immune functions through storage and release of monoamines [87]. The thin papillary and the thicker reticular layers of dermis contain fibroblasts which produce collagen, elastin and structural proteoglycans, among others [87]. The blood and lymphatic vessels, as nervous cells and fibers, can be located in the hypodermis or subcutis, which is a thick layer of fat and connective tissue below the dermis. This layer conserves body heat and absorbs shock to protect underlying tissues and organs from injury [88].

TPH can be found in a variety of tissues throughout the body, particularly in skin where the protein itself may be detected in extracts of whole skin, blood vessels, activated T cells, mast cells, normal epidermal melanocytes and melanoma cells, cultured normal epidermal and immortalized HaCaT, SCC and dermal fibroblasts [3,5]. Additionally, it has already been evidenced by enzymatic activity assays, Western Blot analysis and other techniques, the rapid turnover of TPH in skin, and its presence along with 5-HT in extracts of human skin cells, emphasizing the predominance of this enzyme in normal and malignant melanocytes along with its metabolic products [3,84]. The presence of L-aromatic AAD mRNA in epidermal keratinocytes and melanocytes was also reported [89].

5-HT and its major degradation product 5-HIAA (**Fig. 5**), as well as the presence of 5-HT transporter, were detected in immortalized HaCaT keratinocytes through the use of immunocytochemistry and reversed phase HPLC techniques [3]. 5-HT was also found in skin mast cells by positive immunohistochemical staining [5]. Nevertheless, there is a propensity to find TPH protein and 5-HT in normal and malignant melanocytes under *in vivo* conditions, which may suggest that 5-HT synthesis pathway in the skin might occur mainly in *melanocytic cells* [3]. Secretion of 5-HT by melanocytes and mast cells is important for local cell communication in the skin [5]. Although the involvement of intracellular granules and a Ca^{2+} influx in 5-HT secretion by mast cells has already been evidenced, other forms of 5-HT release are possible, including the reuptake by SERT [5], a 5-HT transporter (5-HTT). SERT is a protein with 12 membrane-spanning domains and a sodium and chloride-dependent transporter with the capability of release and reuptake 5-HT, especially the last one [5]. The extracellular levels of 5-HT must be firmly controlled by SERT in order to achieve its crucial functions in the body [81]. However, in cases where SERT is knocked out, other molecules can assist as a low affinity backup 5-HT transporter, such as the dopamine transporter (DAT) and polyspecific organic cation transporters OCT-1 and OCT-3 [90].

Merkel cells, which are highly specialized cells in contact with many axon terminals that express SERT, also secrete 5-HT, and indirectly regulate sensory neurons through 5-HTRs [5]. Similarly, dendritic cells express functional SERT according to their phase of maturation: mature dendritic cells transport 5-HT between activated and naïve T cells, thereby amplifying immune responses [91]. The expression of SERT in malignant melanoma was also detected [5], as well as in Langerhans cells, lymphocytes, macrophages and HaCaT cells [2].

5-HT and other monoamines are transported from the cytoplasm into vesicles via *vesicular monoamine transporter* (VMAT) using the proton gradient generated by an ATPase [7,92]. There are two VMAT isoforms: VMAT1, which is found exclusively in neuroendocrine cells and VMAT2, the neuronal isoform [7]. In previous studies [92], it was reported that VMAT2 mediates the storage of monoamines in vesicles of normal and tumor cutaneous mast cells. Unlike to what it would be expected, VMAT2 and not VMAT1, was also expressed in Langerhans cells [92].

In the plasma, 5-HT is mostly confined to *platelets*, which have an active 5-HT transport system through the action of SERT and storage of 5-HT in dense granules [93]. The membrane of platelets contains two different 5-HT binding sites, one mediating the uptake of 5-HT, and the other one causing platelet aggregation [93]. There is also a passive uptake mechanism that occurs at a high extracellular 5-HT concentration [93].

Serotonin Receptors in the Skin and their Serotonergic Actions

Serotonergic membrane-bound receptors can be found throughout the entire body and are categorized into seven general families from 5-HT1R to 5-HT7R, with at least 21 subtypes [5,93]. 5-HTRs are coupled to G-proteins, except 5-HT3R, which is a ligand-gated ion channel [2,5,93]. The physiological processes in which 5-HTRs are involved, considering that its signal transduction pathways are different, lead to opposite effects. Thus, 5-HT4, 5-HT6, and 5-HT7R are capable of stimulating cAMP production, whereas 5-HT1R, including 1A, 1B, 1D, 1E, and 1F subtypes, inhibits this production [2]. Additionally, 5-HT1AR is involved in membrane hyperpolarization by coupling to potassium channels [2]. 5-HT2R, including 2A, 2B, and 2C subtypes, can promote phosphatidylinositol hydrolysis [2]. 5-HT5R (5A and 5B subtypes) is thought to be an orphan receptor [2].

The *regulatory activity of 5-HTRs* is modulated by RNA editing, endogenous lipids acting as allosteric modulators, 5-HT moduline acting as an endogenous tetrapeptide with an antagonistic effect on the inhibition of 5-HT release [94], and lastly by 5-HT transporters, whose function affects the levels of 5-HT inside and outside the cells [2]. The structural and functional diversity of these receptors is due to alternative splicing and a different combination of subunits into the receptor complex [2].

Particularly in human skin, it has already been detected the expression of genes coding for 5-HTRs, such as 5-HT-1AR, 1BR, 2AR, 2BR, 2CR, and 7R genes (**Table**

1). The pattern of expression is cell type-specific and can be altered by skin pathology [2].

Table 1. Serotonergic receptors or serotonin transporter (SERT) distribution in human skin of atopic dermatitis patients (adapted from Kim et al. [80]).

Skin Cell	Receptors
$CD3^+$Tcell	5-HT1A, 5-HT2A, SERT, H4R(?)
Langerhans cells	5-HT2A, SERT
Keratonocyte	5-HT1A
Melanocyte	5-HT1A, 5-HT2A
Mast cell	5-HT1A, 5-HT2A, SERT
Fibroblast	5-HT1A
Macrophage	5-HT1A, 5-HT2A, SERT
Dendritic cell	5-HT1A, 5-HT2A, SERT
Langerhans cells	5-HT2A, SERT
Nerve	5-HT1A, 5-HT2A, 5-HT3, SERT

5-HT1 Receptors

5-HT1AR is the best characterized within this family, and is related with the effects of 5-HT in murine and human mast cells [8,95]. The expression of 5-HT1AR in human mastocytoma was also previously found [5]. In addition, mast cells adhesion and migration are mediated by this receptor [96] during the process of inflammation.

Since 5-HT1AR is also expressed in the apical region of epidermis, chronic stress (e.g., in eczema) can contribute to decrease its levels, and consequently, affect the skin barrier [5]. Besides mast cells, 5-HT1AR has already been found in keratinocytes, melanocytes, dermal vasculature, malignant melanomas, and rodent skin dermal and epidermal immune cells [2]. The presence of 5-HT1AR on the upper epidermis suggests a vital role in the differentiation process of keratinocytes, including Ca^{2+} flux, phospholipase activation, adenylate cyclase activity, and prevention of apoptosis [97].

5-HT1AR is involved in fundamental events within the serotonergic system, such as modulation of inflammatory cells behavior; induction of phagocytosis; protection of NK cells from OS; promotion of cell survival and proliferation of mitogen-activated T and B lymphocytes; induction of eosinophils migration; modulation of cytokine secretion from dendritic cells; and inhibition of apoptosis in neuronal cells [96,97].

5-HT2 Receptors

5-HT2AR are present in dermal lymphocytes, fibrocytes, vasculature, sensory nerve endings, malignant melanomas, and in rodent skin dermal and epidermal immune cells [2]. Activation of this receptor results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate, a minor membrane phospholipid, by phospholipase C (PLC), leading to the formation of two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [98]. The first reaction product is involved in numerous physiological events by releasing Ca^{2+} from intracellular storage, and the second product activates protein kinase C (PKC), resulting in the phosphorylation of target proteins in cell [98]. Additionally, 5-HT2R might mediate the chemoattractive potency of 5-HT for eosinophils in various mammalian species [5].

The 5-HT2R family is divided into three different subtypes: 2A, 2B, and 2C. 5-HT2AR is the most extensively studied, whereas 5-HT2CR is the most predominant subtype in human skin [5]. In previous studies [2,99], it was revealed the presence of 5-HT2CR in fibroblasts, epidermal Langerhans cells and melanocytes, as well as in rodent skin dermal and epidermal immune cells. It was also found an alternatively spliced form of 5-HT2CR in human melanoma [2]. The activation of 5-HT2CR causes an enhanced intracellular buildup of inositol phosphates and Ca^{2+}, which results in cellular excitation [99]. In addition, it was suggested that 5-HT2CR activation promotes inflammation *in vivo* and the maturation process of dendritic cells, such as Langerhans cells [99].

The 5-HT2BR subtype is expressed in rodent skin and melanomas, as well as in immortalized mouse follicular melanocytes [2,5]. It was also found in normal human skin and skin affected by BCC [2].

The presence of 5-HT2AR in the upper epidermis of normal and eczematous skin was observed in previous published studies [3]. As already mentioned, the levels of 5-HT1AR may decrease in inflammatory skin disorders, whereas 5-HT2AR levels may increase in this situation [2]. Regarding treatments with 5-HT2AR antagonists, a decline of contact allergic reactions in mice has been observed [100].

Despite keratinocytes being an important target for the action of *cis*-UCA, Kaneko et al. [64] demonstrated the existence of a 5-HT2A receptor-independent pathway for this photoreceptor in human keratinocytes. Additionally, it is believed that the activation of epidermal growth factor receptor is involved in the process, as it leads to the expression of COX-2 followed by PGE_2 production through UV-induced ROS [64]. Furthermore, it has already been shown that immunomodulatory mediators from keratinocytes are less influent in UV-induced immunosuppression than those form bone marrow-derived dendritic cells and monocytes [101]. Thus, keratinocytes may not be the main target for *cis*-UCA induced immunosuppression, remaining undefined the role of 5-HT2AR in other cell types in the skin [64].

Therapeutic Potential of Serotonergic Drugs in the Photocarcinogenesis Context

Mechanism of Action of 5-HT1/2R Agonists and Antagonists

Immunomodulation

There is a connection between UV-induced immunosuppression and skin carcinogenesis, and therefore, drugs that block the first case should also prevent UV-induced skin cancer [61].

5-HTRs also participate in skin reaction to UVR [2,5], particularly 5-HT2AR. Walterscheid et al. [61] demonstrated that *cis*-UCA competitively binds to 5-HT2AR due to its chemical similarity with 5-HT [64], and activates cells via the same receptor. This binding has been further confirmed *in silico* [102] and experimentally in multiple sclerosis patients [103], as well as through saturation studies and competitive binding assays to determine the capacity of radiolabeled *cis*-UCA to bind to 5-HTR. The results obtained revealed that excess *cis*-UCA displaced the binding of ^{14}C-*cis*-UCA or ^{14}C-5-HT in Sf9 insect cells expressing human 5-HTR [61]. Additionally, it was demonstrated that specific 5-HTR and PAF antagonists, namely PCA-4248, obstructed the binding of radiolabeled *cis*-UCA and 5-HT [61]. The confirmation of a structural resemblance between *cis*-UCA and 5-HT was assessed via immunoprecipitation studies, showing that the ring-like structure obtained by *trans*-to-*cis* isomerization of UCA is immunologically equivalent to the epitope recognized by the anti-5-HT antibody [61]. It was also previously demonstrated [104] that *cis*-UCA (not *trans*-UCA) was capable of mobilizing intracellular Ca^{2+} storages within cells expressing 5-HTRs. Subsequent *in vivo* experiments have demonstrated that UVR or *cis*-UCA-induced immunosuppression was blocked in mice previously treated either with anti-*cis*-UCA or anti-5-HT antibodies [61]. In fact, these results show that activation of 5-HT2AR either by endogenous 5-HT or *cis*-UCA has an important role in UV-induced immunosuppression [12]. In addition, *cis*-UCA has the ability to inhibit cellular immune reactions, including DTH responses to HSV [32]. Nevertheless, it was also proposed that *cis*-UCA and

5-HT regulate UV induced immunomodulation through independent pathways [2,5].

Unlike Slominski et al. [105], Kaneko and colleagues showed that 5-HT2AR mRNA was absent in 11 primary human keratinocytes lines, even 6 h after UVR exposure [64]. In addition, it was demonstrated that 5-HT stimulated IL-6 release via interactions with 5-HT1R, 5-HT2R and/or 5-HT7R, but had no effect on PGE_2 or TNF-α release [64]. Similarly, *cis*-UCA also enhanced IL-6 release, which in turn was not prevented by a 5-HTR antagonist, suggesting that 5-HT and *cis*-UCA promote IL-6 release through different processes in human keratinocytes [64]. *Cis*-UCA increased PGE_2 and TNF-α release, but none was inhibited by a 5-HTR antagonist [64]. These findings advocate the existence of a 5-HT2AR-independent pathway for *cis*-UCA in human keratinocytes, being possible that the immunomodulatory mediators from other cells types, such as dendritic cells and monocytes [101], play a more imperative role in UV-induced immunosuppression.

Since there are numerous 5-HTR subtypes, it is important to know whether blocking or activating them has an influence on *cis*-UCA-induced immunosuppression, as their activation may lead to different immunological consequences [61]. For instance, 5-HT1R is involved in the *induction of T and B cells proliferation*, whereas 5-HT3, 5-HT4, and 5-HT7 receptors *stimulate monocytes to release cytokines* [61]. Thus, it has been revealed that 5-HT2AR antagonists prevent UV- and *cis*-UCA-induced immunosuppression, and that treating mice previously injected by *cis*-UCA with a 5-HT1B/DR agonist (zolmitriptan) also blocked immunosuppression [61]. Likewise, 1-(1-Naphthyl)piperazine (1-NPZ) both a 5-HT2AR antagonist and a 5-HT1R agonist, prevented immunosuppression due to the suppression of *cis*-UCA binding to the 5-HT2AR or to the activation of immune cells through the 5-HT1R [61].

Other studies conducted by Sreevidya et al. [13] showed that PAF and 5-HT2AR antagonists prevent skin cancer induction and progression. When PAF and 1-NPZ were injected into mice treated with a single UV dose, UV-induced skin lesions, including apoptosis and cytokine secretion, were blocked. In addition, it was demonstrated that both molecules only act synergistically (when injected as a mixture of 500 pmol each) to block skin cancer induction [13]. These findings indicate that PAF and 5-HT2AR antagonists affect UV-induced carcinogenesis by firstly blocking UV-induced immunosuppression (**Fig. 4**), and secondly by preventing UV-induced lesions on the skin [13].

DNA Repair

Among the several studies performed until now, one finding stands out. Sreevidya et al. [11] have demonstrated that, apart from reverting UV-induced immunosuppression and photocarcinogenesis, PAF and 5-HT2AR antagonists are also capable of modulating DNA repair. Thus, both CPDs and 6-4 photoproducts were removed upon treatment with PAF and 5-HTR antagonists [11]. Considering that this is a NER-dependent mechanism, it was also found that these agents led to acceleration in the unscheduled DNA synthesis, which in turn accelerated NER [11]. Hence, UV-induced immunosuppressive mediators, including PAF and *cis*-UCA, prevent DNA repair *in vivo* [11].

In addition, treatment with PAF, *cis*-UCA and 5-HT in mice induces ROS, which leads to the formation of oxygen radical and DNA damage, as it has already been shown by the 8-oxodG formation [11]. In fact, inhibition of *cis*-UCA and PAF binding to their receptors suppresses ROS and 8-oxo-dG formation, suggesting that PAF and 5-HTR antagonists revert UV-induced immunosuppression and photocarcinogenesis by modulating DNA repair [11]. Therefore, these agents can *induce DNA repair* in mice by two different paths: accelerating the repair of both CPD and 6-4 photoproducts and by decreasing the ROS-induced lesions [11]. Indeed, both paths might be connected, as it has already been revealed that ROS can adversely affect the NER mechanism [11].

Cells Proliferation

The proliferation of cells is a process on which 5-HT has also an important effect. 5-HT induces the growth of dermal fibroblasts in a dose-dependent manner, which is consistent with its established mitogenic activity in mesenchymal cells, including non-skin fibroblasts [105]. On the other hand, 5-

HT has contradictory effects in the case of immortalized epidermal melanocytes, as it stimulates their growth in the absence of melanocytes growth supplements, and inhibits melanocytes proliferation in the presence of these factors [105]. This effect could be regulated through stimulation of intracellular accumulation of cAMP, as this is an essential second messenger for the proliferation of cultured human melanocytes [105]. The latter effect suggests that 5-HT might also antagonize proproliferative growth factors signaling in melanocytes [105]. This paradox can be explained based on the role of 5-HTR on both proliferation and apoptosis regulation [3,5]. In addition, it has been suggested that 5-HT influences melanogenesis due to the observed inhibition of this process in a human melanoma cell line and the inhibition of melanoma cells melanization through the actions of 5-HT-uptake inhibitors [3,5]. 5-HT also affects the proliferation of murine keratinocytes, whereas the metabolites N-acetylserotonin (NAS) and 5-methoxytryptamine (5-MT) were able to stimulate and inhibit melanoma cell proliferation, respectively, at very high ligand concentrations [5]. In general, 5-HT affects the skin cells proliferation, sustaining receptor mediated and/or non-receptor mediated mechanisms of action.

Examples of 5-HT1/2R Agonists and Antagonists

The use of *agonists* and/or *antagonists* directed towards specific 5-HTR may be valuable for the treatment of skin diseases, as this organ produces 5-HT. There are several examples of 5-HT1 and 5-HT2R agonists/antagonists with different binding affinities and physicochemical properties, as described in **Tables 1** and **2** (**Supplementary files**).

To potentially treat photocarcinogenesis through the use of these agents, its topical administration through ultradeformable vesicles (e.g., transfersomes or ethosomes) would be preferential in order to promote a direct action at UV-induced skin lesions. Among the several 5-HT2R antagonists, ketanserin is the most widely used one. This selective 5-HT2AR antagonist is also present in numerous topical formulations with different applications within the large spectrum of action of 5-HT. In previous studies [106,107], ketanserin formulated in a mixture of gelatin, glycerol, and kaolin dissolved in distilled water has been successfully delivered via topical application onto the skin of rats. Nevertheless, topical application of 5-HTR agonists and/or antagonists for the treatment of skin diseases, including skin cancer, still awaits further potential development.

On the other hand, the reuptake process of 5-HT can be inhibited by several antidepressant drugs (e.g., paroxetine, fluoxetine, etc.) named selective serotonin reuptake inhibitors (SSRIs) that represent an important therapy for a number of conditions involving 5-HT levels [5,108]. The effects of SSRIs can be altered by the common 5-HT promoter polymorphism 5-hydroxytryptamine transporter-linked polymorphic region (5-HTTLPR) [109]. The short (S) allele in the 5-HTTLPR is associated with reduced transcription of the promoter compared with the long (L) allele [109,110], which comprises an A/G single nucleotide polymorphism (SNP) that results in L-like and S-like allelic variants [109]. Furthermore, it was found that the 5-HTTLPR S-like allelic variants are associated with poor SSRI receptiveness, which may be due to reduced 5-HT availability in S-like allelic variant carriers [109].

Conclusions and Future Directions

Serotonin or 5-HT is a crucial molecule that mediates a wide range of physiological mechanisms, and therefore, it has been associated with numerous diseases. As herein revised, specific serotonergic drugs may play a crucial role regarding the photocarcinogenesis management. In fact, the therapeutic application of 5-HT2AR antagonists is based on blocking the binding of *cis*-UCA, an UV photoreceptor present in skin, and thus preventing not only immunosuppression, but also UV-induced skin lesions. The mechanism by which 5-HT2AR antagonists prevents immunosuppression involves the modulation of DNA repair through an acceleration of NER, as well as their intervention in blocking ROS formation. 5-HT1AR agonists also block UV-induced immunosuppression and modulate the inflammatory process.

As future perspectives, it would be interesting to test the effects of some 5-HT1A agonists and 5-HT2AR antagonists for photocarcinogenesis management. This therapeutic application will require a better understanding of 5-HTRs, their signal transduction, and the functional outcomes of their interactions. Finally, the topical administration of those agents would be an interesting and challenging field of research.

Authors Contributions

ACM did the main bibliographic search and wrote the main sections of the manuscript. AA/SS and SR/HR/HO revised the photocarcinogenesis and serotonin sections, respectively. All authors participated in review’s design, coordination, and final revision.

Conflict of Interests

All authors declare that there are no conflicts of interest.

Supplementary Files

Table 1. 5-HT1R Agonists: chemical, biological and therapeutic properties of new and commercial drugs [111-118]. NF = Not Found; NA = Not Applicable.

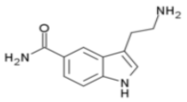
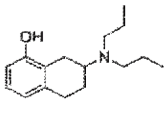
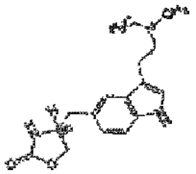
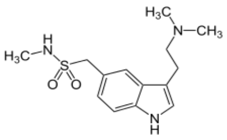
Generic Name	**IUPAC Name**	**Molecular Formula**	**Chemical Structure**	**Molecular Weight (g/mol)**	**Solubility (~25°C)**			**Log P**	**IC50/ Ki**	**Biological Activity**	**Stage of Drug Development**	**Therapeutic Action**	**Route of Administration**
5-Carboxamidotryptamine (5-CT)	3-(2-aminoethyl)-1H-indole-5-carboxamide	$C_{11}H_{13}N_3O$		203	DMSO NF	Water 10 mg/mL	Ethanol NF	0.34	IC50= 0.95 nM	Agonist at **5-HT1A**, **5-HT1B**, **5-HT1D**, **5-HT5** and **5-HT7** receptors	*In vitro* and *in vivo* studies	NA	Topical, intravenous, intrathecal and subcutaneous
8-OH-DPAT	7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol	$C_{16}H_{25}NO$		247	DMSO NF	Water NF	Ethanol NF	3.54	IC50=117 nM	Selective 5-HT1 agonist with high affinity for subtype 5-**HT1A** receptor	*In vitro* and *in vivo* studies	NA	Systemic
Zolmitriptan	(*S*)-4-({3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl}methyl)-1,3-oxazolidin-2-one	$C_{16}H_{21}N_3O_2$		287	DMSO 58 mg/mL	Water < 1 mg/mL	Ethanol 58 mg/mL	1.6 - 2.25	Ki =0.6 nM (5-HT1BR) 5 nM (5-HT1DR) 63 nM (5-HT1FR)	Potent, selective 5-HT1B/1D/1F receptor agonist. Acts as a **full agonist** at the 5-**HT1B** receptor and as a **partial agonist** at 5-**HT1D** and **5-HT1F**. Shows sensory nerve specific restorative effects. Shows antispasm effects *in vivo*	On the market since 1997: AscoTop®, Nomi®, Zolmiles®, zolmiptriptan®, Zolmit®, Zomig®, Zomig Rapimelt®, Zomigon®, Zomigoro®, Zomitan®	Acute treatment of adult migraine with or without auras	Nasal (5 mg) and oral (tablets 2.5, 3.5 and 5 mg)
Sumatriptan	1-[3-(2-Dimethylaminoethyl)-1*H*-indol-5-yl]-*N*-methyl-methanesulfonamide	$C_{14}H_{21}N_3O_2S$		295	DMSO 83 mg/mL	Water 83 mg/mL	Ethanol < 1 mg/mL	0.74 - 1.17	Ki= 17 nM (5-HT1DR) 27 nM (5-HT1BR) 100 nM (5-HT1AR) EC50 = 247 nM (5-HT1FR)	**5-HT1 receptor agonist**. Also shows affinity for 5-HT1F receptor. Reduces vascular inflammation associated with migraine	On the market since 1993: Adracon®, ALSUMA®, Altaxa®, Apigrane®, Boots Pharmacy Migraine Relief®, Cinie®, Frimig®, Illument®, Imigen®, Imigran®, Imigran mite®, Imigran Radis®, Imigrane®, Imitrex®, Imitrex Oral®, Migralgin®, Migraneitor®, Nazdav®, Sapphirex®, Sumatran®, Sumax®, Suminat®, Youshu®, Zecuity®	Treatment of migraine attacks with or without aura	Subcutaneous (4 mg/0.5 mL and 6 mg/0.5 mL), transdermal, nasal (5 and 20 mg; 5 and 20 mg/act solution) and oral (tablets 25, 50 and 100 mg)

Table 2a. 5-HT2R Antagonists: chemical, biological and therapeutic properties of new drugs [111-118]. NF = Not Found; NA = Not Applicable. Note: some solubility data may vary depending on the literature source.

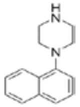
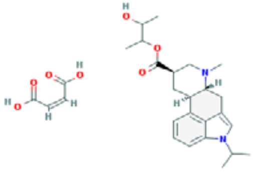
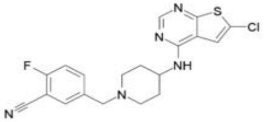
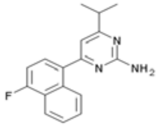
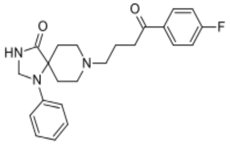
Generic Name	**IUPAC Name**	**Molecular Formula**	**Chemical Structure**	**Molecular Weight (g/mol)**	**Solubility (~25°C)**			**Log P**	**IC50/ Ki**	**Biological Activity**	**Stage of Drug Development**	**Therapeutic Action**	**Route of Administration**
1-NPZ	1-(1-naphthyl)piperazine	$C_{14}H_{16}N_2$		212.29	DMSO	Water	Ethanol	2.53	IC50= 1 nM (rat)	**5-HT1A receptor agonist** and **5-HT2A/C receptor antagonist.** Derivative of quipazine.	*In vitro* and *in vivo* studies	NA	Oral
					NF	Soluble	Slightly soluble						
LY 53857	(Z)-but-2-enedioic acid;3-hydroxybutan-2-yl (6aR,9R,10aR)-7-methyl-4-propan-2-yl-6,6a,8,9,10,10a-hexahydroindolo[4,3-fg]quinolone-9-carboxylate	$C_{27}H_{36}N_2O_7$		500.584 (LY 53857 maleate salt)	0.1 M HCl	Water	Ethanol	NF	IC50 = 26 nM (pyramidal cells) 364 nM (interneurons)	Potent and selective **5-HT2 receptor antagonist**	*In vitro* and *in vivo* studies	NA	Systemic
					25 mg/mL	5.9 mg/mL	Soluble						
PRX-08066	5-((4-(6-chlorothieno[2,3-d]pyrimidin-4-ylamino)piperidin-1-yl)methyl)-2-fluorobenzonitrile	$C_{19}H_{17}ClFN_5S$		402	DMSO	Water	Ethanol	3.88	3.4 nM	Selective **5-HT2B receptor antagonist** that prevents the severity of pulmonary arterial hypertension in the MCT rat model.	*In vitro* and *in vivo* studies Tested in clinical trials for pulmonary hypertension and chronic obstructive pulmonary disease. Phase 2	For pulmonary hypertension and chronic obstructive pulmonary disease.	Oral
					104 mg/mL	104 mg/mL	98 mg/mL						
RS-127445	4-(4-fluoro-1-naphthyl)-6-isopropylpyrimidin-2-amine	$C_{17}H_{16}FN_3$		281.327	DMSO	Water	Ethanol	4.44	pIC50 = 10.4 pKi = 9.5	Selective and potent **5-HT2B antagonist**. 1000-fold selectivity over 5-HT2A, 5-HT2C, 5-HT5, 5-HT6 and 5-HT7	*In vitro* and *in vivo* studies	NA	Oral for 2.5 h, intraperitoneal and intravenous for 0.08 h
					56 mg/mL	< 1 mg/mL	8 mg/mL						
Spiperone	8-[4-(4-fluorophenyl)-4-oxo-butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one	$C_{23}H_{26}FN_3O_2$		395	0.1 M HCl	Water	Ethanol	3.07	1.6 nM (5-HT2R) 15 nM (5-HT1CR)	**5-HT** and D2-like receptor **antagonist**. Potently enhances intracellular Ca^{2+} levels. Additionally activates Ca^{2+}- Cl^- channels to induce Cl^-secretion	It is licensed for clinical use in Japan as a treatment for schizophrenia	Treatment for schizophrenia	Systemic (Intramuscular and intraperitoneal)
					0.3 mg/mL	0.2 mg/mL	1.5 mg/mL						

Table 2b. 5-HT2R Antagonists: chemical, biological and therapeutic properties of commercialized drugs [111-118]. NF = Not Found; NA = Not Applicable.

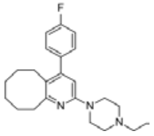
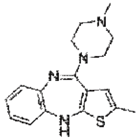
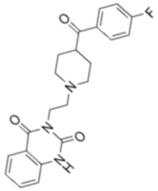
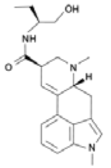
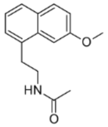
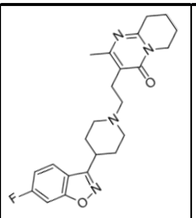
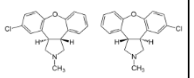
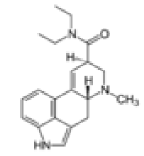
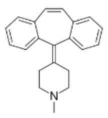
Generic Name	IUPAC Name	Molecular Formula	Chemical Structure	Molecular Weight (g/mol)	Solubility (~25°C)			Log P	IC50/ Ki	Biological Activity	Stage of Drug Development	Therapeutic Action	Route of Administration
Blonanserin	2-(4-ethylpiperazin-1-yl)-4-(4-fluorophenyl)-5,6,7,8,9,10-hexahydrocycloocta[b]pyridine	$C_{23}H_{30}FN_3$		368	DMSO	Water	Ethanol	5.67	Ki = 3.98 nM	Novel atypical antipsychotic agent with potent dopamine D2 and serotonin **5-HT2 receptors antagonist** properties	On the market since 2008: Lonasen®	Treatment of schizophrenia	Oral (tablets 2, 4 and 8 mg)
					< 1 mg/mL	< 1 mg/mL	< 1 mg/mL						
Olanzapine	2-Methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine	$C_{17}H_{20}N_4S$		312	DMSO	Water	Ethanol	2 - 3.6	Ki= 4 (5HT2A), 11 (5HT2C), 57 (5HT3) nM	**5-HT and D2 antagonist.** Additionally displays activity at: M1, M2, M3, M4 (muscarinic receptors), H1 (Histamine receptor), α1 and α2 receptors. Atypical antipsychotic. Displays anxiolytic activity.	On the market since 1996: Zyprexa®, Zypadhera® and Lanzek®	For the acute and maintenance treatment of schizophrenia and related psychotic disorders. Acute treatment of manic or mixed episodes of bipolar 1 disorder. Intramuscular olanzapine is indicated for the rapid control of agitated patients	Intramuscular (powder for solution) and oral (tablets 2.5; 5; 7.5; 10 and 15 mg)
					63 mg/mL	< 1 mg/mL	9 mg/mL						
Ketanserin (R41468)	3-{2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl}quinazoline-2,4(1*H*,3*H*)-dione	$C_{22}H_{22}FN_3O_3$		395	DMSO	Water	Ethanol	3.61	Ki = 2.5 nM (5-HT2A) 50 nM (rat 5-HT2C) 100 nM (human 5-HT2C)	**5-HT2AR antagonist** without partial agonist properties. Moderate affinity and selectivity for human 5-HT1Dα over 5-HT1Dβ receptor, and weak affinity at α1 adrenoceptors. Antihypertensive *in vivo*	On the market since 1987: Sufrexal® ; Ketensin® ; Vulketan® (veterinary use)	Antihypertensive; Radioligand	Oral (tablets 20mg), systemic (5mg/ mL) and topical (ointment/ gel 2%); vaginal ovules
					2 mg/mL (5 mM)	< 1 mg/mL (< 1 mM)	< 1 mg/mL (< 1 mM)						
Methysergide (UML-491)	(6a*R*,9*R*)-*N*-[(2*S*)-1-Hydroxybutan-2-yl]-4,7-dimethyl-6,6a,8,9-tetrahydroindolo[4,3-*fg*]quinoline-9-carboxamide	$C_{21}H_{27}N_3O_2$		353	DMSO	Water	Ethanol	1.5 - 2.2	Ki= 10nM (5-HT2A) 2.5 nM (5-HT2C)	**5-HT1/2R antagonist.** Displays anti-migraine activity	On the market since 1962: Deseril®, Sansert®	Treatment of vascular headache (CHMP are reviewing the use of methysergide in the treatment of migraine and may decide to revoke it from use)	Oral (tablets 1 and 2 mg)
					> 10 mg/mL	2 mg/mL	NF						
Agomelatine	*N*-[2-(7-methoxynaphthalen-1-yl)ethyl]acetamide	$C_{15}H_{17}NO_2$		243	DMSO	Water	Ethanol	2.04 - 2.83	270 nM	Potent MT1/MT2 agonist; **5-HT2C antagonist.** Shows antidepressant effects *in vivo*	On the market since 2009: Melitor®, Thymanax®, Valdoxan®	Treatment of major depressive episodes in adults	Oral administration (tablets 25 mg)
					49 mg/mL	< 1 mg/mL	49 mg/mL						

Table 2b. (Continuation)

Risperidone	3-(2-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)ethyl)-2-methyl-6,7,8,9-tetrahydropyrido[1,2-a]pyrimidin-4-one	$C_{23}H_{27}FN_4O_2$		410	DMSO	Water	Ethanol	2.5 - 3.3	Ki = 0.4 nM	Atypical antipsychotic agent. Potent **5-HT2A antagonist**. Also displays high affinity at D2 receptors.	On the market since 1993: Risperdal®	Treatment of schizophrenia in adults and in adolescents, and for the short-term treatment of manic or mixed episodes of bipolar I disorder in children and adolescents. Manage symptoms of inappropriate behavior due to aggression and/or psychosis in patients with severe dementia	Intramuscular (injection, powder for suspension - 12.5; 25; 37.5 and 50 mg) and oral (solution 1mg/mL or tablets 0.25; 0.5; 1; 2 and 3 mg)
					3 mg/mL	< 1 mg/mL	4 mg/mL						
Asenapine	(3aRS,12bRS)-rel-5-Chloro-2,3,3a,12b-tetrahydro-2-methyl-1H-dibenz[2,3:6,7]oxepino[4,5-c]pyrrole maleate salt	$C_{17}H_{16}ClNO$		286	DMSO	Water	Ethanol	3,72	pK$_i$ = 8.60 (5-HT1A), 8.40 (5-HT1B), 10.15 (5-HT2A), 9.75 (5-HT2B), 10.46 (5-HT2C), 8.84 (5-HT5A), 9.60 (5-HT6), 9.94 (5-HT7)	Novel **multiple receptor antagonist**. Displays activity at 5-HT, dopamine, adrenoceptors and histamine receptors. Increases dopamine, norepinephrine and acetylcholine efflux. Anti-psychotic *in vivo*	On the market since 2009: Saphris®, Sycrest®	Treatment of psychosis, schizophrenia and schizoaffective disorders, manic disorders, and bipolar disorders as monotherapy or in combination	Sublingual (tablets 5 and 10 mg)
					80 mg/mL	< 1 mg/mL	< 1 mg/mL						
Lysergic acid diethylamide (LSD)	(6a*R*,9*R*)-*N*,*N*-diethyl-7-methyl-4,6,6a,7,8,9-hexahydroindolo-[4,3-*fg*]quinoline-9-carboxamide	$C_{20}H_{25}N_3O$		323	DMSO	Water	Ethanol	2.28 - 3.3	[3H]LSD appeared to have a slightly higher affinity for dopamine-sensitive binding (Kd = 0.5 nM) than for 5HT-sensitive binding (Kd = 1.2 nM).	Semisynthetic derivative of ergot (Claviceps purpurea). Effects on serotonergic systems including antagonism at some peripheral serotonin receptors, both agonist and antagonist actions at central nervous system serotonin receptors, and possibly effects on serotonin turnover. Potent hallucinogen, but the mechanisms of that effect are not well understood	It was introduced commercially in 1947, but it was later prohibited: Delysid®	Analytical psychotherapy and experimental studies	Oral (primary route) it can be also inhaled, injected and transdermally applied
					NF	2.70 e-01 g/L	NF						
Cyproheptadine	4-(5*H*-dibenzo[a,d]cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride	$C_{21}H_{21}N$		287	DMSO	Water	Ethanol	4.38 - 5.02	Ki=10 nM pKi= 1.4 nM	Potent H1 and **5-HT2B antagonist**. Non-selective receptor antagonist (shows high potency at other 5-HT, dopamine and muscarinic acetylcholine receptors. Displays anti-histaminergic and anti-serotonergic effects	Apeplus®, Apitup®, Biohept®, Ciplactin®, Cipractine®, Ciproheptadina®, Ciprovit®, Ciptadine®, Cyheptine®, Cyllermin®, Periactin®, Periactine®, Periatin®, Reactin®	Treatment of perennial and seasonal allergic rhinitis, vasomotor rhinitis, allergic conjunctivitis due to inhalant allergens and foods, mild uncomplicated allergic skin manifestations of urticaria and angioedema, amelioration of allergic reactions to blood or plasma, cold urticaria, dermatographism, and as therapy for anaphylactic reactions adjunctive to epinephrine	Oral (syrup 2 mg/5 mL; tablets 4 mg)
					65 mg/mL (200 mM)	< 1 mg/mL (< 1 mM)	20 mg/mL (61 mM)						

References

1. Slominski A, Wortsman J (2000) Neuroendocrinology of the skin. Endocrine reviews 21 (5):457-487. doi:10.1210/edrv.21.5.0410

2. Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD (2012) Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. Advances in anatomy, embryology, and cell biology 212:v, vii, 1-115

3. Slominski A, Wortsman J, Tobin DJ (2005) The cutaneous serotoninergic/melatoninergic system: securing a place under the sun. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19 (2):176-194. doi:10.1096/fj.04-2079rev

4. Zmijewski MA, Slominski AT (2011) Neuroendocrinology of the skin: An overview and selective analysis. Dermato-endocrinology 3 (1):3-10. doi:10.4161/derm.3.1.14617

5. Nordlind K, Azmitia EC, Slominski A (2008) The skin as a mirror of the soul: exploring the possible roles of serotonin. Experimental dermatology 17 (4):301-311. doi:10.1111/j.1600-0625.2007.00670.x

6. Baganz NL, Blakely RD (2013) A dialogue between the immune system and brain, spoken in the language of serotonin. ACS chemical neuroscience 4 (1):48-63. doi:10.1021/cn300186b

7. Fidalgo S, Ivanov DK, Wood SH (2013) Serotonin: from top to bottom. Biogerontology 14 (1):21-45. doi:10.1007/s10522-012-9406-3

8. Froberg GK, Lindberg R, Ritter M, Nordlind K (2009) Expression of serotonin and its 5-HT1A receptor in canine cutaneous mast cell tumours. Journal of comparative pathology 141 (2-3):89-97. doi:10.1016/j.jcpa.2008.08.002

9. Abdala-Valencia H, Berdnikovs S, McCary CA, Urick D, Mahadevia R, Marchese ME, Swartz K, Wright L, Mutlu GM, Cook-Mills JM (2012) Inhibition of allergic inflammation by supplementation with 5-hydroxytryptophan. American journal of physiology Lung cellular and molecular physiology 303 (8):L642-660. doi:10.1152/ajplung.00406.2011

10. Hauso O, Gustafsson BI, Loennechen JP, Stunes AK, Nordrum I, Waldum HL (2007) Long-term serotonin effects in the rat are prevented by terguride. Regulatory peptides 143 (1-3):39-46. doi:10.1016/j.regpep.2007.02.009

11. Sreevidya CS, Fukunaga A, Khaskhely NM, Masaki T, Ono R, Nishigori C, Ullrich SE (2010) Agents that reverse UV-Induced immune suppression and photocarcinogenesis affect DNA repair. The Journal of investigative dermatology 130 (5):1428-1437. doi:10.1038/jid.2009.329

12. Ullrich SE (2007) Sunlight and skin cancer: lessons from the immune system. Molecular carcinogenesis 46 (8):629-633. doi:10.1002/mc.20328

13. Sreevidya CS, Khaskhely NM, Fukunaga A, Khaskina P, Ullrich SE (2008) Inhibition of photocarcinogenesis by platelet-activating factor or serotonin receptor antagonists. Cancer research 68 (10):3978-3984. doi:10.1158/0008-5472.CAN-07-6132

14. http://www.who.int/en/. 2014

15. Ferrandiz L, Ruiz-de-Casas A, Trakatelli M, de Vries E, Ulrich M, Aquilina S, Saksela O, Majewski S, Ranki A, Proby C, Magnoni C, Pitkanen S, Kalokasidis K, Siskou S, Hinrichs B, Altsitsiadis E, Stockfleth E, Moreno-Ramirez D, Group E (2012) Assessing physicians' preferences on skin cancer treatment in Europe. The British journal of dermatology 167 Suppl 2:29-35. doi:10.1111/j.1365-2133.2012.11084.x

16. http://www.skincancer.org. 2014

17. Saager RB, Cuccia DJ, Saggese S, Kelly KM, Durkin AJ (2013) A light emitting diode (LED) based spatial frequency domain imaging system for optimization of photodynamic therapy of nonmelanoma skin cancer: quantitative reflectance imaging. Lasers in surgery and medicine 45 (4):207-215. doi:10.1002/lsm.22139

18. http://www.uv-damage.org/. 2014

19. Gruber F, Zamolo G, Kastelan M, Massari LP, Cabrijan L, Peharda V, Batinac T (2007) Photocarcinogenesis--molecular mechanisms. Collegium antropologicum 31 Suppl 1:101-106

20. Black HS, deGruijl FR, Forbes PD, Cleaver JE, Ananthaswamy HN, deFabo EC, Ullrich SE, Tyrrell RM (1997) Photocarcinogenesis: an overview. Journal of photochemistry and photobiology B, Biology 40 (1):29-47

21. Afaq F, Katiyar SK (2011) Polyphenols: skin photoprotection and inhibition of photocarcinogenesis. Mini reviews in medicinal chemistry 11 (14):1200-1215

22. Kadekaro AL, Kavanagh RJ, Wakamatsu K, Ito S, Pipitone MA, Abdel-Malek ZA (2003) Cutaneous photobiology. The melanocyte vs. the sun: who will win the final round? Pigment cell research / sponsored by the European Society for Pigment Cell Research and the International Pigment Cell Society 16 (5):434-447

23. Vaid M, Katiyar SK (2010) Molecular mechanisms of inhibition of photocarcinogenesis by silymarin, a phytochemical from milk thistle (Silybum marianum L. Gaertn.) (Review). International journal of oncology 36 (5):1053-1060

24. Mittal A, Elmets CA, Katiyar SK (2003) Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. Carcinogenesis 24 (8):1379-1388. doi:10.1093/carcin/bgg095

25. Goodsell DS (2001) The molecular perspective: ultraviolet light and pyrimidine dimers. The oncologist 6 (3):298-299

26. Yamamoto J, Martin R, Iwai S, Plaza P, Brettel K (2013) Repair of the (6-4) photoproduct by DNA photolyase requires two photons. Angewandte Chemie 52 (29):7432-7436. doi:10.1002/anie.201301567

27. Kamiya H, Iwai S, Kasai H (1998) The (6-4) photoproduct of thymine-thymine induces targeted substitution mutations in mammalian cells. Nucleic acids research 26 (11):2611-2617

28. Rochette PJ, Therrien JP, Drouin R, Perdiz D, Bastien N, Drobetsky EA, Sage E (2003) UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine-thymine dipyrimidines and correlate with the mutation spectrum in rodent cells. Nucleic acids research 31 (11):2786-2794

29. Maverakis E, Miyamura Y, Bowen MP, Correa G, Ono Y, Goodarzi H (2010) Light, including ultraviolet. Journal of autoimmunity 34 (3):J247-257. doi:10.1016/j.jaut.2009.11.011

30. Li J, Uchida T, Todo T, Kitagawa T (2006) Similarities and differences between cyclobutane pyrimidine dimer photolyase and (6-4) photolyase as revealed by resonance Raman spectroscopy: Electron transfer from the FAD cofactor to ultraviolet-damaged DNA. The Journal of biological chemistry 281 (35):25551-25559. doi:10.1074/jbc.M604483200

31. Kim TH, Moodycliffe AM, Yarosh DB, Norval M, Kripke ML, Ullrich SE (2003) Viability of the antigen determines whether DNA or urocanic acid act as initiator molecules for UV-induced suppression of delayed-type hypersensitivity. Photochemistry and photobiology 78 (3):228-234

32. Beissert S, Ruhlemann D, Mohammad T, Grabbe S, El-Ghorr A, Norval M, Morrison H, Granstein RD, Schwarz T (2001) IL-12 prevents the inhibitory effects of cis-urocanic acid on tumor antigen presentation by Langerhans cells: implications for photocarcinogenesis. Journal of immunology 167 (11):6232-6238

33. Mittal A, Piyathilake C, Hara Y, Katiyar SK (2003) Exceptionally high protection of photocarcinogenesis by topical application of (--)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: relationship to inhibition of UVB-induced global DNA hypomethylation. Neoplasia 5 (6):555-565

34. Chial H (2008) Proto-oncogenes to oncogenes to cancer. Nat Educ 1 (1)::33

35. Goukassian D, Gad F, Yaar M, Eller MS, Nehal US, Gilchrest BA (2000) Mechanisms and implications of the age-associated decrease in DNA repair capacity. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 14 (10):1325-1334

36. Narayanapillai S, Agarwal C, Tilley C, Agarwal R (2012) Silibinin is a potent sensitizer of UVA radiation-induced oxidative stress and apoptosis in human keratinocyte HaCaT cells. Photochemistry and photobiology 88 (5):1135-1140. doi:10.1111/j.1751-1097.2011.01050.x

37. Kawachi Y, Xu X, Taguchi S, Sakurai H, Nakamura Y, Ishii Y, Fujisawa Y, Furuta J, Takahashi T, Itoh K, Yamamoto M, Yamazaki F, Otsuka F (2008) Attenuation of UVB-induced sunburn reaction and oxidative DNA damage with no alterations in UVB-induced skin carcinogenesis in Nrf2 gene-deficient mice. The Journal of investigative dermatology 128 (7):1773-1779. doi:10.1038/sj.jid.5701245

38. Kryston TB, Georgiev AB, Pissis P, Georgakilas AG (2011) Role of oxidative stress and DNA damage in human carcinogenesis. Mutation research 711 (1-2):193-201. doi:10.1016/j.mrfmmm.2010.12.016

39. Wondrak GT, Cabello CM, Villeneuve NF, Zhang S, Ley S, Li Y, Sun Z, Zhang DD (2008) Cinnamoyl-based Nrf2-activators targeting human skin cell photo-oxidative stress. Free radical biology & medicine 45 (4):385-395. doi:10.1016/j.freeradbiomed.2008.04.023

40. Halliday GM (2005) Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. Mutation research 571 (1-2):107-120. doi:10.1016/j.mrfmmm.2004.09.013

41. Song EJ, Gordon-Thomson C, Cole L, Stern H, Halliday GM, Damian DL, Reeve VE, Mason RS (2013) 1alpha,25-Dihydroxyvitamin D3 reduces several types of UV-induced DNA damage and contributes to photoprotection. The Journal of steroid biochemistry and molecular biology 136:131-138. doi:10.1016/j.jsbmb.2012.11.003

42. Kitsera N, Stathis D, Luhnsdorf B, Muller H, Carell T, Epe B, Khobta A (2011) 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1. Nucleic acids research 39 (14):5926-5934. doi:10.1093/nar/gkr163

43. Kunisada M, Sakumi K, Tominaga Y, Budiyanto A, Ueda M, Ichihashi M, Nakabeppu Y, Nishigori C (2005) 8-Oxoguanine formation induced by chronic UVB exposure makes Ogg1 knockout mice susceptible to skin carcinogenesis. Cancer research 65 (14):6006-6010. doi:10.1158/0008-5472.CAN-05-0724

44. Huang XX, Scolyer RA, Abubakar A, Halliday GM (2012) Human 8-oxoguanine-DNA glycosylase-1 is downregulated in human basal cell carcinoma. Molecular genetics and metabolism 106 (1):127-130. doi:10.1016/j.ymgme.2012.02.017

45. Wolfle U, Haarhaus B, Schempp CM (2013) The photoprotective and antioxidative properties of luteolin are synergistically augmented by tocopherol and ubiquinone. Planta medica 79 (11):963-965. doi:10.1055/s-0032-1328716

46. Kripke ML (1974) Antigenicity of murine skin tumors induced by ultraviolet light. Journal of the National Cancer Institute 53 (5):1333-1336

47. Katiyar SK (2007) Interleukin-12 and photocarcinogenesis. Toxicology and applied pharmacology 224 (3):220-227. doi:10.1016/j.taap.2006.11.017

48. Nasti TH, Iqbal O, Tamimi IA, Geise JT, Katiyar SK, Yusuf N (2011) Differential roles of T-cell subsets in regulation of ultraviolet radiation induced cutaneous photocarcinogenesis. Photochemistry and photobiology 87 (2):387-398. doi:10.1111/j.1751-1097.2010.00859.x

49. Schwarz T, Schwarz A (2011) Molecular mechanisms of ultraviolet radiation-induced immunosuppression. European journal of cell biology 90 (6-7):560-564. doi:10.1016/j.ejcb.2010.09.011

50. Romani N, Brunner PM, Stingl G (2012) Changing views of the role of Langerhans cells. The Journal of investigative dermatology 132 (3 Pt 2):872-881. doi:10.1038/jid.2011.437

51. Beissert S, Loser K (2008) Molecular and cellular mechanisms of photocarcinogenesis. Photochemistry and photobiology 84 (1):29-34. doi:10.1111/j.1751-1097.2007.00231.x

52. Justo GZ, Shishido SM, Machado D, Silva RA, Ferreira CV (2011) Druggable Targets for Skin Photoaging: Potential Application of Nanocosmetics and Nanomedicine. In: Beck R, Guterres S, Pohlmann A (eds) Nanocosmetics and Nanomedicines. Springer Berlin Heidelberg, pp 197-227. doi:10.1007/978-3-642-19792-5_10

53. Kautz-Neu K, Noordegraaf M, Dinges S, Bennett CL, John D, Clausen BE, von Stebut E (2011) Langerhans cells are negative regulators of the anti-Leishmania response. The Journal of experimental medicine 208 (5):885-891. doi:10.1084/jem.20102318

54. Obhrai JS, Oberbarnscheidt M, Zhang N, Mueller DL, Shlomchik WD, Lakkis FG, Shlomchik MJ, Kaplan DH (2008) Langerhans cells are not required for efficient skin graft rejection. The Journal of investigative dermatology 128 (8):1950-1955. doi:10.1038/jid.2008.52

55. Schwarz A, Noordegraaf M, Maeda A, Torii K, Clausen BE, Schwarz T (2010) Langerhans cells are required for UVR-induced immunosuppression. The Journal of investigative dermatology 130 (5):1419-1427. doi:10.1038/jid.2009.429

56. Dinkova-Kostova AT (2008) Phytochemicals as protectors against ultraviolet radiation: versatility of effects and mechanisms. Planta medica 74 (13):1548-1559. doi:10.1055/s-2008-1081296

57. Loser K, Apelt J, Voskort M, Mohaupt M, Balkow S, Schwarz T, Grabbe S, Beissert S (2007) IL-10 controls ultraviolet-induced carcinogenesis in mice. Journal of immunology 179 (1):365-371

58. Katiyar SK (2007) UV-induced immune suppression and photocarcinogenesis: chemoprevention by dietary botanical agents. Cancer letters 255 (1):1-11. doi:10.1016/j.canlet.2007.02.010

59. Katiyar S, Elmets CA, Katiyar SK (2007) Green tea and skin cancer: photoimmunology, angiogenesis and DNA repair. The Journal of nutritional biochemistry 18 (5):287-296. doi:10.1016/j.jnutbio.2006.08.004

60. Schwarz A, Stander S, Berneburg M, Bohm M, Kulms D, van Steeg H, Grosse-Heitmeyer K, Krutmann J, Schwarz T (2002) Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. Nature cell biology 4 (1):26-31. doi:10.1038/ncb717

61. Walterscheid JP, Nghiem DX, Kazimi N, Nutt LK, McConkey DJ, Norval M, Ullrich SE (2006) Cis-urocanic acid, a sunlight-induced immunosuppressive factor, activates immune suppression via the 5-HT2A receptor. Proceedings of the National Academy of Sciences of the United States of America 103 (46):17420-17425. doi:10.1073/pnas.0603119103

62. Schwarz A, Maeda A, Kernebeck K, van Steeg H, Beissert S, Schwarz T (2005) Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair. The Journal of experimental medicine 201 (2):173-179. doi:10.1084/jem.20041212

63. Norval M, Gibbs NK, Gilmour J (1995) The role of urocanic acid in UV-induced immunosuppression: recent advances (1992-1994). Photochemistry and photobiology 62 (2):209-217

64. Kaneko K, Travers JB, Matsui MS, Young AR, Norval M, Walker SL (2009) cis-Urocanic acid stimulates primary human keratinocytes independently of serotonin or platelet-activating factor receptors. The Journal of investigative dermatology 129 (11):2567-2573. doi:10.1038/jid.2009.129

65. Gibbs NK, Norval M (2011) Urocanic acid in the skin: a mixed blessing? The Journal of investigative dermatology 131 (1):14-17. doi:10.1038/jid.2010.276

66. Kaneko K, Smetana-Just U, Matsui M, Young AR, John S, Norval M, Walker SL (2008) cis-Urocanic acid initiates gene transcription in primary human keratinocytes. Journal of immunology 181 (1):217-224

67. Ullrich SE (2005) Mechanisms underlying UV-induced immune suppression. Mutation research 571 (1-2):185-205. doi:10.1016/j.mrfmmm.2004.06.059

68. Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD, Gonzalez-Estrada A, Ullrich SE (2011) Mast cell-derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. Journal of immunology 186 (1):25-31. doi:10.4049/jimmunol.1001657

69. Ullrich SE, Byrne SN (2012) The immunologic revolution: photoimmunology. The Journal of investigative dermatology 132 (3 Pt 2):896-905. doi:10.1038/jid.2011.405

70. Hart PH, Gorman S, Finlay-Jones JJ (2011) Modulation of the immune system by UV radiation: more than just the effects of vitamin D? Nature reviews Immunology 11 (9):584-596. doi:10.1038/nri3045

71. Raj D, Brash DE, Grossman D (2006) Keratinocyte apoptosis in epidermal development and disease. The Journal of investigative dermatology 126 (2):243-257. doi:10.1038/sj.jid.5700008

72. Erb P, Ji J, Kump E, Mielgo A, Wernli M (2008) Apoptosis and pathogenesis of melanoma and nonmelanoma skin cancer. Advances in experimental medicine and biology 624:283-295. doi:10.1007/978-0-387-77574-6_22

73. Ji J, Kump E, Wernli M, Erb P (2008) Gene silencing of transcription factor Gli2 inhibits basal cell carcinomalike tumor growth in vivo. International journal of cancer Journal international du cancer 122 (1):50-56. doi:10.1002/ijc.23023

74. Adhami VM, Syed DN, Khan N, Afaq F (2008) Phytochemicals for prevention of solar ultraviolet radiation-induced damages. Photochemistry and photobiology 84 (2):489-500. doi:10.1111/j.1751-1097.2007.00293.x

75. Afaq F, Adhami VM, Mukhtar H (2005) Photochemoprevention of ultraviolet B signaling and photocarcinogenesis. Mutation research 571 (1-2):153-173. doi:10.1016/j.mrfmmm.2004.07.019

76. Svobodova A, Psotova J, Walterova D (2003) Natural phenolics in the prevention of UV-induced skin damage. A review. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 147 (2):137-145

77. Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD (2007) The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. Current biology : CB 17 (13):1140-1145. doi:10.1016/j.cub.2007.05.074

78. Yaar M, Gilchrest BA (2007) Photoageing: mechanism, prevention and therapy. The British journal of dermatology 157 (5):874-887. doi:10.1111/j.1365-2133.2007.08108.x

79. Narayanan DL, Saladi RN, Fox JL (2010) Ultraviolet radiation and skin cancer. International journal of dermatology 49 (9):978-986. doi:10.1111/j.1365-4632.2010.04474.x

80. Kim K (2012) Neuroimmunological mechanism of pruritus in atopic dermatitis focused on the role of serotonin. Biomolecules & therapeutics 20 (6):506-512. doi:10.4062/biomolther.2012.20.6.506

81. Maximino C (2012) Serotonin in the nervous system of vertebrates. In: Serotonin and Anxiety. Springer-Verlag New York, pp 15-36. doi:10.1007/978-1-4614-4048-2

82. Johansen PA, Jennings I, Cotton RG, Kuhn DM (1996) Phosphorylation and activation of tryptophan hydroxylase by exogenous protein kinase A. Journal of neurochemistry 66 (2):817-823

83. Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J, Szczesniewski A, Slugocki G, McNulty J, Kauser S, Tobin DJ, Jing C, Johansson O (2002) Serotoninergic and melatoninergic systems are fully expressed in human skin. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 16 (8):896-898. doi:10.1096/fj.01-0952fje

84. Slominski A, Pisarchik A, Johansson O, Jing C, Semak I, Slugocki G, Wortsman J (2003) Tryptophan hydroxylase expression in human skin cells. Biochimica et biophysica acta 1639 (2):80-86

85. Semak I, Korik E, Naumova M, Wortsman J, Slominski A (2004) Serotonin metabolism in rat skin: characterization by liquid chromatography-mass spectrometry. Archives of biochemistry and biophysics 421 (1):61-66

86. Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J (2003) Characterization of the serotoninergic system in the C57BL/6 mouse skin. European journal of biochemistry / FEBS 270 (16):3335-3344

87. Bensouilah J, Buck P (2006) Skin structure and function. In: Aromadermatology: aromatherapy in the treatment and care of

common skin conditions. Radcliffe Publishing Ltd,

88. http://www.dermnetnz.org. 2014

89. Gillbro JM, Marles LK, Hibberts NA, Schallreuter KU (2004) Autocrine catecholamine biosynthesis and the beta-adrenoceptor signal promote pigmentation in human epidermal melanocytes. The Journal of investigative dermatology 123 (2):346-353. doi:10.1111/j.0022-202X.2004.23210.x

90. Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD (2001) Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity

serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. The Journal of neuroscience : the official journal of the Society for Neuroscience 21 (16):6348-6361

91. Thorslund K, Amatya B, Dufva AE, Nordlind K (2013) The expression of serotonin transporter protein correlates with the severity of psoriasis and chronic stress. Archives of dermatological research 305 (2):99-104. doi:10.1007/s00403-012-1303-8

92. Anlauf M, Schafer MK, Depboylu C, Hartschuh W, Eiden LE, Kloppel G, Weihe E (2004) The vesicular monoamine transporter 2 (VMAT2) is expressed by normal and tumor cutaneous mast cells and Langerhans cells of the skin but is absent from Langerhans cell histiocytosis. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 52 (6):779-788

93. Kema IP, de Vries EG, Muskiet FA (2000) Clinical chemistry of serotonin and metabolites. Journal of chromatography B, Biomedical sciences and applications 747 (1-2):33-48

94. Maes M, Kenis G, Bosmans E (2002) The negative immunoregulatory effects of serotonin (5-HT) moduline, an endogenous 5-HT1B receptor antagonist with anti-anxiety properties. Cytokine 19 (6):308-311

95. Ritter M, El-Nour H, Hedblad MA, Butterfield JH, Beck O, Stephanson N, Holst M, Giscombe R, Azmitia EC, Nordlind K (2012) Serotonin and its 5-HT1 receptor in human mastocytosis. Immunopharmacology and immunotoxicology 34 (4):679-685. doi:10.3109/08923973.2011.651222

96. Kushnir-Sukhov NM, Gilfillan AM, Coleman JW, Brown JM, Bruening S, Toth M, Metcalfe DD (2006) 5-hydroxytryptamine induces mast cell adhesion and migration. Journal of immunology 177 (9):6422-6432

97. Nordlind K, Thorslund K, Lonne-Rahm S, Mohabbati S, Berki T, Morales M, Azmitia EC (2006) Expression of serotonergic receptors in psoriatic skin. Archives of dermatological research 298 (3):99-106. doi:10.1007/s00403-006-0652-6

98. Akin D, Manier DH, Sanders-Bush E, Shelton RC (2004) Decreased serotonin 5-HT2A receptor-stimulated phosphoinositide signaling in fibroblasts from melancholic depressed patients. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 29 (11):2081-2087. doi:10.1038/sj.npp.1300505

99. El-Nour H, Lundeberg L, Boman A, Abramowski D, Holst M, Nordlind K (2007) The expression and functional significance of the serotonin(2C) receptor in murine contact allergy. Experimental dermatology 16 (8):644-650. doi:10.1111/j.1600-0625.2007.00573.x

100. Ameisen JC, Meade R, Askenase PW (1989) A new interpretation of the involvement of serotonin in delayed-type hypersensitivity. Serotonin-2 receptor antagonists inhibit contact sensitivity by an effect on T cells. Journal of immunology 142 (9):3171-3179

101. Zhang Q, Yao Y, Konger RL, Sinn AL, Cai S, Pollok KE, Travers JB (2008) UVB radiation-mediated inhibition of contact hypersensitivity reactions is dependent on the platelet-activating factor system. The Journal of investigative dermatology 128 (7):1780-1787. doi:10.1038/sj.jid.5701251

102. Shen L, Ji HF (2009) Molecular basis for cis-urocanic acid as a 5-HT(2A) receptor agonist. Bioorganic & medicinal chemistry letters 19 (18):5307-5309. doi:10.1016/j.bmcl.2009.07.143

103. Correale J, Farez MF (2013) Modulation of multiple sclerosis by sunlight exposure: role of cis-urocanic acid. Journal of neuroimmunology 261 (1-2):134-140. doi:10.1016/j.jneuroim.2013.05.014

104. Gibbs NK, Tye J, Norval M (2008) Recent advances in urocanic acid photochemistry, photobiology and photoimmunology. Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology 7 (6):655-667. doi:10.1039/b717398a

105. Slominski A, Pisarchik A, Zbytek B, Tobin DJ, Kauser S, Wortsman J (2003) Functional activity of serotoninergic and melatoninergic systems expressed in the skin. Journal of cellular physiology 196 (1):144-153. doi:10.1002/jcp.10287

106. Hong Y, Ji H, Wei H (2006) Topical ketanserin attenuates hyperalgesia and inflammation in arthritis in rats. Pain 124 (1-2):27-33. doi:10.1016/j.pain.2006.03.010

107. Wang D, Gao Y, Ji H, Hong Y (2010) Topical and systemic administrations of ketanserin attenuate hypersensitivity and expression of CGRP in rats with spinal nerve ligation. European journal of pharmacology 627 (1-3):124-130. doi:10.1016/j.ejphar.2009.11.011

108. Zhu CB, Lindler KM, Owens AW, Daws LC, Blakely RD, Hewlett WA (2010) Interleukin-1 receptor activation by systemic lipopolysaccharide induces behavioral despair linked to MAPK regulation of CNS serotonin transporters. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35 (13):2510-2520. doi:10.1038/npp.2010.116

109. Kroeze Y, Zhou H, Homberg JR (2012) The genetics of selective serotonin reuptake inhibitors. Pharmacology & therapeutics 136 (3):375-400. doi:10.1016/j.pharmthera.2012.08.015

110. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R (2003) Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301 (5631):386-389. doi:10.1126/science.1083968

111. http://www.chemicalize.org/. 2014

112. http://pubchem.ncbi.nlm.nih.gov. 2014

113. http://www.abcam.com. 2014

114. http://en.chembase.cn. 2014

115. http://www.sigmaaldrich.com. 2014

116. http://www.drugbank.ca. 2014

117. http://www.selleckchem.com/. 2014

118. https://clinicaltrials.gov. 2014

Chapter 2
CELL BIOLOGY STUDIES

Effect of 1-(1-Naphthyl)Piperazine on Human MNT-1 Melanoma Cells

Ana Catarina Menezes[1], Manuela Carvalheiro[2], José Miguel P. Ferreira de Oliveira[3], Andreia Ascenso[2], Helena Oliveira[3]

[1]Faculdade de Farmácia da Universidade de Lisboa
Lisbon, Portugal

[2]NanoBB Research Group of iMed.UL
Lisbon, Portugal

[3]Departamento de Biologia, Laboratório de Biotecnologia e Citómica,
CESAM, Universidade de Aveiro
Aveiro, Portugal

Abstract

Serotonin (5-HT) is a key signaling molecule that mediates neurocutaneous communications at different levels. 5-HT can act as a growth factor for several types of human cancer, such as skin cancer, as its receptors may well be implicated in immunosuppression events. Thus, the aim of this work was to study the effect of 1-(1-Naphthyl)piperazine (1-NPZ), both agonist of 5-HT1A and antagonist of 5-HT2A receptors, on human MNT-1 melanoma cells.

The cell viability as well as exposure conditions of 1-NPZ were established by MTT assay. Cell cycle dynamics, ROS production and apoptosis were all evaluated by flow cytometry. The expression levels of genes involved in immunosuppression and cancer progression were analyzed using reverse transcription PCR (RT-PCR).

Pretreatment of MNT-1 cells with 1-NPZ for 24 h decreased cell viability and induced apoptosis in a dose-dependent manner. Simultaneously, 1-NPZ mediated S-phase delay in cell cycle dynamics and increased ROS levels. Furthermore, 1-NPZ significantly increased the expression of cyclooxygenase-2 (COX-2) in MNT-1 cells. These findings suggest that 1-NPZ pretreatment is able to induce oxidative stress, and consequently apoptotic cell death in melanoma cells.

In conclusion, this study showed serotonin receptors, namely 5-HT1/2A, as potential therapeutic targets for the treatment of melanoma skin cancer, identifying for the first time 1-NPZ as a promising chemotherapeutic agent.

Keywords: Melanoma Skin Cancer, 5-HT1/2A Receptors, 1-NPZ, Apoptosis, Oxidative stress

Introduction

Epidermal *melanocytes* have a key role in protecting the skin from the damaging effects of solar UV radiation (UVR) through melanin synthesis. Melanin, the major pigment of skin, is capable of absorbing UV rays that penetrate into the epidermis and prevent DNA damage [1]. Furthermore, melanin acts as a radical scavenger for reactive oxygen species (ROS) [1]. The main consequences of UV exposure are photoaging and photocarcinogenesis [1]. Regarding photocarcinogenesis, the biological impact of UVR is felt through direct cellular damage and variations in immunologic function [2]. Direct effects of UV exposure include the generation of DNA photoproducts, gene mutations, immunosuppression and oxidative damage [2,3]. If these lesions are not repaired before DNA replication, it will lead to mutations in cancer-related genes, such as suppressor genes, proto-oncogenes, and other genes associated with the regulation of cell cycle [3, for a review]. The genes involved in skin pigmentation are also crucial in the development of skin cancer [3, for a review]. On the other hand, indirect effects of UV exposure comprise the cell cycle arrest and melanogenesis. These processes are regulated by numerous UV-induced cytokines and growth factors that mediate the survival, proliferation, and function of epidermal and dermal cells [3, for a review].

Skin cancer is one of the most costly and predominant form of human cancer (according to WHO), and its prevalence is rapidly increasing all over the world in recent years with changes in population lifestyle and deterioration of the ozone layer [3, for a review]. Besides nonmelanoma skin cancers, UVR is also responsible for the induction of malignant melanomas, the deadliest form of skin cancer that arises from melanocytes [3, for a review]. In the last five decades, the prevalence of melanoma skin cancer has been growing at an alarming rate in Caucasian populations around the world [4]. Currently, 132,000 melanoma cases occur worldwide each year [5]. Although, melanoma is the least common skin cancer, it is responsible for about 80 % of deaths related to cutaneous malignancies due to its metastatic potential [6]. Melanoma contrasts in several aspects from other types of skin tumors, since these are related to episodes of sunburn in childhood as opposed to the overall accumulation of UV exposure in life [7]. Another differentiating factor consist on the most recurrently mutated genes [7]. Melanoma is one of the most challenging malignancies to address therapeutically. Several mechanisms are involved in chemoresistance of melanoma cells, such as counteracting the harmful effects of DNA-damaging drugs and promoting antioxidant adaptive mechanisms [1,2]. Therefore, there is a need of discovering new therapies that may overcome such obstacles and improve quality of life.

Besides its carcinogenic nature in terms of irreversible alterations in cell genetic material, UV-induced immunosuppression is a significant event in skin carcinogenesis [2,3]. One major cause behind UV-induced immunosuppression is the presence of the naturally occurring *trans*-urocanic acid (3-(1H-imidazol-4-ly)-2-propenoic acid, UCA) in the skin. Upon UV exposure, *trans*-UCA isomerizes to the *cis* configuration with immunosuppressive effects [3, for a review]. *In vivo* studies have shown that *cis*-UCA is capable of mimicking several features of UV-induced immunosuppression [8-15]. However, despite being recognized as an immunosuppressive agent many years ago [16], the mechanism of action of *cis*-UCA is still unclear.

Serotonin (5-hydroxytryptamine, 5-HT) is a key mediator between the skin and the neuroendocrine system, as it is present in both central and peripheral nervous system [3, for a review]. 5-HT is synthetized in human skin by different types of cells, particularly by melanocytes, where its secretion is important for local cell communication [3, for a review]. Skin cells express membrane-bound receptors (R) for 5-HT (mainly 5-HT1 and 5-HT2R). In particular, 5-HT2A subtype plays an active role in skin reaction to UVR [3, for a review]. Furthermore, the chemical similarity between 5-HT and the photoreceptor *cis*-UCA leads to a competitive binding for 5-HT2AR [17]. *In vivo* experiments revealed that UV- or *cis*-UCA-induced immunosuppression was inhibited in mice previously treated either with anti-*cis*-UCA or anti-5-HT antibodies [17]. These results showed that activation of 5-HT2AR either by endogenous 5-HT or *cis*-UCA has a significant role in UV-induced immunosuppression.

The resistance of advanced melanoma tumors to chemotherapy and their high metastatic potential demands a continuous search for novel strategies for the management of cutaneous malignancies. 5-HT2AR antagonists are a good example of such, as they are capable of preventing UV- and *cis*-UCA-induced immunosuppression [17]. Apart from 5-HT2AR antagonists, 5-HT1R agonists like zolmitriptan are also capable of preventing immunosuppression [17]. *1-(1-Naphthyl)piperazine* (1-NPZ) is both a 5-HT2AR antagonist and a 5-HT1AR agonist with a dual mechanism of action. Therefore, 1-NPZ will simultaneously prevent the binding of *cis*-UCA to 5-HT2AR and trigger immune cells via 5-HT1AR [3, for a review]. Studies in irradiated mice previously injected with 1-NPZ demonstrated that skin cancer induction was significantly inhibited by preventing UV-induced immunosuppression and modulating DNA repair [17-19]. *In vitro* experiments revealed not only that *cis*-UCA triggers ROS production, but also that 1-NPZ is able to suppress this harmful process [19]. Nevertheless, the effects of 1-NPZ have only been studied so far *in vitro* on a squamous cell carcinoma line [5] or *in vivo* in irradiated mice [3,4]. Therefore, it is still necessary to better elucidate the mechanism of action of these serotonergic drugs in this context. Finally, the treatment with 1-NPZ could be a different strategy for the management of cutaneous malignancies specially attending to certain limitations of the current treatments, including the resistance of advanced melanoma tumors to chemotherapy due to their high metastatic potential. In fact, the new era of immuno-oncology opens a new chapter in skin cancer management: instead or besides radiotherapy and chemotherapy (for tumor elimination), the immunotherapy will stimulate the immune system of cancer patients with lower side effects and high efficacy particularly for immunogenic skin tumors.

Serotonin receptors have been implicated in immunosuppressive events and through them 1-NPZ has shown positive effects in the prevention of photocarcinogenesis [17-19]. Since melanocytes express both 5-HT1/2AR, we aimed to study the antitumoral effect of 1-NPZ, a selective 5-HT2AR antagonist and 5-HT1AR agonist, on human melanoma cells regarding melanoma therapy.

Materials and Methods

Reagents

1-NPZ was obtained from Enzo Life Sciences (Farmingdale, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V–fluorescein isothiocyanate (FITC)/PI apoptosis kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). TRIzol reagent was purchased from Life Technologies (St. Louis, MO, USA). RNA extraction kit was obtained using PureLink RNA Mini Kit from Life Technologies (St. Louis, MO, USA). Omniscript RT Kit was obtained from Qiagen (Hilden, Germany). Primers for human *COX2*, *IL12A*, *PAK1* and *GAPDH* were purchased from Sigma-Aldrich (St. Louis, MO, USA). iTaq Universal SYBR Green Supermix was purchased from Bio-Rad (Hercules, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin–streptomycin, fungizone, TrypLE and phosphate-buffered saline solution (PBS) were all purchased from Gibco (Life Technologies, Grand Island, NY, USA).

Cell culture

Human MNT-1 melanoma cells were cultured in DMEM medium, high glucose, supplemented with 10 % FBS, 2 mM L-glutamine, 1 % penicillin–streptomycin (10,000 U/mL) and 1 % fungizone (250 U/mL) in a humidified incubator at 37 ºC and 5 % CO_2. Transfer of culture was carried out once every 2-3 days with a complete medium, and subcultures were performed when monolayers reached 70 % confluence. Confluent cells were detached by incubation with TrypLE for 5 min. Cell morphology was observed using an inverted microscope Nikon Eclipse 80i (Coyoacán, Mexico). For 1-NPZ treatment, a stock solution of 1-NPZ was prepared by dissolving the powder in sterile PBS.

MTT assay

The cytotoxic effects of 1-NPZ were determined by the colorimetric MTT assay. Briefly, MNT-1 cells were seeded in 96-well plates and allowed to adhere. After cellular adhesion, cells were incubated for 3 and 24 h (6 x 10^3 and 3.5 x 10^3 cells/well, respectively) with a range of six concentrations of 1-NPZ (50, 100, 150, 200, 250 and 300 µg/mL), at 37 ºC in 5 % CO_2. The control received only the culture medium. Upon exposure, 50 µL MTT solution (1 mg/mL in PBS, pH 7.2) was added to each well. After 4 h of incubation, the medium from each well was replaced with 150 µL dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The plate was shaken for about 2 h protected from light. Cell viability was measured by the optical density of reduced MTT at 570 nm using a microplate reader (Synergy HT from BioTeK Instruments Inc., Winooski, VT, USA). The percentage of viable cells was determined as the ratio between the absorbance of treated versus untreated cells. Likewise, IC_{50} was defined as the concentration of 1-NPZ that leads to a 50 % decrease in absorbance of treated cells compared with untreated cells.

Cell cycle analysis

MNT-1 cells were seeded in 6-well plates (3 x 10^5 cells/well) and incubated at 37 ºC for 24 h with two different concentrations of 1-NPZ, 141.8 µg/mL (IC_{20}) and 163.6 µg/mL (IC_{50}). After incubation, adherent cells were harvested and centrifuged at 700 x *g* for 5 min. The supernatant was removed and the cells were washed in PBS and fixed in 85 % cold ethanol until analysis. All samples were centrifuged (700 x *g* for 5 min) to remove ethanol and the cell pellets were resuspended with PBS. Samples were filtrated on a nylon mesh (50 µm pore size) to the test tubes. Cells were then incubated with 50 µL PI, a DNA intercalating fluorochrome, and 50 µL RNase for 20 min, in the dark and at room temperature. Cell cycle distributions were assessed using flow cytometry (Coulter EPICS XL from Beckman Coulter Inc., Brea, CA, USA) and the percentage of cells in sub-G1, G0/G1, S and G2 phases was determined by FlowJo software (FlowJo LLC, Ashland, OR, USA) applying the Watson Pragmatic model.

Analysis of Intracellular levels of ROS

Intracellular ROS accumulation in MNT-1 cells was assessed using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Upon cleavage of the acetate groups and further oxidation, DCFH-DA transforms into the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS (i.e. H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$, $^{\bullet}NO$, $ONOO^-$ [20]). Briefly, cells were seeded in 6-well plates (3 x 10^5 cells/well), followed by treatment as indicated (IC_{20} and IC_{50}) for 24 h. Upon exposure, cells were washed with PBS and then incubated for 30 min with 10 µM DCFH-DA at 37 ºC in the dark. After staining, cells were washed, trypsinized and resuspended in cold DMEM containing 2 % FBS. DCF fluorescence was analyzed by flow cytometry. The fluorescence intensity was proportional to the intracellular ROS levels. Data analysis was performed using FlowJo software.

Cell apoptosis assay

Quantitative assessment of apoptosis was performed by flow cytometry using annexin V-FITC Apoptosis Detection Kit. MNT-1 cells were seeded in 6-well plates (3 x 10^5 cells/well) and allowed to adhere. After a 24 h treatment with doses corresponding to IC_{20} and IC_{50}, cells were collected, washed in PBS twice and resuspended in 1x binding buffer. 5 µL of both annexin V-FITC and PI were then added to 100 µL of cell suspension (10^5 cell/mL), with the samples being left in the dark for 15 min. Then, 400 µL of binding buffer was added to each sample. Unstained cells were also included as controls. The externalization of phosphatidylserine and the permeability to PI were assessed by flow cytometry. Data was acquired using the SYSTEM II™ (v. 2.5) software. Early apoptotic cells were positively stained with annexin V-FITC, whereas late apoptotic cells were positively stained with both annexin V-FITC positive and PI. FlowJo software was used to analyze the data.

RNA extraction and cDNA synthesis

MNT-1 cells were seeded in 6-well plates (3 x 10^5 cells/well) and incubated at 37 ºC for 24 h with 163.6 µg/mL (IC_{50}) of 1-NPZ. Upon exposure, adherent cells were washed twice with PBS and lysed in 1 mL TRIzol reagent. After 5 min, 200 µL chloroform was added to each sample, followed by vortexing and incubation at room temperature for 2 min. Phase separation was achieved by centrifugation at 12,000 x *g* for 5 min at 4 ºC in Phase-Lock Gel Heavy tubes (5 Prime, Inc., Gaithersburg, MD, USA). The aqueous phase was mixed with 1 volume 70 % ethanol and RNA was further purified using RNeasy Mini Kit columns (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted RNA was eluted in 20 µL RNase-free water and its concentration was determined by spectrophotometry at 260-280 and 230-260 nm (Nanodrop Spectrophotometer ND-1000 from Thermo Fisher Scientific, Wilmington, DE, USA). Reversed transcription reaction of 1 µg total RNA was carried out using the Omniscript RT Kit (Qiagen, Hilden, Germany) for 1 h at 37 ºC. The reaction mixture contained 1 µM Oligo(dT)18 primer, 5 mM dNTPs, reaction buffer and RT enzyme. At the end, cDNA samples were prediluted in milliQ water (1:20).

Quantitative real-time PCR (RT-PCR)

For cyclooxygenase-2 (COX-2), interleukin-12 (IL-12), p21-activated kinase (Pak1) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene expression evaluation, quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out using an iQ5 Bio-Rad thermal cycler (Bio-Rad, Hercules, CA, USA). GAPDH was used as the reference gene. The final individual qRT-PCR reactions contained iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 600 nM of each primer and 1:4 (v/v) prediluted cDNA (1:20). Two qRT-PCR technical replicates were performed per sample. The standard PCR conditions were as follows: 1 min denaturation at 95 ºC, followed 60 cycles at 94 ºC for 5 s, 58 ºC for 15 s, and 72 ºC for 15 s. At the end, a melting temperature program was carried out. Mean PCR efficiencies and cycle thresholds were obtained using the algorithm Real-Time PCR Miner. The expression levels of COX-2, IL-12 and Pak1 transcripts were determined by using the Pfaffl method after normalization to GAPDH. The primer sequences used in the qRT-PCR were GAPDH: 5’-GCACCGTCAAGGCTGAGAAC-3’ and 5’-TGGTGAAGACGCCAGTGGA-3’; *PTGS2* (COX-2): 5'-TCCCACAGTCAAAGATACTCAGG-3' and 5'-GCTCATCACCCCATTCAGG-3'; *IL12A* (IL-12 subunit α): 5'-GCAGCCTCCTCCTTGTGG-3' and 5'-GCCCTCAGCAGGTTTTGG-3'; *PAK1* (Pak1): ACCACTCCACCAGATGCTTT and CGCCCACACTCACTATGCT. Primer design was performed using Primer3 and primer specificity was confirmed using the In-Silico PCR UCSC Genome Browser.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. SigmaPlot Version 12.0 for windows was used for the statistical analysis. Data were analyzed by one-way ANOVA, followed by a Holm-Sidak test to evaluate the significance of disparities between the different conditions. A value of $p<0.05$ was considered statistically significant and $p<0.001$ statistically highly significant.

Results

Effect of 1-NPZ on the viability in MNT-1 cells

Treatment of human MNT-1 melanoma cells with 1-NPZ caused distinctive morphological changes, such as roundness and flattening (**Fig. 1**).

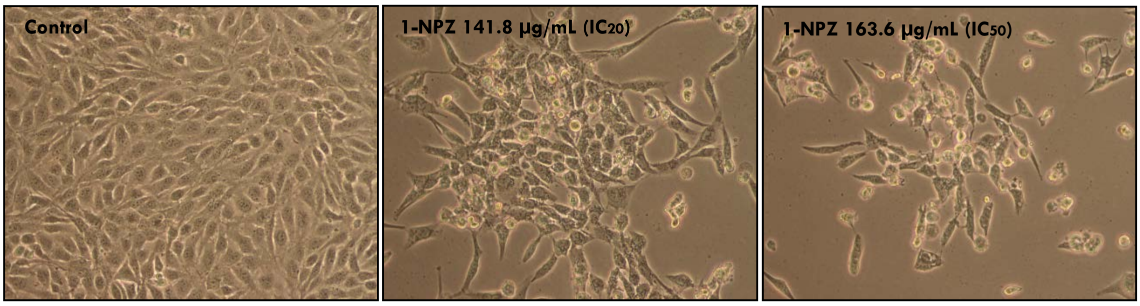


Fig. 1. **1-(1-Naphthyl)piperazine (1-NPZ) induced morphological changes and cell death in human MNT-1 melanoma cells.** Light microscopy images of MNT-1 cells exposed to 1-NPZ (0, 141.8 and 163.6 μg/mL) for 24 h (100x total magnification).

MTT assay was performed to measure MNT-1 cells viability after 3 and 24 h exposure to different 1-NPZ concentrations (50-300 μg/mL). The viability of exposed MNT-1 cells decreased in a dose- and time-dependent manner. Accordingly, there was a significant reduction in cell viability ($p<0.05$) after 3 and 24 h exposure to 1-NPZ concentrations just above 50 and 150 μg/mL, respectively (**Fig 2**). Thus, the exposure time selected for further experiments was 24 h, and the concentrations of 1-NPZ used were 141.8 and 163.6 μg/mL correspondent to IC_{20} and IC_{50}, respectively.

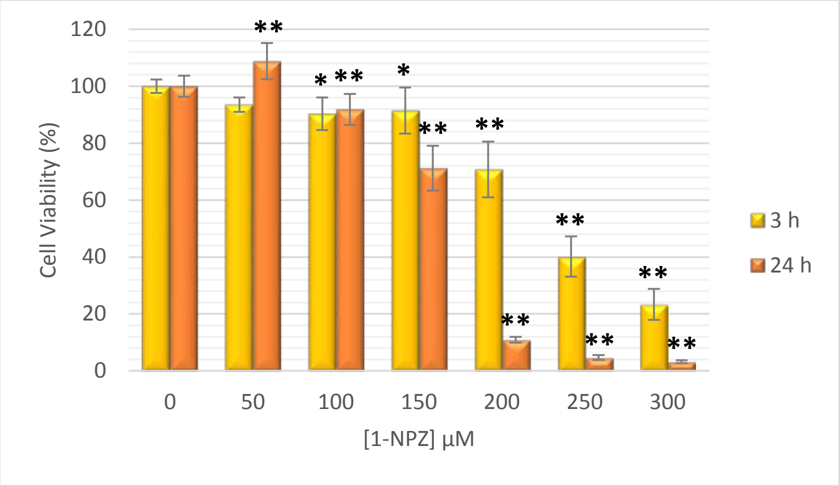


***Fig. 2.* 1-(1-Naphthyl)piperazine (1-NPZ) inhibited the cell viability of human MNT-1 melanoma cells.** MTT cell viability after 3-h and 24-h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: **p*<0.05 (significant), ***p*<0.001(highly significant).

Effect of 1-NPZ on cell cycle distribution in MNT-1 cells

The effect of 1-NPZ on cell cycle was also investigated as a possible cause for the reduction in cell viability as observed above. Briefly, MNT-1 cells were treated with 141.8 µg/mL (IC_{20}) and 163.6 µg/mL (IC_{50}) of 1-NPZ for 24 h and cell cycle profiles were analyzed by flow cytometry. The results are presented in **Fig. 3**.

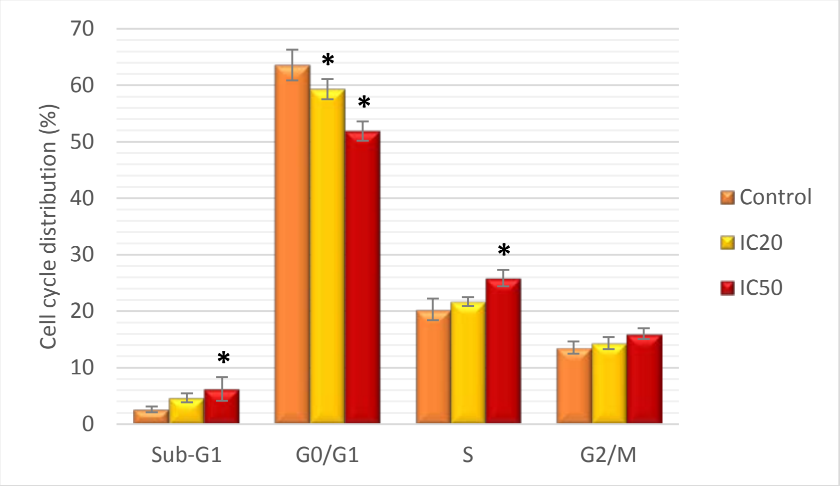


Fig. 3. **1-(1-Naphthyl)piperazine (1-NPZ) inhibited cell cycle progress in human MNT-1 melanoma cells.** Cell cycle distribution after 24-h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: *p<0.05 (significant).

1-NPZ treatment induced a significant accumulation of cells in the sub-G1 phase (hypodiploid cells) from 2.6 % to 6.2 % at IC_{50} ($p<0.05$). Exposed cells to IC_{50} also resulted in a significant decrease in the proportion of cells (51.9 %) in the G0/G1 phase of the cell cycle compared to untreated control (63.6 %). the percentage of cells population in the S phase significantly increased from 20.3 % in the untreated group to 25.9 % in the treated group (IC_{50}). The proportion of cells in the G2/M phase tend to increase with exposure, however differences did not reach statistical significance. These results showed that 1-NPZ inhibited MNT-1 cells proliferation.

Effect of 1-NPZ on the intracellular ROS levels in MNT-1 cells

In order to evaluate the capacity of 1-NPZ to cause oxidative stress, MNT-1 cells were treated with IC_{20} and IC_{50} of 1-NPZ for 24 h, and then analyzed by flow cytometry using DCFH-DA as a fluorescence probe. As shown in **Fig. 4a** and **b**, ROS formation increased in a dose-dependent manner. The generation of ROS was significantly higher for IC_{50} ($p<0.05$) (**Fig. 4a**).

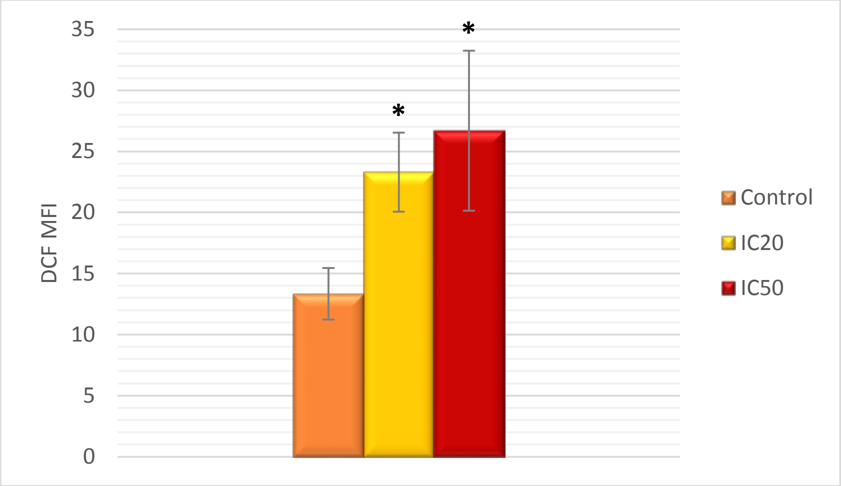


Fig. 4a. **1-(1-Naphthyl)piperazine (1-NPZ) induced the generation of ROS in human MNT-1 melanoma cells.** DCF mean fluorescence intensity (MFI) after 24-h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: $*p<0.05$ (significant).

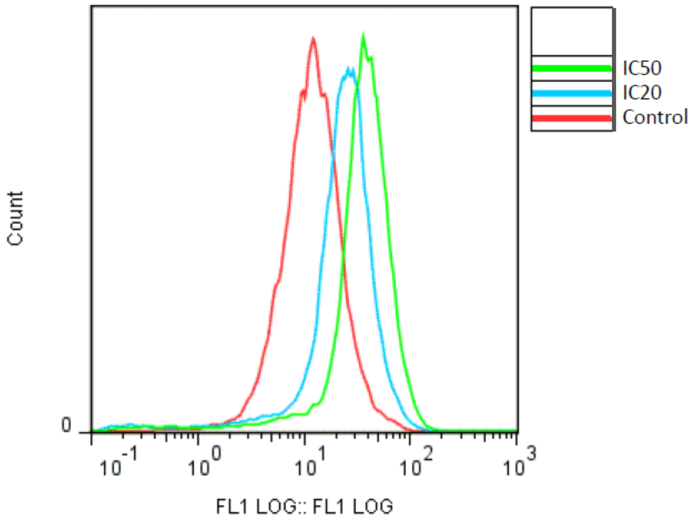


Fig. 4b. **1-(1-Naphthyl)piperazine (1-NPZ) induced the generation of ROS in human MNT-1 melanoma cells.** FSC-SSC density plot (SS LOG *vs* FS LOG).

Effect of 1-NPZ on apoptosis in MNT-1 cells

The extent of apoptosis of 1-NPZ (IC_{20} and IC_{50}) treated and untreated MNT-1 cells further stained with annexin V-FITC and propidium iodide was measured by flow cytometry. As shown in **Fig. 5a** and **b**, the main shift upon 1-NPZ exposure was from viable to apoptotic cells. IC_{20} and IC_{50} of 1-NPZ clearly induced apoptosis after 24 h, increasing the percentages of both early and late apoptotic cells from 1.8 to 19.1 % (IC_{20}) and 29.1 % (IC_{50}) and from 8.2 to 18.5% (IC_{20}) and 23.6 % (IC_{50}), respectively. Viable and non-apoptotic cells also exhibited a decrease in their percentages after 1-NPZ exposure. All differences between control and IC_{50} treatment group were statistically significant (p<0.05).

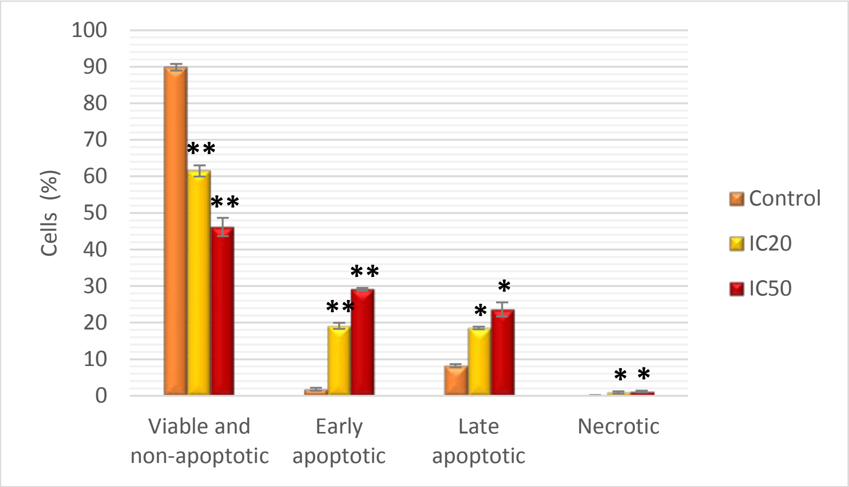


***Fig. 5a*. 1-(1-Naphthyl)piperazine (1-NPZ) increased apoptotic cells in human MNT-1 melanoma cells.** Percentage of apoptotic cells after 24 h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: *p<0.05 (significantly different); **p<0.001(highly significant).

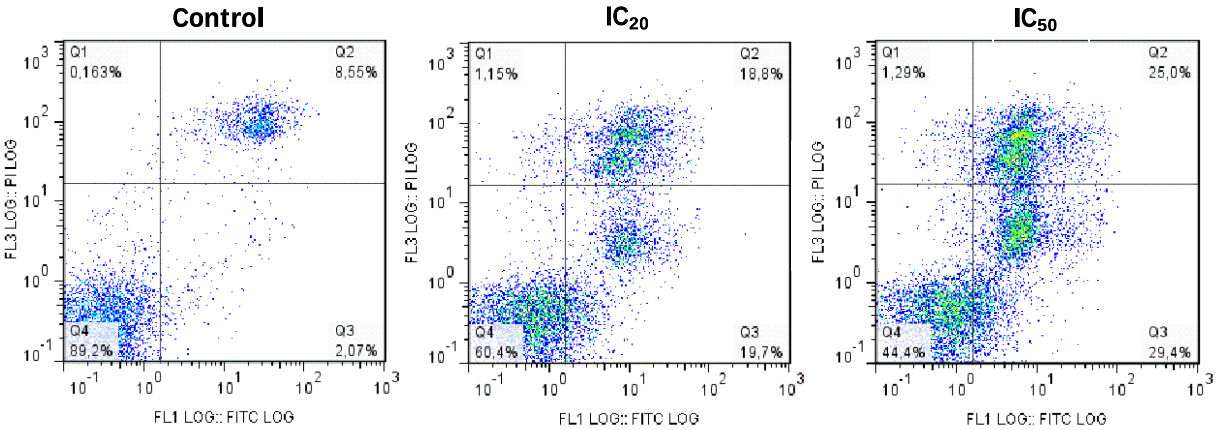


***Fig. 5b*. 1-(1-Naphthyl)piperazine (1-NPZ) increased apoptotic cells in human MNT-1 melanoma cells.** Annexin V-FITC Dot-Plots Gating (FL1 LOG vs FITC LOG): Q1 - Necrotic cells (Annexin-FTIC (-) and PI (+)); Q2 – Late apoptotic cells (FTIC (+) and PI (+)); Q3 – Early apoptotic

cells (Annexin-FTIC (+) and PI (-)); Q4 – Viable and non-apoptotic cells (Annexin-FTIC (-) and PI (-)).

Effects of 1-NPZ on the expression of several genes in MNT-1 cells

mRNA expression of genes related to immunosuppression (*PTGS2 or COX-2* and *IL12A*) and chemoresistance events (*PAK1*) was analyzed by RT-PCR. The expression levels of genes related to apoptosis (*CASP3*, *BAX* and *BCL2*) were also evaluated (**Supplementary data Fig. 1**). The analysis was performed using a lower concentration of 1-NPZ (IC_{20}), considering that increasing cellular damage could mask the effect of 1-NPZ on these cells. Since the plausible mechanism of action of 1-NPZ interferes with UV-induced immunosuppression, genes associated with such events were also investigated including COX-2 (*PTGS2*) and IL-12 genes. Both genes presented an increased expression being *PTGS2* significantly up-regulated (**Fig. 6d** and **e**). In addition, exposure of MNT-1 cells to 1-NPZ led to a decrease in *PAK1* expression (**Fig. 6f**).

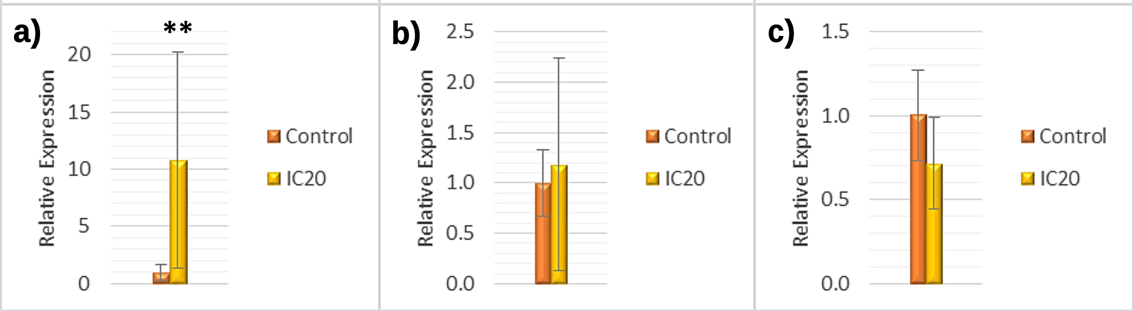


Fig. 6. **1-(1-Naphthyl)piperazine (1-NPZ) induced changes on the expression levels of several genes in human MNT-1 melanoma cells.** *PTGS2* (a), *IL12A* (b) and *PAK1* (c) expression after 24-h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: $**p<0.001$(highly significant).

Discussion

In the present study, the effects of 1-NPZ on human MNT-1 melanoma cells towards the treatment of skin cancer were investigated.

Treatment of MNT-1 cells with 1-NPZ led to visible **morphological changes** and damage, becoming flattened and rounded as the concentration increased (**Fig. 1**). In addition, 1-NPZ exposure for either 3 or 24 h significantly inhibited the **viability** of MNT-1 cells in a dose- and time-dependent manner (**Fig. 2**). Moreover, both exposure time and 1-NPZ concentration were selected from MTT cell viability assay to establish the exposure conditions for the other experiments. Considering the results obtained, an exposure time of 24 h ($p<0.001$) and concentrations of 1-NPZ corresponding to IC_{20} (141.8 µg/mL) and IC_{50} (163.6 µg/mL) were selected.

Further experiments were performed to clarify the mechanism leading to decreased cell viability in MNT-1 cells by 1-NPZ. The most defining feature of cancer may be uncontrolled proliferation, caused by the loss of normal **cell cycle** control [21,22]. The cellular response to DNA damage is a cell cycle arrest via checkpoint mechanisms, which in turn depend on the type of damage and the phase of the cell cycle at which it occurs [22]. It was shown that treatment of MNT-1 cells with 1-NPZ (IC_{50}) induced a significant S-phase delay of cell cycle progression ($p<0.05$) (**Fig. 3**). The most frequently used biochemical marker of apoptotic cell death is the generation of fragmented DNA [23], which is represented on the cell cycle histograms by the sub-G1 peak. As shown, treating MNT-1 cells with 1-NPZ led to an increased percentage of cells in sub-G1 phase, which might be a sign of apoptosis. The upsurge in sub-G1 phase cells was significant again for IC_{50} ($p<0.05$).

ROS production and oxidative stress have been long associated with melanoma and other types of cancer. ROS are capable of modulating the activities and expression of numerous transcription factors and proteins that are implicated in the stress response and cell survival [24]. Indeed, several mechanisms can induce oxidative stress during cancer progression, including activation of oncogenes, mitochondrial dysfunction, loss of functional p53, inflammatory cytokines, among others [24]. Here, the treatment of MNT-1 cells with 1-NPZ significantly increased ROS production in a dose-dependent manner ($p<0.05$) (**Fig. 4a** and **b**). These results supported the hypothesis that treatment with 1-NPZ would lead to ROS production, which might play an essential role in 1-NPZ-induced apoptosis in MNT-1 cells. The results here are different from those obtained by Sreevidya et al. [19], where 1-NPZ treatment blocked ROS production in Pam 212 cells previously exposed to 5-HT, *cis*-UCA or UV irradiated. However, in this case 1-NPZ might have contributed to the recovery of cells upon a minor UV damage. Regardless, the increase of ROS following treatment with 1-NPZ could be due either to an upsurge of ROS production or to a reduction of ROS-scavenging ability by MNT-1 cells [24]. Despite an increase in ROS being convenient for cancer cells, excessive levels of these reactive species can be also toxic to the cells [24]. Thus, cancer cells with an excessive production of ROS are likely to be more vulnerable to damage by oxidative stress [24]. Therefore, targeting these biochemical alterations might be a viable strategy to attain therapeutic activity and selectivity, and even prevent chemoresistance [24].

In more advanced stages, several malignancies develop resistance to **apoptosis** and/or to the cytotoxic effects of chemotherapeutics. Apoptotic cell death is characterized by distinctive physiological changes in cells including surface exposure of phosphatidylserine, which can be detected by its affinity for a phospholipid binding protein, annexin V [25]. The membrane integrity is not affected during this process [25]. Therefore, the effects of 1-NPZ on the induction of apoptosis in MNT-1 cells were investigated. As shown in **Fig. 5a** and **b**, treating cells with 1-NPZ caused a highly significant reduction in viable and non-apoptotic cells ($p<0.001$) as well as a significant increase in early apoptotic cells at both IC_{20} and IC_{50} ($p<0.001$). At the same time, there was a significant increase in necrotic and late apoptotic cells following 1-NPZ exposure at both concentrations ($p<0.001$). These results suggested that induction of apoptosis in MNT-1 cells following 1-NPZ treatment could explain and be the primary mechanism for the reduced cell viability caused by 1-NPZ. Therefore, treatment of MNT-1 cells with 1-NPZ led to a significant dose-dependent induction of apoptosis, reinforcing the idea that 1-NPZ might be a promising chemotherapeutic agent against melanoma skin cancer.

Apoptosis can be preceded by **upregulation** and/or **downregulation of various genes** in cells. Considering that 1-NPZ has already demonstrated its ability to modulate immunosuppression *in vivo*, here the expression levels of COX-2 and IL-12 genes were also investigated. COX-2 (encoded by the *PTHS* gene) is a ubiquitously expressed inducible enzyme involved in the regulation of prostaglandin synthesis, especially PGE_2 [26,27]. COX-2 is usually associated with cancer progression, and has been shown to significantly enhance carcinogenesis by upregulating phosphoinositide 3-kinase (PI3K) signaling, among others [28]. NF-κB plays an important role in COX-2 promoter activity [28]. Regarding IL-12, this immunomodulatory cytokine is produced mainly by antigen-presenting cells, and it has well-evidenced roles in the inhibition of UV-induced DNA damage and immunosuppression [14,29]. Previous studies in humans have shown that IL-12 remains a potential immunotherapeutic agent [30]. In addition, expression level of Pak1 gene was also analyzed attending that its expression is significantly increased in various types of cancer. Pak1 is a central downstream effector of the Rho family GTPases (Cdc42 and Rac1), playing an essential role in controlling cell motility and apoptosis [31]. In fact, Pak1 has been shown to protect cells from apoptotic cell death induced by chemotherapeutic agents [32]. As shown in **Fig. 6**, the expression levels of COX-2 gene (*PTGS2*) increased significantly following treatment with 1-NPZ in MNT-1 cells ($p<0.001$). Furthermore, 1-NPZ exposure induced both an increase and decrease in expression levels of IL-12*A* and Pak1 genes, respectively (**Fig. 9**). The unexpected increase in COX-2 expression levels might be explained by a similar COX-2-dependent pathway underlying apoptosis elicited by cannabidiol [33]. Previous studies have already shown that cannabidiol is able to inhibit 5-HT reuptake and reduce 5-HT neurotransmission [34]. Moreover, cannabidiol has demonstrated a high affinity for both 5-HT1/2AR, acting as an agonist for 5-HT1AR [34]. Therefore, 1-NPZ could have the same effects on MNT-1 cells by preventing the binding of 5-HT to both 5-HT1/2AR. COX-2-dependent apoptosis has shown to be preceded by activation of the transcription factor PPAR-γ by prostaglandins [33]. In fact, previous studies indicate that PPAR-γ has a crucial role in eliciting apoptosis on other types of cancer cells [35]. Notwithstanding, further work will be required to fully understand the effects of COX-2 upregulation in MNT-1 cells. Regarding IL-12 and Pak1 expression levels, the results obtained were as theoretically expected. IL-12 is an anti-inflammatory cytokine that promotes the reversal of immunosuppression and Pak1 has been identified as a key modulator of melanoma sensitivity to DNA damage [36]. *In vitro* experiments demonstrated that Pak1 is involved in a signaling cascade that ends in decreased apoptosis and increased expression of prosurvival genes following DNA damage in melanoma cells [36].

Conclusion

In summary, this study showed that 1-NPZ is capable of inhibiting cellular growth by inducing S-phase cell cycle delay, ROS generation and apoptosis in human MNT-1 melanoma cells. Additionally, 1-NPZ treatment was capable of inducing changes on important signaling cascades attending to different expression levels of various genes in exposed cells. It was also demonstrated that the mechanism leading to apoptosis in MNT-1 cells by 1-NPZ might not be associated with *cis*-UCA signaling pathway.

This study demonstrated the potential of 5-HT1/2A receptors and 1-NPZ as promising therapeutic target and chemotherapeutic agent, respectively, for the treatment of melanoma skin cancer. Further *in vitro* and *in vivo* studies are essential to fully understand the mechanism of action of 1-NPZ, and to determine whether it could be an efficient chemotherapeutic agent for the treatment of such malignancy.

Acknowledgments

The authors acknowledge Drª Manuela Gaspar for kindly providing human MNT-1 melanoma cells for this work.

Supplementary Data

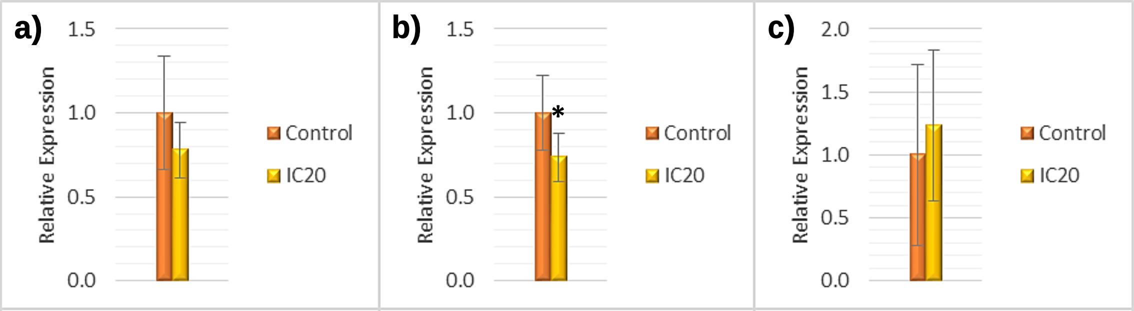


Fig. 1. *CASP3* (a), *BAX* (b) and *BCL2* (c) expression after 24-h treatment. Results are expressed as mean ± SD of three independent experiments. Statistical analysis: **p*<0.05 (significant).

References

1. Kadekaro AL, Kavanagh R, Kanto H, Terzieva S, Hauser J, Kobayashi N, Schwemberger S, Cornelius J, Babcock G, Shertzer HG, Scott G, Abdel-Malek ZA (2005) alpha-Melanocortin and endothelin-1 activate antiapoptotic pathways and reduce DNA damage in human melanocytes. Cancer Res 65 (10):4292-4299. doi:10.1158/0008-5472.CAN-04-4535

2. Narayanan DL, Saladi RN, Fox JL (2010) Ultraviolet radiation and skin cancer. International journal of dermatology 49 (9):978-986. doi:10.1111/j.1365-4632.2010.04474.x

3. Menezes AC, Raposo S, Simoes S, Ribeiro H, Oliveira H, Ascenso A (2015) Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors. Mol Neurobiol. doi:10.1007/s12035-014-9068-z

4. Belanger F, Rajotte V, Drobetsky EA (2014) A majority of human melanoma cell lines exhibits an S phase-specific defect in excision of UV-induced DNA photoproducts. PLoS One 9 (1):e85294. doi:10.1371/journal.pone.0085294

5. Goodsell DS (2001) The molecular perspective: ultraviolet light and pyrimidine dimers. The oncologist 6 (3):298-299

6. Strickland LR, Pal HC, Elmets CA, Afaq F (2015) Targeting drivers of melanoma with synthetic small molecules and phytochemicals. Cancer Lett 359 (1):20-35. doi:10.1016/j.canlet.2015.01.016

7. Mouret S, Forestier A, Douki T (2012) The specificity of UVA-induced DNA damage in human melanocytes. Photochem Photobiol Sci 11 (1):155-162. doi:10.1039/c1pp05185g

8. Ross JA, Howie SE, Norval M, Maingay J, Simpson TJ (1986) Ultraviolet-irradiated urocanic acid suppresses delayed-type hypersensitivity to herpes simplex virus in mice. J Invest Dermatol 87 (5):630-633

9. Kurimoto I, Streilein JW (1992) cis-urocanic acid suppression of contact hypersensitivity induction is mediated via tumor necrosis factor-alpha. J Immunol 148 (10):3072-3078

10. Kondo S, Sauder DN, McKenzie RC, Fujisawa H, Shivji GM, El-Ghorr A, Norval M (1995) The role of cis-urocanic acid in UVB-induced suppression of contact hypersensitivity. Immunol Lett 48 (3):181-186. doi:10.1016/0165-2478(95)02462-X

11. Lauerma AI, Aioi A, Maibach HI (1995) Topical cis-urocanic acid suppresses both induction and elicitation of contact hypersensitivity in BALB/C mice. Acta Derm Venereol 75 (4):272-275

12. Gibbs NK, Tye J, Norval M (2008) Recent advances in urocanic acid photochemistry, photobiology and photoimmunology. Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology 7 (6):655-667. doi:10.1039/b717398a

13. Norval M, Gilmour JW, Simpson TJ (1990) The effect of histamine receptor antagonists on immunosuppression induced by the cis-isomer of urocanic acid. Photodermatol Photoimmunol Photomed 7 (6):243-248

14. Beissert S, Ruhlemann D, Mohammad T, Grabbe S, El-Ghorr A, Norval M, Morrison H, Granstein RD, Schwarz T (2001) IL-12 prevents the inhibitory effects of cis-urocanic acid on tumor antigen presentation by Langerhans cells: implications for photocarcinogenesis. Journal of immunology 167 (11):6232-6238

15. Beissert S, Mohammad T, Torri H, Lonati A, Yan Z, Morrison H, Granstein RD (1997) Regulation of tumor antigen presentation by urocanic acid. J Immunol 159 (1):92-96

16. De Fabo EC, Noonan FP (1983) Mechanism of immune suppression by ultraviolet irradiation in vivo. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. J Exp Med 158 (1):84-98

17. Walterscheid JP, Nghiem DX, Kazimi N, Nutt LK, McConkey DJ, Norval M, Ullrich SE (2006) Cis-urocanic acid, a sunlight-induced immunosuppressive factor, activates immune suppression via the 5-HT2A receptor. Proceedings of the National Academy of Sciences of the United States of America 103 (46):17420-17425. doi:10.1073/pnas.0603119103

18. Sreevidya CS, Khaskhely NM, Fukunaga A, Khaskina P, Ullrich SE (2008) Inhibition of photocarcinogenesis by platelet-activating factor or serotonin receptor antagonists. Cancer research 68 (10):3978-3984. doi:10.1158/0008-5472.CAN-07-6132

19. Sreevidya CS, Fukunaga A, Khaskhely NM, Masaki T, Ono R, Nishigori C, Ullrich SE (2010) Agents that reverse UV-Induced immune suppression and photocarcinogenesis affect DNA repair. The Journal of investigative dermatology 130 (5):1428-1437. doi:10.1038/jid.2009.329

20. Gomes A, Fernandes E, Lima JL (2005) Fluorescence probes used for detection of reactive oxygen species. Journal of biochemical and biophysical methods 65 (2-3):45-80. doi:10.1016/j.jbbm.2005.10.003

21. Kamb A (1995) Cell-cycle regulators and cancer. Trends in genetics : TIG 11 (4):136-140

22. Pavey S, Spoerri L, Haass NK, Gabrielli B (2013) DNA repair and cell cycle checkpoint defects as drivers and therapeutic targets in melanoma. Pigment cell & melanoma research 26 (6):805-816. doi:10.1111/pcmr.12136

23. Kastan MB, Canman CE, Leonard CJ (1995) P53, cell cycle control and apoptosis: implications for cancer. Cancer metastasis reviews 14 (1):3-15

24. Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 8 (7):579-591. doi:10.1038/nrd2803

25. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31 (1):1-9

26. Kale S, Raja R, Thorat D, Soundararajan G, Patil TV, Kundu GC (2014) Osteopontin signaling upregulates cyclooxygenase-2 expression in tumor-associated macrophages leading to enhanced angiogenesis and melanoma growth via alpha9beta1 integrin. Oncogene 33 (18):2295-2306. doi:10.1038/onc.2013.184

27. Fu L, Chen W, Guo W, Wang J, Tian Y, Shi D, Zhang X, Qiu H, Xiao X, Kang T, Huang W, Wang S, Deng W (2013) Berberine Targets AP-2/hTERT, NF-kappaB/COX-2, HIF-1alpha/VEGF and Cytochrome-c/Caspase Signaling to Suppress Human Cancer Cell Growth. PloS one 8 (7):e69240. doi:10.1371/journal.pone.0069240

28. Gowda R, Madhunapantula SV, Desai D, Amin S, Robertson GP (2013) Simultaneous targeting of COX-2 and AKT using selenocoxib-1-GSH to inhibit melanoma. Molecular cancer therapeutics 12 (1):3-15. doi:10.1158/1535-7163.MCT-12-0492

29. Narayanapillai S, Agarwal C, Deep G, Agarwal R (2014) Silibinin inhibits ultraviolet B radiation-induced DNA-damage and apoptosis by enhancing interleukin-12 expression in JB6 cells and SKH-1 hairless mouse skin. Molecular carcinogenesis 53 (6):471-479. doi:10.1002/mc.22000

30. Lee P, Wang F, Kuniyoshi J, Rubio V, Stuges T, Groshen S, Gee C, Lau R, Jeffery G, Margolin K, Marty V, Weber J (2001) Effects of interleukin-12 on the immune response to a multipeptide vaccine for resected metastatic melanoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 19 (18):3836-3847

31. Ong CC, Jubb AM, Haverty PM, Zhou W, Tran V, Truong T, Turley H, O'Brien T, Vucic D, Harris AL, Belvin M, Friedman LS, Blackwood EM, Koeppen H, Hoeflich KP (2011) Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. Proceedings of the National Academy of Sciences of the United States of America 108 (17):7177-7182. doi:10.1073/pnas.1103350108

32. Deacon K, Mistry P, Chernoff J, Blank JL, Patel R (2003) p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. Molecular biology of the cell 14 (5):2071-2087. doi:10.1091/mbc.E02-10-0653

33. Ramer R, Heinemann K, Merkord J, Rohde H, Salamon A, Linnebacher M, Hinz B (2013) COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells. Molecular cancer therapeutics 12 (1):69-82. doi:10.1158/1535-7163.MCT-12-0335

34. Russo EB, Burnett A, Hall B, Parker KK (2005) Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochemical research 30 (8):1037-1043. doi:10.1007/s11064-005-6978-1

35. Keshamouni VG, Reddy RC, Arenberg DA, Joel B, Thannickal VJ, Kalemkerian GP, Standiford TJ (2004) Peroxisome proliferator-activated receptor-gamma activation inhibits tumor progression in non-small-cell lung cancer. Oncogene 23 (1):100-108. doi:10.1038/sj.onc.1206885

36. Ho H, Aruri J, Kapadia R, Mehr H, White MA, Ganesan AK (2012) RhoJ regulates melanoma chemoresistance by suppressing pathways that sense DNA damage. Cancer research 72 (21):5516-5528. doi:10.1158/0008-5472.CAN-12-0775

Chapter 3

PHARMACEUTICAL THECNOLOGY STUDIES

Development and Characterization of Novel 1-(1-Naphthyl)Piperazine-loaded Lipid Vesicles for Dermal Delivery

Ana Catarina Menezes[1], Sandra Simões[2], Andreia Ascenso[2]

[1]Faculdade de Farmácia da Universidade de Lisboa
Lisbon, Portugal

[2]NanoBB Research Group of iMed.UL
Lisbon, Portugal

Abstract

Topical administration of anticancer drugs represents a potential therapeutic approach for the efficient treatment of skin cancer. 1-(1-Naphthyl)piperazine (1-NPZ) has shown promising effects by inhibiting UV-induced immunosuppression, and therefore, photocarcinogenesis. Ultradeformable vesicles (UDV) are new advantageous systems capable of improving the dermal and/or transdermal delivery of numerous drugs. Thus, the aim of the present study was to investigate 1-NPZ-loaded transethosomes (NPZ-TE) and dmsosomes (NPZ-DM) as novel topical delivery nanosystems for the treatment of this malignancy.

The physicochemical properties of both vesicle formulations were evaluated, including the mean vesicles size and zeta potential by dynamic and electrophoretic light scattering, respectively. Vesicle deformability was assessed by pressure driven transport, rheology by viscometry and both lipid and drug entrapment yields by spectrophotometry and HPLC, respectively. *In vitro* topical delivery studies were also performed in order to evaluate the penetration and permeation profiles.

Either NPZ-TE or NPZ-DM exhibited positive results in terms of mean size, zeta potential, deformability and rheology. 1-NPZ entrapment yield was 90.6 % for NPZ-TE and 95.8 % for NPZ-DM. *In vitro* data also revealed an improved penetration of 1-NPZ into newborn pig skin, especially by NPZ-TE (0.31 μg/cm^2).

The successful results here obtained encourage us to carry on the studies on both formulations for the topical treatment of skin cancer.

Keywords: Skin cancer, 1-NPZ, transethosomes, dmsosomes, topical delivery

Introduction

Skin cancer is the most common malignancy worldwide and as deadly as other forms of cancer [1,2]. Nowadays, surgical excision is the most common treatment for these localized malignancies, even though it is no longer feasible when they spread to distant sites [2,3]. For metastatic melanoma, nanomedicine offers a powerful drug delivery system, allowing a site-specific drug delivery to cancer cells, which significantly increases treatment efficiency [2]. In addition, topical administration of anticancer drugs as an alternative to other routes of administration and surgery can be an extremely pertinent option that deserves to be explored, especially for skin cancer [3].

1-(1-Naphthyl)piperazine (1-NPZ) is a serotonergic (5-hydroxytryptamine, 5-HT) derivative of quipazine [4]. It is both a 5-HT2A receptor antagonist and 5-HT1A receptor agonist, comprising a dual mechanism of action in the prevention of immunosuppression and photocarcinogenesis. Hence, 1-NPZ will block the binding of *cis*-urocanic acid to 5-HT2A receptor while eliciting immune cells through 5-HT1A receptor [1]. These receptors (R) are primarily present in melanocytes [1]. Both *in vitro* and *in vivo* experiments have shown promising results with this serotonergic drug [5,6]. In fact, we have already observed such beneficial effects caused by the action of 1-NPZ on photocarcinogenesis through several *in vitro* studies with human MNT-1 melanoma cell line (results not published yet).

Several drug nanodelivery systems have been developed over the years for the treatment of skin cancer, such as liposomes, ultradeformable vesicles (UDV), polymersomes, protein-based nanoparticles, carbon-based nanoparticles, dendrimers and inorganic nanoparticles [2]. The inherent benefits range from increased drug absorption through the skin with a sustained release to decreased degradation of the entrapped drug [7]. Conventional liposomes were the first nanocarriers used to enhance the permeation rate of the entrapped active compounds across the skin [8]. However, their use was limited by their confinement in the *stratum corneum* (SC) due to their large minimum size and absence of flexibility [9]. To overcome such limitation, pioneering vesicular systems have been developed with increased permeation rates of topically administered drugs [8].

Recent strategies in modulating the composition of nanocarriers have resulted in several designs of novel vesicular drug delivery systems, including transfersomes and ethosomes, among others. Transfersomes were the first generation of flexible liposomes introduced by Cevc and Blune in the early 1990s [10]. They contain phospholipids and an edge activator, which is often a single-chain surfactant that destabilizes the lipid bilayers in order to increase vesicles flexibility [11]. The assembly of all these components explain the improved efficiency of transfersomes, whose hydration energy of polar head groups is of great importance in their mechanism of penetration across the skin [12]. In fact, the main force responsible for the penetration of these deformable vesicles is an osmotic gradient created by variances in water concentration among different skin layers [12]. Thus, these vesicles must be topically applied under non-occlusive conditions [13]. Ethosomes were developed by Touitou et al. in 2000 [14], and are formulated with phospholipids, water and ethanol (20-45 %) [13]. High ethanol content allows ethosomes to have a much smaller size than liposomes and greater flexibility, disrupting the organization of SC and improving its fluidity [12,15]. *In vitro* and *in vivo* experiments have already demonstrated that both ethosomes and transfersomes are capable of enhancing skin delivery of numerous drugs [15-18]. Given the advantages that transfersomes and ethosomes may provide, liposomal formulations comprising both a surfactant and an alcohol would be desirable as a flexible carrier to deliver drugs into deeper skin layers. Transethosomes (TE) emerged a few years ago and were developed by Song et al. [19]. They consist of phospholipids, water, ethanol and an edge activator or a penetration enhancer [19]. These novel vesicular nanocarriers have shown increased skin permeation and hence superior characteristics compared to conventional liposomes, transfersomes and ethosomes [19,20]. We have also obtained similar results in a previous study [21].

Our research group has recently developed another type of innovative vesicles containing dimethyl sulfoxide (DMSO) termed Dmsosomes (DM) for a simplified designation. DMSO is a powerful aprotic solvent and one of the most popular skin penetration enhancer, being capable of promoting the transdermal delivery of numerous drugs [22]. It increases skin permeability by interacting with the intercellular lipid domains of SC and interfering with the packing geometry [22]. DMSO is generally used as a tissue/organ preservative, penetration enhancer and solubilizing agent [23]. In fact, 1-NPZ has a higher solubility in DMSO rather than in water, which clearly justifies the use of DMSO. In addition, it has also biological activity, being a free radical scavenger and exhibiting anti-inflammatory analgesic effects [24]. As a radical scavenger, DMSO acts by neutralizing free radicals, stabilizing cellular membranes and slowing down or stopping cell death in traumatized areas as those affected by extravasation of chemotherapy into the tissues surrounding the administration site [25,26]. Apart from these applications, DMSO has already shown promising results as a chemotherapeutic agent [27]. However, its use in cancer therapy continues to generate an intense debate due to its side effects [22].

The aim of the current study was to formulate and fully characterize 1-NPZ-loaded transethosomes and 1-NPZ-loaded dmsosomes for topical administration regarding skin cancer context.

Materials and Methods

Materials

1-NPZ was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Soybean phosphatidylcholine (S100) was obtained from Lipoid AG (Steinhausen, Switzerland). Sodium cholate, dimethyl sulfoxide (DMSO), dimethylformamide (DMF) and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Perchloric acid and ammonium molybdate were purchased from Merck (Darmstadt, Germany) and Riedel-de Haen (Seelze, Germany), respectively. Citric acid monohydrate and trisodium citrate dihydrate were purchased from Panreac Quimica, SA (Barcelona, Spain). Ultrapure water was obtained from a MILLI-Q System by Millipore (Billerica, MA, USA). All other reagents were of analytical or high performance liquid chromatography (HPLC) grade.

Preparation of Lipid Vesicles Formulations

1-NPZ-loaded Transethosomes (NPZ-TE) were formulated according to **Table 1** and prepared by the "classic cold method", as described in literature [14,21]. Briefly, SPC (at 10 % w/v final concentration) and sodium cholate were first dissolved in absolute ethanol under constant stirring and immersed in a water bath maintained at 30 ± 2 ºC. 1-NPZ previously dissolved in purified water by sonication (at 0.04 % w/v final concentration) was then slowly added through a syringe needle to the prior mixture under stirring in a well-sealed container. The formulation was continuously mixed for an additional 5 min at 30 ºC and left to cool at room temperature. NPZ-TE were dimensioned by pressure filtration through track-etched polycarbonate membranes of 100 nm pore size (Millipore, USA).

1-NPZ-loaded Dmsosomes (NPZ-DM) were also formulated according to **Table 1**. The composition of these vesicles was optimized in previous studies (results not published yet). 1-NPZ previously dissolved in DMSO by sonication (0.04 % w/v) was added to SPC (20 % w/v) and citrate buffer 50 mM pH 5 under vigorous stirring for about 24 h at room temperature. NPZ-DM were then filtered through track-etched polycarbonate membranes of 400 and 100 nm pore size under nitrogen stream.

Empty vesicles (TE and DM) were used as controls for each formulation.

Table 1. Composition of lipid vesicles formulations.

Ingredient	Class	Main Action	Composition (% w/v)	
			TE	DM
1-NPZ	Drug	5-HT1AR agonist/5-HT2AR antagonist	0.04	0.04
Soybean phosphatidylcholine (SPC)	Phospholipid	Vesicles forming component	10	20
Sodium cholate (NaCo)	Anionic surfactant	Vesicles flexibility	3.75:1 (SPC: NaCo, Molar ratio)	–
DMSO	Polar aprotic solvent	Solvent Skin enhancer	–	30
Ethanol	Polar protic solvent	Solvent Skin enhancer	30	-
Purified Water	Polar protic solvent	Hydrating medium	q.s. ad 100	-
Citrate buffer 50 mM solution	Buffering agent (pH 5)	Hydrating medium	-	q.s. ad 100

Physical Characterization of Lipid Vesicles Formulations

Vesicles Size and Zeta Potential

Particle size and polydispersity index (PDI) of both NPZ-TE and NPZ-DM formulations were determined by dynamic light scattering using a Zetasizer Nano-S (Malvern Instruments Lda., Worcestershire, UK). Zeta potential was measured by electrophoretic light scattering using a Zetasizer Nano-Z (Malvern Instruments Lda., Worcestershire, UK). Both formulations were properly diluted with 3-4 mL ultrapure water to escape multiple scattering phenomena. Measurements were performed in triplicate for each sample.

Deformability Index

Deformability index of both vesicles formulations was performed by pressure driven transport with a stainless steel pressure holder of 1 mL capacity. Accordingly, previously diluted NPZ-TE and NPZ-DM (at 1 % w/v final concentration of SPC) were forced to pass through a 30 nm pore size track-etched polycarbonate membrane under 10 bar pressure for at least 5 min. Data were collected with WinWedge software (TAL Technologies Inc., Philadelphia, USA) and carried out in triplicate. The amount of each formulation collected was weighed and plotted against time in order to determine the deformability index (DI) according to **Eq. 1** [28]:

$$DI = J\left(\frac{d_0}{p}\right)\left(\frac{d_0}{|d_1 - d_0|}\right) \qquad (\mathbf{1})$$

Where J is the flux of vesicles throughout pressure filtration (g/s), d_0 and d_1 are the vesicle mean sizes (nm) before and after pressure filtration respectively, and p is the pore size of the membrane (nm).

Rheology

The rheology study was performed using a DV-II Brookfield Digital Viscometer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) with a spindle number 21. Flow curves were generated by ramping the shear rate from 6.12 to 122.36 s^{-1} (upward curve) and then from 122.36 to 6.12 s^{-1} (downward curve) for 30 s each. All tests were carried out on 10 mL samples at 25 ± 1 ºC. The shear stress was calculated using Newton’s law of viscosity according to **Eq. 2**:

$$\tau = \eta \times \gamma \qquad (\mathbf{2})$$

Where τ is the shear stress (Pa), η is the viscosity (Pa.s) and γ is the shear rate (s^{-1}).

Chemical Characterization of Lipid Vesicles Formulations

Phospholipid Content

The lipid content of both NPZ-TE and NPZ-DM before and after the purification step was measured using a method introduced by Rouser et al. [29], which is based on the colorimetric determination of inorganic phosphate. After diluting all samples in ultrapure water (1:50 for NPZ-TE and 1:100 for NPZ-DM), 25 µL of each sample was added to the test tubes in triplicate and then heated in a dry bath (Block heater SBH200D/3, Stuart, UK) until complete dryness. After complete cooling to room temperature, 300 µL of perchloric acid 70% was added to each tube in order to hydrolyze phospholipids into inorganic phosphate, and heated at 170 ºC for about 45 min. After cooling, 1 mL water, 400 µL ammonium molybdate 1.25 % and 400 µL ascorbic acid solution 5% were added to each tube and further homogenized. Then, all tubes were heated in a water bath at 100ºC for 5 min. The phospholipid content was determined spectrophotometrically at 797 nm (Shimadzu UV-160 Spectrophotometer, Kyoto, Japan). A calibration curve was also performed in the same experiment in order to determine the lipid content of the samples.

Drug Entrapment Yield

The quantification of 1-NPZ entrapped in TE and DM was performed after the purification step by ultracentrifugation of diluted samples at 180,000 x *g* and 15 ºC for 2 h (L8-60M ultracentrifuge Beckman Coulter, Brea, CA, USA).

1-NPZ quantification was confirmed in both pellet and supernatant by HPLC using a Midas Spark 1.1 autoinjector (Spark, AJ Emmen, The Netherlands), a Diode-Array 168 detector (Beckman Coulter, Brea, CA, USA), and a PurospherStar RP-18, 5 µm 150-4.6 column (Merck, Darmstadt, Germany). The HPLC system was monitored by a 32 Karat software (Beckman Coulter, Brea, CA, USA). The mobile phase was composed of methanol and water (40:60 v/v) acidized to pH 4.7, and eluted at a flow rate of 1.0 mL/min. Both vesicle formulations were diluted and destroyed with DMF in order to quantify 1-NPZ, which was detected at 221 nm with an injected loop of 10 µL.

pH evaluation

The pH of each formulation was evaluated by potentiometry (Metrohm pH Meter 744 with a glass electrode; Herisau, Switzerland).

Chemical Stress Stability Study

NPZ-TE and NPZ-DM were subjected to a chemical stability study under stress light conditions, as reported earlier [30]. The formulations were well sealed and exposed to incandescent light (60 watt-lamp, 230 V) at a distance of 15 cm for 24 h. After this period, the amount of 1-NPZ remaining in each sample was determined by HPLC.

Topical Delivery Studies

In Vitro Skin Permeation and Penetration

Topical delivery studies were carried out according to OECD Guideline 428 [31] using Franz diffusion cells with a diffusion area of 1.0 cm^2 and fresh newborn pig skin obtained from a local slaughterhouse. Both formulations (300 µL, an infinite dose) were applied onto the skin surface under non-occlusive conditions in the donor compartment. Receptor chamber was filled with 3.7 mL distilled water, assuring the maintenance of sink conditions at 37 ± 3 ºC and under stirring at 200 rpm (Julabo U3 Thermostat and Multimagnetic stirrer SBS, Labexchange, Paris, France). The receptor medium and skin samples from each experiment were analyzed 24 h later in order to evaluate the permeation and penetration profiles, respectively.

After 24 h, the skin surface was rinsed to remove excess formulation and dried with filter paper. Then, the intact skin samples were cut into small sections and the tissue was destroyed with 2 mL DMSO using a Polytron PT 3000 homogenizer at 30,000 rpm for 1 min (Kinematica AG, Lucerne, Switzerland). The tissue suspension was sonicated for 45 min to complete the drug extraction process. The final solution was centrifuged (3,650 x *g* for 20 min) and the supernatant was filtered (0.2 µm) and analyzed for 1-NPZ skin retention by HPLC as described before.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of three independent batches of each formulation. Statistical analysis was assessed by one-way analysis of variance (ANOVA) with all pairwise multiple comparison procedures (Holm-Sidak method) using the SigmaPlot software. Values of $p<0.05$ and $p<0.001$ were considered statistically significant and statistically highly significant, respectively.

Results

Physical Characterization of Lipid Vesicles

Vesicles Size and Zeta Potential

The assessment of physicochemical parameters of vesicle formulations is a crucial aspect to be investigated, since they may affect the biopharmaceutical characteristics of the entrapped drugs.

Accordingly, the mean size, polydispersity index (PDI) and zeta potential were determined and represented in **Table 2**. NPZ-DM showed statistically significant differences in mean size ($p<0.05$) and zeta potential ($p<0.001$) compared to NPZ-TE.

Table 2. Short composition and physical features of the prepared lipid vesicles.

	Composition (% w/v)		**Size (nm)**	**PDI**	**Zeta potential (mV)**
	Ethanol	*SPC*			
Empty TE	30	10	102.90 ± 0.70	0.083 ± 0.010	-39.3 ± 1.3
NPZ-TE	30	10	105.30 ± 18.82	0.126 ± 0.036	-22.3 ± 1.8
Empty DM	-	20	* 197.40 ± 0.72	0.226 ± 0.010	-13.3 ± 0.1 **
NPZ-DM	-	20	212.20 ± 15.56	0.189 ± 0.013	3.3 ± 0.1

Values are expressed as mean ± SD (n = 3). Statistical analysis: *$p<0.05$, **$p<0.001$.

The final mean sizes obtained for NPZ-TE and NPZ-DM formulations were approximately 105.30 ± 18.82 nm and 212.20 ± 15.56 nm, respectively. PDI values were 0.126 ± 0.036 for NPZ-TE and 0.189 ± 0.013 for NPZ-DM, which indicates the presence of a homogenous vesicle population for both formulations (PDI < 0.2). 1-NPZ-loaded vesicles also showed a slightly increased mean size compared to empty formulations (controls).

Zeta potential consists in the electric potential of the vesicles, and it is dependent on the presence of an edge activator and the concentration of ethanol in TE. NPZ-TE containing both sodium cholate and ethanol displayed a negative zeta potential (-22.3 ± 1.8 mV). On the other hand, NPZ-DM containing DMSO and citrate buffer 50 mM pH 5 showed a slightly positive zeta potential (3.3 ± 0.1 mV). In addition, zeta potential of empty formulations was more negative compared to NPZ-loaded vesicles, which means that 1-NPZ disturbed the surface charge properties of the vesicles.

Vesicles Deformability and Rheology

Topical application and delivery of lipid vesicles is extremely dependent on their deformability index (DI) or elasticity and their rheological profile. The DI of both 1-NPZ-loaded and unloaded TE and DM formulations was determined by pressure driven transport, and are represented in **Table 3** and **Fig.1**. TE exhibited statistically highly significant differences in flux and DI values compared to DM (*p*<0.001). On the contrary, these parameters were quite similar between empty and respective loaded vesicles. Mean sizes of NPZ-DM before and after the experiment diminished very significantly (*p*<0.001) (data not shown).

Table 3. Flux under pressure, deformability index and viscosity of the lipid formulations.

	Flux under pressure (g/s)	**Deformability index (g/s)**	**Viscosity (Pa.s) (at 25 ± 1ºC)**
Empty TE	0.061 ± 0.006	1.123 ± 0.150	0.064
NPZ-TE	0.064 ± 0.009	1.140 ± 0.251	
Empty DM	0.015 ± 0.005	0.298 ± 0.102	0.225
NPZ-DM	0.013 ± 0.002	0.248 ± 0.039	

TE diluted 1:10 and DM diluted 1:20. Values are expressed as mean ± SD (n = 3). Statistical analysis: ***p*<0.001.

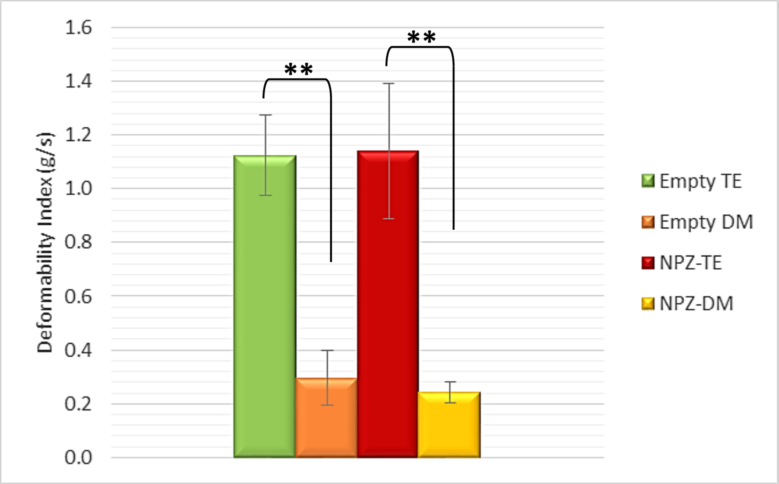


Fig. 1. Deformability index of NPZ-TE and NPZ-DM formulations and respective controls (empty vesicles). TE diluted 1:10 and DM diluted 1:20. Values are expressed as mean ± SD (n = 3). Statistical analysis: $^{**}p<0.001$.

Rheology studies are an important tool to assess the spreadability of topical formulations as well as their physical stability. The viscosity and rheogram of both NPZ-TE and NPZ-DM formulations were obtained by viscometry and are presented in **Table 3** and **Fig. 2**, respectively. NPZ-DM exhibited higher viscosity compared to NPZ-TE. In addition, NPZ-TE and NPZ-DM displayed a non-Newtonian time-independent pseudoplastic behavior, in which the apparent viscosity decreases with the shear rate increase (upward curve), returning it to original values with the shear rate decrease (downward curve). Both curves were almost overlapping, especially for NPZ-DM.

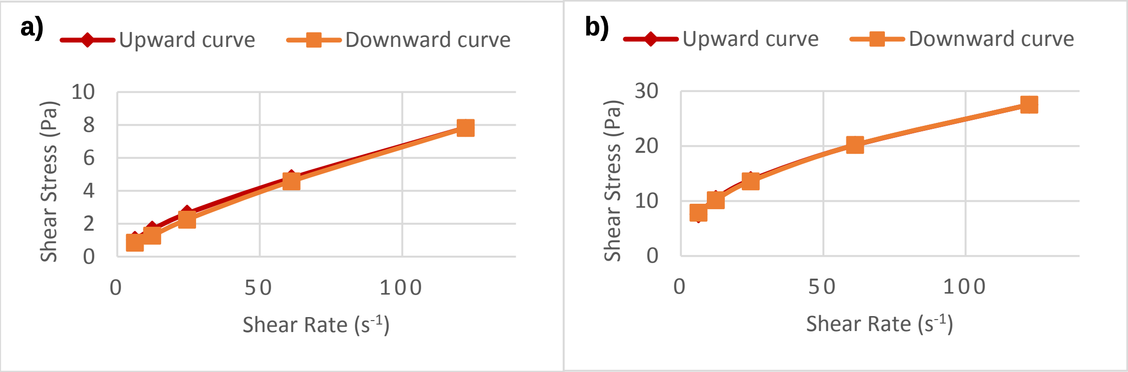


Fig. 2. Rheograms of NPZ-TE (a) and NPZ-DM (b) formulations.

Chemical Characterization of Lipid Vesicles

Phospholipids Content, Drug Entrapment Yield and pH

The main chemical parameters of NPZ-TE and NPZ-DM are presented in **Table 4.** The lipid concentration of these vesicles before and after the ultracentrifugation step was assessed using a method based on the colorimetric determination of inorganic phosphate [29]. NPZ-DM exhibited a minor lipid loss after ultracentrifugation compared to NPZ-TE. A very high drug entrapment yield determined by HPLC was obtained for both vesicles as well. It should be noted that empty vesicles did not interfere with 1-NPZ quantification. Finally, it was possible to load 4.33 ± 0.33 and 2.16 ± 0.58 µg 1-NPZ per mg SPC in TE and DM, respectively. Active loadings of NPZ-TE before and after centrifugation were significantly higher compared to NPZ-DM (*p*<0.05). These results revealed to be quite appropriate for the therapeutic purpose of these formulations.

The pH obtained was consistent with each formulation composition and suitable for topical application (especially pH 5).

Table 4. Chemical characterization of the lipid formulations.

	SPC Yield (%)	**Drug Entrapment Yield (%)**		**Active loading (µg/mg SCP)**			**pH**
				Before centrifugation		*After centrifugation*	
NPZ-TE	74.3 ± 10.7	90.6 ± 6.0	*	3.57 ± 0.67	*	4.33 ± 0.33	8.5
NPZ-DM	82.1 ± 13.4	95.8 ± 1.2		1.95 ± 0.07		2.16 ± 0.58	5.0

Values are expressed as mean ± SD (n = 3). Statistical analysis: *p<0.05.

Chemical Stress Stability Study

The chemical stability of both vesicle formulations was investigated under stress light conditions after 24 h, as previously described [30]. Although the samples were well sealed, NPZ-TE indicated signals of ethanol evaporation, thereby concentrating the samples (results not shown). On the other hand, NPZ-DM presented a good degree of stability only decreasing 7.8 fold ± 5.2.

Topical Delivery Studies

In Vitro Skin Permeation and Penetration Studies

Many formulation properties, including vesicles composition, structure, surface charge, elasticity and drug solubility can affect the skin penetration and permeation profiles of NPZ-TE and NPZ-DM. Thus, *in vitro* topical delivery studies using vertical Franz diffusion cells were conducted under non-occlusive conditions to assess the influence of such elements on 1-NPZ transport across newborn pig skin. After 24 h, the amount of 1-NPZ that penetrated the intact skin (SC and epidermis/dermis) was quantified (**Fig. 3**). NPZ-TE revealed a statistically highly significant increase in penetration into the skin layers in comparison with NPZ-DM (*p*<0.001). Although it was detected 1-NPZ in the receptor phase, the data of these permeation studies were below the quantification limit of HPLC method.

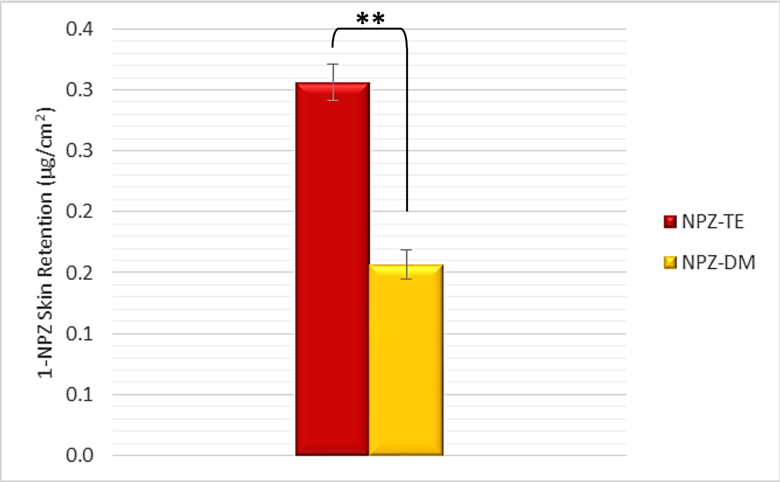


Fig. 3. Skin retention of 1-NPZ for both NPZ-TE and NPZ-DM formulations. Values are expressed as mean ± SD (n = 3). Statistical analysis: ***p*<0.001.

Discussion

In the present study, two different lipid vesicle formulations loaded with 1-NPZ were fully analyzed and compared to identify the best carrier towards the development of a novel topical formulation for the management of skin cancer. Accordingly, two new UDV were formulated attending to their advantages: **Transethosomes** (TE) containing both an edge activator (surfactant) and a skin enhancer (alcohol) [19] and **Dmsosomes** (DM) including DMSO as a skin enhancer and a good solvent to dissolve 1-NPZ. Therefore, both physical and chemical properties of NPZ-TE and NPZ-DM were analyzed besides their topical delivery.

Regarding vesicles mean **size**, NPZ-TE showed a smaller size compared to NPZ-DM (**Table 2**), as expected due to the corresponding increase in the phospholipid content (10 % to 20 % w/v, respectively). In addition, the presence of ethanol in NPZ-TE composition is also an important factor for reducing the mean size of vesicles [14]. Touitou et al. have demonstrated that vesicles size increases with decreasing ethanol concentrations as well as with increasing phospholipid content [14]. Sodium cholate as an edge activator also influenced the size of NPZ-TE [32]. Nevertheless, both vesicles sizes were within the expected range (150 ± 50 nm) and suitable for topical administration.

The **charge** of vesicle formulations is an essential parameter that can influence vesicle properties including stability and skin-vesicle interactions [14]. Soybean phosphatidylcholine (SPC) is a phospholipid frequently used in such formulations due to the presence of oleic and linoleic acids, which are both permeation enhancers [9]. SPC is also a zwitterionic molecule, and therefore will have no effect on the vesicles charge. Thus, any variance in zeta potential of both TE and DM formulations would be due to their different components. Zeta potential of all formulations exhibited negative values except for NPZ-DM (**Table 2**). TE formulations presented higher absolute values, which would contribute for a higher electrostatic repulsion and consequent stabilization of vesicles size. The negative charge was due to the presence of both ethanol and sodium cholate in TE composition. Sodium cholate is a negatively charged and nontoxic surfactant, whereas ethanol has proven to be capable of inducing a transition in the charge of the vesicles from positive to negative [14]. On the other hand, empty DM also displayed negative values of zeta potential, which could be due to the presence of citrate ion from citrate buffer pH 5 in its composition. At pH 5, citrate (pKa = 3.05 - 5.39) has one protonated carboxyl group and two others deprotonated, which confer an overall negative charge [33]. Moreover, the 1-NPZ loading decreased the absolute charge of both formulations due to the presence of a protonated amino group (pKa = 8.87) in its structure [33].

Deformability of lipid vesicles is a fundamental factor for the permeation-enhancing effect across the skin. The deformability index (DI) is calculated from the flux of vesicles through a membrane filter of known size pores (30 nm) driven by an external pressure according to **Eq.1** [30]. As shown in **Table 3** and **Fig. 1**, the flux and DI of NPZ-TE, either loaded or unloaded, were significantly higher than those of NPZ-DM ($p<0.001$). In fact, the mean sizes of NPZ-TE and NPZ-DM decreased approximately 26 % and 37 %, respectively, after filtration under pressure. The reason behind NPZ-TE higher deformability is associated with a lower percentage of SPC and with the synergistic presence of ethanol and sodium cholate. In fact, surfactants are capable of changing the packing characteristics of the lipids in the membrane bilayer, which increases vesicles flexibility [19].

Both NPZ-TE and NPZ-DM formulations showed a non-Newtonian time-independent **pseudoplastic behaviour** (**Fig. 2**) revealing a good spreadability, which makes them quite suitable for topical application. Attending to the viscosity values obtained, the addition of a polymer would not be required especially for NPZ-DM.

NPZ-TE and NPZ-DM were also chemically characterized (**Table 4**). The **phospholipid yield** of both types of vesicles was much higher than 50 %, especially for NPZ-DM. The reason behind this is the higher lipid concentration and mean size of NPZ-DM, which in turn contributed to an easier separation. **Drug entrapment yield** of both formulations was also very high (> 90 %). In the case of NPZ-TE, the drug loading might be increased due to its solubilization by the presence of both co-solvent and surfactant in TE [14,34]. Furthermore, the high ethanol concentration (30 %) in NPZ-TE composition lowers the water content outside the vesicles, increasing the drug entrapment. Regarding NPZ-DM, the high value of this parameter is related with the extremely high solubility of 1-NPZ in DMSO, as observed during the formulation development. On the other hand, 1-NPZ is an amphipathic molecule (*Log* P = 2.53) [1], and therefore, it could be located in both aqueous and/or lipid phase of these vesicles.

Regarding **chemical photostability** after 24 h, NPZ-DM showed a good stability. However, inconclusive results were obtained for NPZ-TE due to ethanol evaporation during this assay.

In vitro **permeation/penetration** studies were performed under non-occlusive conditions in order to facilitate the transport of vesicles across skin following its hydration gradient (particularly for TE) [35]. In addition, newborn pig skin was used as a skin model due to the resemblances with human skin regarding hair follicle density, biochemical features and thickness of SC [35]. As shown in **Fig. 3**, NPZ-TE presented significantly higher penetration into skin layers than NPZ-DM ($p<0.001$). This improved profile could be due to numerous factors, such as: lower SPC content, viscosity and vesicles size, as well as higher DI compared to NPZ-DM. In addition, the synergistic action of permeation enhancers (surfactant and alcohol) of this formulation also contributed for the obtained results. In particular, two different mechanisms of action have been suggested to explain the skin enhancing effect caused by the edge activator [18]. The intracellular mechanism involves the fusion of the vesicles with skin lipids, which leads to changes in the enthalpy of the lipid-related transitions of the SC [18]. Regarding the extracellular pathway, the edge activator would be able to reduce the energy necessary for the deformation of the vesicles, allowing them to squeeze through skin pores [18]. In addition, ethanol has well-known skin enhancing properties besides increasing the flexibility of the vesicles as well [18]. Therefore, the combination of different TE properties and mechanisms contributed to their improved penetration into deeper skin layers, as obtained in previous work [21]. Although NPZ-DM have revealed a lower skin penetration profile as already explained, DMSO contributed as a potent skin enhancer. In fact, DMSO is capable of displacing bound water from keratin, extracting skin lipids, changing keratin conformation and/or interacting with lipid alkyl chains in SC [36]. In general, topical delivery was successfully achieved by both formulations as intended given the location of both melanoma and nonmelanoma skin cancer in epidermis layer.

Conclusions

Two innovative 1-NPZ-loaded UDV were developed and successfully characterized in order to compare their potential as topical delivery nanosystems for the management of skin cancer. Both NPZ-TE and NPZ-DM were tested to assess not only their physicochemical properties, but also their effective capacity to deliver 1-NPZ into skin layers. All tested parameters were suitable either for NPZ-TE or NPZ-DM due to the synergistic combination of surfactant and ethanol or DMSO, respectively. Therefore, both formulations should be good candidates for therapeutic use.

Future work will mainly cover the assessment of long-term stability of both formulations, as well as *in vitro* (3D skin models) and *in vivo* (mouse models) studies concerning the toxicity and therapeutic effect of 1-NPZ on skin cancer.

Acknowledgments

The authors acknowledge Carla Euletério and Joana Marto for technical support in HPLC analysis and topical delivery studies, respectively).

References

1. Menezes AC, Raposo S, Simoes S, Ribeiro H, Oliveira H, Ascenso A (2015) Prevention of Photocarcinogenesis by Agonists of 5-HT1A and Antagonists of 5-HT2A Receptors. Mol Neurobiol. doi:10.1007/s12035-014-9068-z

2. Dianzani C, Zara GP, Maina G, Pettazzoni P, Pizzimenti S, Rossi F, Gigliotti CL, Ciamporcero ES, Daga M, Barrera G (2014) Drug delivery nanoparticles in skin cancers. BioMed research international 2014:895986. doi:10.1155/2014/895986

3. Chinembiri TN, Gerber M, du Plessis L, du Preez J, du Plessis J (2015) Topical Delivery of 5-Fluorouracil from Pheroid Formulations and the In Vitro Efficacy Against Human Melanoma. AAPS PharmSciTech. doi:10.1208/s12249-015-0328-7

4. Haleem DJ, Saify ZS, Siddiqui S, Batool F, Haleem MA (2002) Pre- and postsynaptic responses to 1-(1-naphthylpiperazine) following adaptation to stress in rats. Progress in neuro-psychopharmacology & biological psychiatry 26 (1):149-156

5. Sreevidya CS, Khaskhely NM, Fukunaga A, Khaskina P, Ullrich SE (2008) Inhibition of photocarcinogenesis by platelet-activating factor or serotonin receptor antagonists. Cancer research 68 (10):3978-3984. doi:10.1158/0008-5472.CAN-07-6132

6. Sreevidya CS, Fukunaga A, Khaskhely NM, Masaki T, Ono R, Nishigori C, Ullrich SE (2010) Agents that reverse UV-Induced immune suppression and photocarcinogenesis affect DNA repair. The Journal of investigative dermatology 130 (5):1428-1437. doi:10.1038/jid.2009.329

7. Severino P, Fangueiro JF, Ferreira SV, Basso R, Chaud MV, Santana MH, Rosmaninho A, Souto EB (2013) Nanoemulsions and nanoparticles for non-melanoma skin cancer: effects of lipid materials. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico 15 (6):417-424. doi:10.1007/s12094-012-0982-0

8. Cosco D, Paolino D, Maiuolo J, Marzio LD, Carafa M, Ventura CA, Fresta M (2015) Ultradeformable liposomes as multidrug carrier of resveratrol and 5-fluorouracil for their topical delivery. International journal of pharmaceutics 489 (1-2):1-10. doi:10.1016/j.ijpharm.2015.04.056

9. Ascenso A, Cruz M, Euleterio C, Carvalho FA, Santos NC, Marques HC, Simoes S (2013) Novel tretinoin formulations: a drug-in-cyclodextrin-in-liposome approach. Journal of liposome research 23 (3):211-219. doi:10.3109/08982104.2013.788026

10. Cevc G, Blume G (1992) Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochimica et biophysica acta 1104 (1):226-232

11. Cevc G (2004) Lipid vesicles and other colloids as drug carriers on the skin. Advanced drug delivery reviews 56 (5):675-711. doi:10.1016/j.addr.2003.10.028

12. Pierre MB, Dos Santos Miranda Costa I (2011) Liposomal systems as drug delivery vehicles for dermal and transdermal applications. Archives of dermatological research 303 (9):607-621. doi:10.1007/s00403-011-1166-4

13. Zhang JP, Wei YH, Zhou Y, Li YQ, Wu XA (2012) Ethosomes, binary ethosomes and transfersomes of terbinafine hydrochloride: a comparative study. Archives of pharmacal research 35 (1):109-117. doi:10.1007/s12272-012-0112-0

14. Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M (2000) Ethosomes - novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. Journal of controlled release : official journal of the Controlled Release Society 65 (3):403-418

15. Ghanbarzadeh S, Arami S (2013) Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. BioMed research international 2013:616810. doi:10.1155/2013/616810

16. Zhang YT, Shen LN, Wu ZH, Zhao JH, Feng NP (2014) Comparison of ethosomes and liposomes for skin delivery of psoralen for psoriasis therapy. International journal of pharmaceutics 471 (1-2):449-452. doi:10.1016/j.ijpharm.2014.06.001

17. Shen LN, Zhang YT, Wang Q, Xu L, Feng NP (2014) Enhanced in vitro and in vivo skin deposition of apigenin delivered using ethosomes. International journal of pharmaceutics 460 (1-2):280-288. doi:10.1016/j.ijpharm.2013.11.017

18. Bragagni M, Mennini N, Maestrelli F, Cirri M, Mura P (2012) Comparative study of liposomes, transfersomes and ethosomes as carriers for improving topical delivery of celecoxib. Drug delivery 19 (7):354-361. doi:10.3109/10717544.2012.724472

19. Song CK, Balakrishnan P, Shim CK, Chung SJ, Chong S, Kim DD (2012) A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: characterization and in vitro/in vivo evaluation. Colloids and surfaces B, Biointerfaces 92:299-304. doi:10.1016/j.colsurfb.2011.12.004

20. Guo F, Wang J, Ma M, Tan F, Li N (2015) Skin targeted lipid vesicles as novel nano-carrier of ketoconazole: characterization, in vitro and in vivo evaluation. Journal of materials science Materials in medicine 26 (4):175. doi:10.1007/s10856-015-5487-2

21. Ascenso A, Raposo S, Batista C, Cardoso P, Mendes T, Praça FG, Bentley MV, Simões S (2015 (accepted)) Development, Characterization and Skin Delivery Studies of Related Ultradeformable Vesicles: Transfersomes, Ethosomes and Transethosomes. International journal of nanomedicine

22. Williams AC, Barry BW (2004) Penetration enhancers. Advanced drug delivery reviews 56 (5):603-618. doi:10.1016/j.addr.2003.10.025

23. Capriotti K, Capriotti JA (2012) Dimethyl sulfoxide: history, chemistry, and clinical utility in dermatology. The Journal of clinical and aesthetic dermatology 5 (9):24-26

24. Conde-Estevez D, Mateu-de Antonio J (2014) Treatment of anthracycline extravasations using dexrazoxane. Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico 16 (1):11-17. doi:10.1007/s12094-013-1100-7

25. Perez Fidalgo JA, Garcia Fabregat L, Cervantes A, Margulies A, Vidall C, Roila F, Group EGW (2012) Management of chemotherapy extravasation: ESMO-EONS Clinical Practice Guidelines. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 23 Suppl 7:vii167-173. doi:10.1093/annonc/mds294

26. Casiraghi A, Ardovino P, Minghetti P, Botta C, Gattini A, Montanari L (2007) Semisolid formulations containing dimethyl sulfoxide and alpha-tocopherol for the treatment of extravasation of antiblastic agents. Archives of dermatological research 299 (4):201-207. doi:10.1007/s00403-007-0746-9

27. Cyran CC, Sennino B, Chaopathomkul B, Fu Y, Rogut V, Shames DM, Wendland MF, McDonald DM, Brasch RC (2008) Magnetic resonance imaging assays for dimethyl sulfoxide effect on cancer vasculature. Investigative radiology 43 (5):298-305. doi:10.1097/RLI.0b013e318164b71d

28. van den Bergh BA, Wertz PW, Junginger HE, Bouwstra JA (2001) Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. International journal of pharmaceutics 217 (1-2):13-24

29. Rouser G, Fkeischer S, Yamamoto A (1970) Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5 (5):494-496

30. Ascenso A, Pinho S, Eleuterio C, Praca FG, Bentley MV, Oliveira H, Santos C, Silva O, Simoes S (2013) Lycopene from tomatoes: vesicular nanocarrier formulations for dermal delivery. Journal of agricultural and food chemistry 61 (30):7284-7293. doi:10.1021/jf401368w

31. OECD guideline 428. Guidance document for the conduct of skin absorption studies (2004). Paris

32. Lee EH, Kim A, Oh YK, Kim CK (2005) Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials 26 (2):205-210. doi:10.1016/j.biomaterials.2004.02.020

33. http://en.chembase.cn. 2014

34. Dayan N, Touitou E (2000) Carriers for skin delivery of trihexyphenidyl HCl: ethosomes vs. liposomes. Biomaterials 21 (18):1879-1885

35. Ascenso A, Salgado A, Euleterio C, Praca FG, Bentley MV, Marques HC, Oliveira H, Santos C, Simoes S (2014) In vitro and in vivo topical delivery studies of tretinoin-loaded ultradeformable vesicles. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV 88 (1):48-55. doi:10.1016/j.ejpb.2014.05.002

36. Lane ME (2013) Skin penetration enhancers. International journal of pharmaceutics 447 (1-2):12-21. doi:10.1016/j.ijpharm.2013.02.040

Final Remarks

In the present study, we sought to investigate the potential of 1-NPZ as a chemotherapeutic agent for the treatment of melanoma skin cancer. In addition, two novel 1-NPZ-loaded UDV formulations were developed and subsequently characterized in order to evaluate their ability as topical delivery nanocarriers for the effective treatment of this malignancy.

As expected, 1-NPZ proved to be able to reduce the viability of MNT-1 cells as well as inhibit their growth. Possible causes for such might be the S-phase arrest of cell cycle and induction of apoptosis observed in these cells following treatment with 1-NPZ. Furthermore, we also demonstrated that the mechanism leading to apoptosis in MNT-1 cells by 1-NPZ might not be associated with *cis*-UCA signaling pathway. Therefore, this study identified for the first time 1-NPZ as a promising chemotherapeutic agent for the management of melanoma skin cancer.

Regarding topical formulations, both NPZ-TE and NPZ-DM appeared to have suitable physicochemical properties (including a very high entrapment yield) and a successful skin penetration profile. Hence, both formulations should be appropriate for therapeutic use.

Notwithstanding, further work will be needed to fully understand the mechanism of action of 1-NPZ both under *in vitro* and *in vivo* conditions. The therapeutic index of 1-NPZ should also be evaluated with a specific mouse model for skin cancer. In addition, this research work could be further extended to both prevention and treatment approaches of melanoma and nonmelanoma skin cancers.