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Bruno Pacheco Fernandes

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Professor Doutor Artur Manuel Cavaco Paulo

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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MODIFICAÇÃO DO CABELO HUMANO: MODELADORES DA MELANOGÉNESE COMO AGENTES DE MUDANÇA DA COR

RESUMO

A cor do cabelo é um elemento marcante do nosso visual e tem um impacto social irrefutável. Em virtude da importância dada ao aspeto físico, a coloração do cabelo é hoje uma prática bastante comum. Todavia, os efeitos adversos do recurso frequente a tais práticas exigem que novos métodos sejam desenvolvidos para alteração da cor do cabelo. Neste sentido, esta tese teve como objetivo principal demonstrar que, interferindo com o processo fisiológico da pigmentação do cabelo, é possível alterar a sua cor natural a partir do folículo, de forma segura.

O tom natural do cabelo é determinado pelo seu conteúdo em melaninas, pigmentos sintetizados por melanócitos do folículo capilar através de um processo conhecido como melanogénese. O presente trabalho foi concebido com base na hipótese de escurecer ou clarear o cabelo via aplicação tópica de modeladores da melanogénese no couro cabeludo. Primeiro, desenvolveu-se um método com base na fluorescência dos produtos de oxidação da melanina, mais expedito que os métodos de quantificação deste pigmento já estabelecidos e com igual ou melhor precisão, exatidão, sensibilidade e especificidade que o método mais usado na literatura. Este novo método foi essencial para a procura de modeladores da produção *in vitro* de melaninas numa linha celular humana pigmentada. De entre 1200 fármacos com perfil toxicológico bem definido e longo historial de uso seguro em humanos, 23 fármacos apresentaram efeitos significativos. Os quatro com propriedades pro- ou anti-melanogénicas mais acentuadas foram otimizados tendo em vista a sua toxicidade e relação dose-resposta; a atividade da tirosinase foi também avaliada para esclarecer os respetivos mecanismos de ação. Atendendo aos resultados obtidos, um indutor e dois inibidores da melanogénese foram selecionados para testes *in vivo* em voluntários humanos. Como previsto, o cabelo dos voluntários cresceu mais escuro ou mais claro devido à aplicação tópica do Composto A+ ou Composto B-, respetivamente. O Composto C- diminuiu o teor de melaninas, mas não alterou a cor visualmente percetível dos cabelos. Nenhum efeito adverso foi reportado. A eficiência de um sistema de entrega folicular particulado face a uma solução não particulada foi ainda avaliada *ex vivo* para o fármaco ciclosporina A, como modelo de tratamento capilar, neste caso, para alopecia. Os resultados confirmaram que nanopartículas de ácido polilático são um excelente veículo de entrega folicular, em particular, para fármacos com baixa permeabilidade cutânea.

De acordo com o objetivo inicial, esta tese institui o uso tópico de moduladores da melanogénese como uma inovadora abordagem segura e eficaz na alteração da cor natural do cabelo, sendo o ponto de partida para um novo tipo de cosméticos de coloração capilar.

PALAVRAS-CHAVE: Cabelo; Cor; Cosméticos; Fármacos; Melanogénese

MODIFICATION OF THE HUMAN HAIR: MODULATORS OF MELANOGENESIS AS AGENTS OF COLOUR CHANGING

ABSTRACT

Hair colour is an undeniable element of body image with great social impact. Given the importance attributed to hair look, the dyeing of hair fibres is today a common practice. Nonetheless, the adverse effects of long-term usage of such fibre colouring procedures demand the development of other methods for colour change. Therefore, the major purpose of this Ph.D. thesis was the development of a new method for hair colour modification by safely interfering with the physiological process of hair pigmentation at the hair follicle level.

The natural colour shade of hair is largely defined by its content in melanins, a class of pigments synthesized by follicular melanocytes through a biochemical process known as melanogenesis. The current work was built upon the hypothesis of hair darkening/lightening through the topical treatment of the scalp with modulators of melanin synthesis. First, a new methodology was developed for melanin quantification based on the fluorescence of its oxidation products. This method was more expeditious than other established assays, performing equally or better than the most used method in terms of sensitivity, precision, accuracy, and specificity. This new method was essential for the *in vitro* search of melanogenesis modulators. A collection of 1200 generic drugs with well-defined toxicological profile and a long history of safe usage in humans was screened regarding the ability to alter the melanin contents of a melanin-producing human cell line. Among the 23 drugs that showed highly significant effects, the top four inducers and inhibitors were selected for further validation and optimization regarding their cytotoxicities and dose-response activities. Additionally, the activity of tyrosinase was also assessed to gain some insight into the mechanisms of action. Taking into consideration the *in vitro* data generated, three drugs were chosen for a clinical trial with intervention of cosmetics. As predicted, the hair of human volunteers grew darker or lighter due to the topical treatment of their scalps with Compound A+ or Compound B-, respectively. Compound C- also decreased the melanin content of hairs but without visually perceptible colour changes. No adverse effects were reported. The efficiency of a particulate follicular delivery system was also evaluated *ex vivo* by comparing it to a non-particulate vehicle. Cyclosporine A was used as a model drug of hair treatment, in this case, for alopecia. The results confirmed that polylactic acid nanoparticles are an excellent vehicle for follicular delivery of drugs with low skin permeability.

According to its major purpose, this thesis provides pioneer evidence that melanogenesis modulators, applied topically, can safely and effectively change the colour of human hair from the inside out, opening a new window of opportunities for the development of ground-breaking hair colouring cosmetics.

KEYWORDS: Colour; Cosmetics; Drugs; Hair; Melanogenesis.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	**AC**	Adenylyl cyclase
	ACh	Acetylcholine
	AChE	Acetylcholinesterase
	ACTH	Adrenocorticotropin
	ADAM17	ADAM metallopeptidase domain 17
	ADP	Adenosine diphosphate
	Amel-Mc	Amelanotic melanocyte
	AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
	ANOVA	Analysis of variance
	AP	Adaptor-related protein complex
	approx.	Approximately
	AR	Adrenergic receptor
	Arg	Arginine
	ASIP	Agouti signalling protein
	Asn	Asparagine
	ATC	Anatomical therapeutic chemical
	ATP	Adenosine triphosphate
	ATP7A	ATPase copper transporting alfa
B	**BCL2**	B-cell lymphoma 2
	BLOC	Biogenesis of lysosomal organelles complex
	BMP	Bone morphogenetic protein
	BMPR	Bone morphogenetic protein receptor
	BSA	Bovine serum albumin
C	**cAMP**	Cyclic adenosine monophosphate
	CB1	Cannabinoid receptor 1
	CBP	CREB-binding protein
	CD	Cysteinyldopa
	cGMP	Cyclic guanosine monophosphate
	cm^2	Square centimetre
	CO_2	Carbon dioxide
	COX	Cyclooxygenase

C	**CRE**	cAMP responsive element
	CREB	cAMP responsive-element-binding protein
	CRF	Corticotropin-releasing factor
	CRFR	Corticotropin-releasing factor receptor
	CRTC	CREB-regulated transcription coactivator
	CsA	Cyclosporin A
	CTNS	Cystinosin
	CV	Coefficient of variation
	Cys	Cysteine
	CySS	Cystine
D	**DAG**	Diacylglycerol
	DCT	Dopachrome tautomerase
	DHI	5,6-dihydroxyindole
	DHICA	5,6-dihydroxyindole-2-carboxylic acid
	DHPR	Dihydropteridine reductase
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	DP	Dermal papilla
	DQ	Dopaquinone
E	**e.g.**	For example
	EDNRB	Endothelin receptor type B
	EMA	European medicines agency
	Epi	Epinephrine
	ER	Endoplasmic reticulum
	ERK	Extracellular signal-regulated kinase
	ESCRT	Endosomal sorting complexes required for transport
	ET	Endothelin
	EtOH	Ethanol
F	**FDA**	Food and drug administration
	FSCN1	Fascin actin-bundling protein 1
	Fsk	Forskolin
	FZD	Frizzled

G	**g**	Gram
	GFRP	GTP-cyclohydrolase I feedback regulatory protein
	Glu	Glutamate
	GPER	G protein-coupled oestrogen receptor
	GPR143	G protein-coupled receptor 143
	GSH	Glutathione, reduced form
	GSK3β	Glycogen synthase kinase 3β
	GTP	Guanosine triphosphate
	GTP-CH-I	GTP-cyclohydrolase I
H	**h**	Hour
	H_2O_2	Hydrogen peroxide
	H_2R	Histamine H_2 receptor
	His	Histidine
	Hist	Histamine
	hpf	Hours post fertilization
	HPLC	High performance liquid chromatography
	HSP	Heat shock protein
	HTS	High throughput screening
I	**IBMX**	3-isobutyl-1-methyl-xanthine
	IC50	Half maximal inhibitory concentration
	IFD	Infundibulum
	ILV	Intralumenal vesicle
	IP_3	Inositol–triphosphate
	IPM	Isopropyl myristate
	ISO	International organization for standardization
J	**JNK**	c-Jun N-terminal kinase
K	**KA**	Kojic acid
	kDa	Kilodalton
	Ker	Keratinocyte
	KIT	KIT proto-oncogene receptor tyrosine kinase
	KITLG	KIT ligand

L	**L**	Litre
	LAMP	Lysosomal-associated membrane protein
	L-DOPA	L-3,4-dihydroxyphenylalanine
	LEF	Lymphoid enhancer factor
	lncRNA	Long non-coding RNA
	L-Tyr	L-tyrosine
M	**M**	Molar
	MAPK	Mitogen-activated protein kinase
	MC(1/4)R	Melanocortin (1/4) receptor
	Mel	Melanocyte
	Mel-Mc	Melanogenic melanocyte
	MelSC	Melanocyte stem cell
	mg	Milligram
	mGluR6	Metabotropic glutamate receptor 6
	min	Minute
	miRNA	Micro RNA
	MITF	Microphthalmia-associated transcription factor
	mL	Millilitre
	MLANA	Protein melan-a
	MLPH	Melanophilin
	mm	Millimetre
	mM	Millimolar
	M(1-5)R	Muscarinic (1-5) receptor
	mRNA	Messenger RNA
	MTN	Melatonin
	MTNR	Melatonin receptor
	MTT	Thiazolyl blue tetrazolium bromide
	Mw	Molecular weight
	MYO	Myosin
N	**NaOH**	Sodium hydroxide
	ncRNA	Non-coding RNA
	ND	Not detectable

N	**NEpi**	Norepinephrine
	ng	Nanogram
	NHE	Sodium hydrogen exchanger
	NHM	Normal human melanocyte
	NK1R	Neurokinin 1 receptor
	nm	Nanometre
	nM	Nanomolar
	NMDA	N-methyl-D-aspartate
	NO	Nitric oxide
	NOS	Nitric oxide synthase
O	**OCA2**	Oculocutaneous albinism 2 protein
	ORS	Outer root sheath
P	**PA**	Palmitic acid
	PAH	Phenylalanine hydroxylase
	PAR2	Protease-activated receptor 2
	PAX3	Paired box 3
	PBS	Phosphate buffered saline solution
	PC	Proprotein convertase
	PDE	Phosphodiesterase
	PDI	Polydispersity index
	PGC	Peroxisome proliferator-activated receptor coactivator
	Phe	Phenylalanine
	PI3K	Phosphatidylinositol 3-kinase
	PKA	Protein kinase A
	PKC	Protein kinase C
	PKG	Protein kinase G
	PLA	Poly (D,L-lactide)
	PLCγ	Phospholipase Cγ
	PMEL	Premelanosome protein
	POMC	Pro-opiomelanocortin
	PPAR	Peroxisome proliferator-activated receptor
Q	**q-BH_2**	Quinonoid dihydropterin

R	**RNA**	Ribonucleic acid
	RNEC	Registo nacional de ensaios clínicos
	ROS	Reactive oxygen species
S	**SCF**	Stem cell factor
	SD	Standard deviation
	Ser	Serine
	SIK2	Salt-inducible kinase 2
	SLC7A5	Solute carrier family 7, member 5
	SLC7A11	Solute carrier family 7, member 11
	SLC24A5	Solute carrier family 24, member 5
	SLC45A2	Solute carrier family 45, member 2
	SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
	SOX	SRY (sex determining region Y)-box
	SP	Substance P
	SSRI	Selective serotonin reuptake inhibitor
T	**TCF**	T-cell factor
	TGF	Transforming growth factor
	TGN	trans-Golgi network
	TPC2	Two pore segment channel 2
	TRH	Thyrotropin-releasing hormone
	TRHR	Thyrotropin-releasing hormone receptor
	Trp	Tryptophan
	TRPM1	Transient receptor potential cation channel, subfamily M, member 1
	TRPML3	Transient receptor potential cation channel, mucolipin subfamily, member 3
	TYR	Tyrosinase
	TYRP1	Tyrosinase related protein 1
	TYRP2	Tyrosinase related protein 2
U	**UV**	Ultraviolet
V	**v/v**	Volume/volume
	VAMP	Vesicle-associated membrane protein
	V-ATPase	Vacuolar ATPase

W	**w/w**	Weight/weight
	w/v	Weight/volume

4a-OH-BH_4	4a-hydroxy-tetrahydrobiopterin
4a-OH-BH_4 DH	4a-hydroxy-tetrahydrobiopterin dehydratase
5-HT	Serotonin
5-HTR	Serotonin receptor
6BH_4	(6R)-L-erythro 5,6,7,8 tetrahydrobiopterin
7-BH_4	(7R)-L-erythro-5,6,7,8-tetrahydrobiopterin
°C	Degree Celsius
α-MSH	Alpha melanocyte-stimulating hormone
β-END	Beta-endorphin
β-MSH	Beta melanocyte-stimulating hormone
σ	Standard deviation
ζ-potential	Zeta-potential
λ	Wavelength
μ	Mean
μg	Microgram
μL	Microlitre
μm	Micrometre
μM	Micromolar
Ψm	Membrane potential

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PROLOGUE

FRAMEWORK OF THE THESIS

SCOPE AND GOALS

Hair colour is one of the most distinctive traits of our physical appearance, greatly affecting the way we are perceived by others and ourselves. Today, more than ever, our looks are a predicate for social success and career opportunities, with millions of individuals routinely changing the colour of hair to enhance their own idea of self-beauty and/or to follow fashion trends. In this context, the hair colouring market has thrived by supplying a variety of hair fibre dyeing systems that comply with distinct aesthetic requirements of consumers. On the downside, most of those systems are known to cause extensive damage to the hair fibre structure and their long-term use has been associated with several adverse health effects like acute to mild dermatitis and hair loss. Consequently, the development of strategies for hair colour modification more focused on healthy hair and consumers wellbeing and safety is an important issue to be addressed.

While most of the upfront research on safer solutions for colour modification is devoted to act on the external portion of hair fibres, the innovative factor of this Ph.D. thesis consists in demonstrating the cosmetic potential of transiently interfering with the biological process of pigmentation, that occurs at hair follicle, to change the natural colour of hair from the inside out. To do so, two major goals were defined:

1. Discover new modulators of the *in vitro* synthesis of melanins, the natural pigments responsible for hair colour, among pharmaceutical drugs already approved for human usage;

2. Provide evidence that topical application of those drugs to the human scalp can safely and effectively modulate the *in vivo* production of melanin by follicular melanocytes, changing the colour of hair fibres as they are produced at the bulb.

The journey for achieving these goals is reported over the next chapters, as outlined.

THESIS OUTLINE

This thesis is divided into five chapters, with chapters II to IV detailing the experimental work. The content of each chapter is summarized below.

Chapter I. Paving the way for innovative hair colour-changing cosmetics: a comprehensive review of human hair pigmentation

The introductory chapter of this thesis provides a comprehensive overview of the natural process of human hair pigmentation. Additionally, current cosmetic procedures employed in hair colour modification

are briefly described. At last, case reports of hair darkening, lightening or repigmentation as a side effect of the therapeutic usage of many drugs are mentioned, substantiating the feasibility to tune hair colour by interfering with the follicular pigmentary unit. This review gives an overall perspective from which it becomes clear that being able to influence melanogenesis (the synthesis of melanins) is crucial for changing the hair colour phenotype. Outstandingly, the biochemical pathway of melanin synthesis presents countless molecular targets for doing so.

Chapter II. Fluorescent quantification of melanin

The basis of many studies dealing with the discovery of melanogenesis modulators involves the *in vitro* measurement of melanins in cultured cells. Although absorption spectroscopy is the most widely used method, it does not always provide the sensitivity and specificity required to evaluate the significance of such modulators. As a result, in this chapter, the fluorometric method is revisited to establish a protocol for easy and accurate quantification of melanins. Besides cell samples, the usefulness of this protocol is assessed in more complex pigmented matrices like zebrafish embryos and human hair.

Chapter III. Discovery of new melanogenesis modulators for hair colour modification: an *in vitro* to *in vivo* study

The core chapter of this Ph.D. thesis starts by reporting the quest for new modulators of melanogenesis in drugs already approved for human usage. Given that animal testing for cosmetic purposes is prohibited in the E.U. since 2013, looking for new cosmetic ingredients among compounds with known toxicological profile is, today, a reliable approach to ensure that they pose no to low risk for humans. Thus, the data gathered in the screening of Prestwich Chemical Library® regarding the ability to change the *in vitro* production of melanin is presented. Then, the results of validation and optimization assays performed with the top hit inducers and inhibitors are discussed. Lastly, to gain some insight regarding their mechanism of action, the effects of the top hit modulators on the intracellular activity of tyrosinase (rate-limiting enzyme of the biochemical pathway of melanin synthesis) are analysed. In the second part of this chapter, the feasibility of using some selected drugs as topically applied ingredients for the darkening or lightening of hair is debated, considering the findings of a pilot study performed in human volunteers.

Chapter IV. Cyclosporin A-loaded Poly (D, L-lactide) nanoparticles: a promising tool for treating alopecia

Changing the natural colour of hair from the inside out is a biphasic challenge. After finding the best modulators of melanin production, we still have to efficiently deliver them to their cellular targets. Follicular

drug delivery is a hot topic in dermatology and hair cosmetic sciences. Cyclosporin A (CsA), an immunosuppressant drug, has hair growth-inducing properties but its poor cutaneous absorption undermines its topical use in the treatment of alopecia. This chapter addresses the ability of Poly (D,L-lactide) (PLA) nanoparticles to improve the dermal permeation of CsA. Conceptually, this work is aligned with the main scope of the present thesis because it reinforces the suitability of nanoparticles-based systems for the efficient and selective delivery of drugs into hair follicles upon topical application. In this context, the PLA nanoparticles here reported will certainly be of interest in future studies that aim to improve the hair colour-changing effects of the modulators of melanogenesis found in the previous chapter.

Chapter V. Pharmacological modulation of melanogenesis: where do we stand now and which drugs to repurpose next?

Drugs with melanogenic effects have long been studied in view of their implications for skin pigmentation-related disorders. Thus, as a way of concluding, the last chapter places together the findings of the current thesis work with existing literature to generate an up-to-date overview on pharmacological modulators of pigmentation. In doing so, not only the important contribution of this thesis to the field of innovative hair colouring cosmetics are highlighted, but also the profound connotations between the data generated and the development of new therapeutic approaches to deal with diseases related with abnormal production of melanin in the skin are pointed out.

CHAPTER I

PAVING THE WAY FOR INNOVATIVE HAIR COLOUR-CHANGING COSMETICS: A COMPREHENSIVE REVIEW OF HUMAN HAIR PIGMENTATION

Chapter I*

Paving the way for innovative hair colour-changing cosmetics: a comprehensive review of human hair pigmentation

ABSTRACT

Hair colour is one of the most distinctive human phenotypic traits, contributing significantly to our visual perception of other individuals. The natural colouration of hairs takes place in the bulb of hair follicles and it is strictly couple to the hair growth cycle. Three critical steps must proceed in perfectly synchrony for an efficient pigmentation of hair shafts: 1) melanosomes biogenesis in neural crest-derived melanocytes, 2) biochemical synthesis of melanins (melanogenesis) inside melanosomes, and 3) the transfer of melanin granules to surrounding pre-cortical keratinocytes for incorporation into the forming fibres. All these steps are under complex genetic control, with the array of natural hair shades (ranging from black, brown, and blond to red) being ascribed to polymorphisms in several pigmentary genes. A myriad of factors acting via autocrine, paracrine, and endocrine mechanisms also contributes greatly for the pigmentation of hair and its diversity. By scrutinizing hair pigmentation, this review pinpoint key targetable processes for the development of innovative cosmetics that can safely change the hair colour from the inside out.

*** This chapter is based on the following scientific paper:**

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I.1. GENERAL OVERVIEW

Hair colour is one of the most distinctive human phenotypes, with various selective pressures having contributed to the dissemination of an array of natural shades ranging from black, brown, and blond to red. Additionally, grey/white hair is often seen, harbingering the loss of youth. As much of the perceived variation between human sub-groups can be ascribed to the different types of hair pigmentation, the colour of hair has an enormous social and cosmetic impact [1–5].

Hair and skin pigmentation are a manifestation of the presence of pigments called melanins. Although melanins of the epidermis had important evolutionary implications (acting as filter for ultraviolet (UV) light, they protect the skin against UV-induced damage), the reasons behind the evolutionary selective pressures for the development of pigmented scalp hair are less clear. Due to the dominant position of fish in the diet of early humans, pigmented scalp hair might have been important to prevent the build-up of toxic metals from fish species. Since toxins/metals have the ability to selective bind to melanins and hair fibres present a fast growth and turnover, pigmented hair shafts allow the body to rapidly get rid of harmful substances, limiting their access to the living tissue of the highly vascularized scalp. Hair analyses corroborate this theory as significant amounts of various heavy metal ions bound to melanin have been reported. Moreover, as our nakedness draws much attention to our facial and scalp hair, the development of pigmented scalp hairs could also have been favoured as a non-verbal mean of social communication and mate selection [1,3,5,6].

The colouration of hair shafts results from precise and sequential interactions between different cell populations in the hair follicle, the mini-organ responsible for the hair shaft production. First, melanins are synthesized by melanocytes, inside cytoplasmic membrane-bound organelles called melanosomes. Melanins are the product of a complex biochemical pathway called melanogenesis and they can undertake two chemically distinct forms: the black to brown eumelanic form and the reddish-brown to yellow pheomelanic form. Melanins are then transferred to the surrounding keratinocytes that will proliferate and differentiate originating hair shafts. During the differentiation process, the growing hairs become pigmented. The diversity in hair pigmentation relies mainly on the quantity and ratio of the two types of melanin incorporated into the hair shafts, with other physical aspects of hair fibres intervening only as minor or segmental colour modifiers [1–8].

The genetic basis of hair colour variety has been the subject of many studies and led to the identification of several genes involved in the normal variation of human hair colour. The protein products depending on these *loci* and their polymorphisms exhibit different patterns of transcription, translation, and functional

activity during the pigmentation of hairs. Melanogenesis *per se* presents a wide range of potential targets with different functions: enzymes, structural proteins, transcription regulators, transporters, receptors, and their ligands. Besides, many intrinsic hair follicle signals, as the products of keratinocytes, have also been uncovered as intervenient in many aspects of hair pigmentation and its diversity [3,8].

I.2. DEVELOPMENT OF THE HAIR FOLLICLE PIGMENTARY UNIT

Melanocytes that constitute the follicular and epidermal pigmentary units derive from the pluripotent cells of the neural crest, a transient component of the ectoderm located between the neural tube and the epidermis. Besides melanocytes, the neural crest gives rise to several other types of cells including neurons and glia cells of the peripheral nervous system as well as bone and cartilage cells of the head skeleton. The commitment of neural crest cells to the melanocyte lineage and the entire journey of melanoblasts (precursors of melanocytes) during embryogenesis to produce the pigmentary unit is regulated by several factors [6,8–11].

Microphthalmia-associated transcription factor (MITF) appears to be the master regulator of melanocyte identity at the neural crest. MITF regulates the expression of genes that confer melanocyte characteristics to the neural crest cells such as tyrosinase (*TYR*), tyrosinase related protein 1 (*TYRP1*) and dopachrome tautomerase (*DCT*). The transcription of *MITF* is synergistically activated by paired box 3 (PAX3) and SRY-box 10 (SOX10) transcription factors. In turn, forkhead box D3 (FOXD3) and SOX2 transcription factors repress *MITF* expression and thus, their downregulation is a critical initial step in the specification of melanoblasts; the mechanism by which their expression is suppressed is still unclear, but histone deacetylase 1 (HDAC1) seems to be involved. The upregulation of WNT proteins (namely, WNT1 and WNT3A) and downregulation of bone morphogenetic protein 4 (BMP4) also appear to be involved in the commitment of neural crest cells to the melanocyte lineage [12,13,22,23,14–21].

Once melanoblasts begin to express specific markers, they migrate dorsoventrally through the developing embryo and start to proliferate. The migration of melanoblasts is directed by chemotactic signals and patterns of cell surface molecules and receptors expressed by the mobilized melanoblasts or present within the extracellular matrix through which they move. The migration and proliferation of melanoblasts require endothelin receptor type B (EDNRB) and its ligand endothelin 3 (ET-3). The KIT proto-oncogene receptor tyrosine kinase (KIT) and its ligand stem cell factor (SCF, also known as KITLG) are also essential for migration and proliferation as well as for survival of melanoblasts; other factors as B-cell lymphoma 2

(BCL2) antiapoptotic protein also affect proliferation and survival of melanoblasts. Other key players in the migration of melanoblasts and cytoskeletal organization are Rac family small GTPase 1 (RAC1), fascin actin-bundling protein 1 (FSCN1), Lamellipodin and the Scar/WAVE complex. The migration of melanoblasts from the neural crest, across the dermis and into the epidermis also involves changes in the expression of several calcium-dependent adherens junction proteins (E-cadherin, N-cadherin, P-cadherin), regulated by transcription factors as zinc finger protein SNAI1 (snail family transcription repressor 1). By the seventh week of gestational age, melanoblasts are already in the human epidermis, remaining there until hair morphogenesis begins, two weeks later [4,6,29,11,13,16,24–28].

When hair morphogenesis begins some melanoblasts leave the epidermis and distribute in the developing hair follicles – Figure I.1. The C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal-cell derived factor 1 or SDF1) appears to act as a chemoattractant for melanoblasts through its receptor C-X-C motif chemokine receptor 4 (CXCR4). SCF has been shown to act as a chemokinetic factor. Melanoblasts expressing KIT migrate into the SCF-supplying hair follicle epithelium and, once "SCF-positive", differentiated melanocytes (melanogenically active) target the hair bulb, the only site of melanin production for the pigmentation of hair shafts. In the hair bulb, highly melanogenic melanocytes locate above and around the dermal papilla, establishing a functional unit with neighbouring pre-cortical keratinocytes that receive melanin granules and incorporate them into the forming hair shafts. A minor sub-population of poorly differentiated melanocytes (lacking significant pigmentation) is also found in the most proximal and peripheral regions of the hair bulb; however, due to their spatial distribution, they do not transfer melanin to the pre-cortical keratinocytes. Melanogenically active melanocytes are also present at the basal layers of the infundibulum and sebaceous glands. Although the function of these melanocytes is not clear, given their location at the interface with external environment and the known antimicrobial potential of melanin and its intermediates, they may have an important role in innate immunity and defence. Melanoblasts that do not express KIT invade the outer root sheath and bulge regions. These undifferentiated, amelanotic melanoblasts function as a pool of melanocyte stem cells (MelSC) that are recruited for regeneration of the cycling portion of the pigmentary unit, following the events of the catagen phase. This phase of the normal hair growth cycle involves an almost complete loss of differentiated, pigment-producing bulbar melanocytes [1,4,6,8,13,30–34].

Although they derive from the same precursors, the follicular and epidermal pigmentary units are quite different. In the hair follicle, there is approximately one melanocyte for every five keratinocytes while the proportion in the epidermis is one to thirty-six on average. Moreover, active melanocytes in the hair bulb

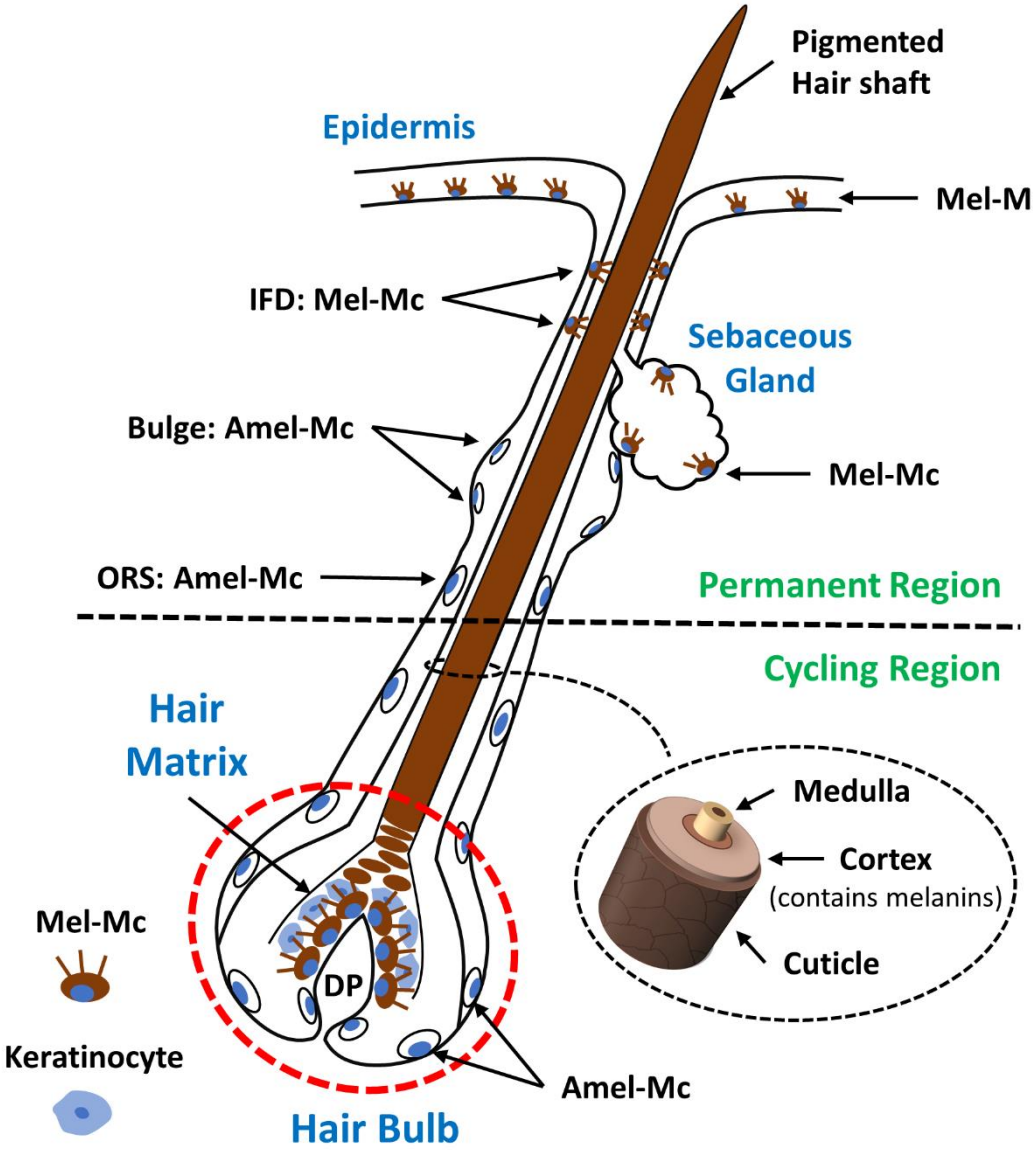


Figure I.1. Distribution of melanocytes in the different regions of the human anagen scalp hair follicle. Amel-Mc: amelanotic melanocytes; DP: dermal papilla; IFD: infundibulum; Mel-Mc: melanogenic melanocytes; ORS: outer root sheath.

are larger, more dendritic, produce larger melanosomes, have longer dendrites, more extensive Golgi apparatus and rough endoplasmic reticulum (ER) compared to the active melanocytes of the epidermis. Melanin produced in epidermis degrades almost completely in the differentiating keratinocyte layers, but melanin granules transferred into follicular cortical keratinocytes are minimally digested providing similar pigmentation to the proximal and distal ends of hair shafts. This difference in the melanin degradation pattern is usually attributed to the significantly larger size of follicular melanosomes that influence their

uptake by keratinocytes and susceptibility to enzymatic degradation. Both pigmentary units exhibit quite different antigenic profiles, and the immunological differences between them reflect the fact that epidermal unit is immunocompetent while the follicular unit resides in the immune-privileged hair bulb. Despite all those, the most striking difference is the fact that hair follicle melanogenesis is cyclic and tightly coupled to the hair growth cycle while epidermal melanogenesis is continuous (although easily stimulated after exposure to UV radiation) [1,4,6,8,35,36].

I.3. HAIR FOLLICLE MELANOGENESIS AND THE HAIR GROWTH CYCLE

The hair follicle is one of the most complex mini organs of the human body. It consists of an upper permanent region and an inferior region (containing the hair bulb) that undergoes dramatic cyclic transformation, renewing itself during our lifetime. The purpose of hair cycling is thought to be related to cleaning the skin surface of debris and parasites, excretion of deleterious chemicals encapsulated within trichocytes, regulation of paracrine and endocrine secretion of hormones and growth modulators produced within the hair follicle, and as a safe-guarding system against malignant degeneration by protecting rapidly dividing keratinocytes from oxidative damage by depletion. Changes in the local milieu are thought to drive the hair cycle transformation through its different phases: anagen, catagen and telogen. The activity of hair bulb melanocytes is tightly coupled to the hair growth cycle and undergoes concomitant transformation – Figure I.2 [4,8,37,38].

The hair shaft synthesis and pigmentation take place during anagen (or growth phase); in human scalp, 85 to 90% of hairs are in the anagen phase which lasts on average 3 years. The mature anagen hair follicle contains a fully formed hair bulb. Embraced by the hair bulb lies the follicular papilla whose volume, number of cells and secretory activity determine the size of hair bulb, the duration of anagen, and the length and thickness of hair shafts [4,8,39]. Anagen is divided into stages I to VI, throughout which melanocytes experience several morphological and biochemical changes. At the start of anagen, immature melanocytes located in the permanent part of the hair follicles are stimulated and respond by increasing their volume, entering mitotic activity and migrating in a coordinate manner, to repopulate the bulb of the new forming hair follicles [1,4,8,38,40]. SCF/KIT signalling was shown to be required for their activation and migration [33,41]. In Anagen I, immature melanocytes already express TYR and TYRP1 at the gene and protein level, but lack enzymatic activity; high levels of TYR allosteric inhibitor (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6-BH_4) have been reported in anagen I. Nevertheless, at the same time, the follicular papilla of the forming hair follicles begins to accumulate L-phenylalanine and there is an increase

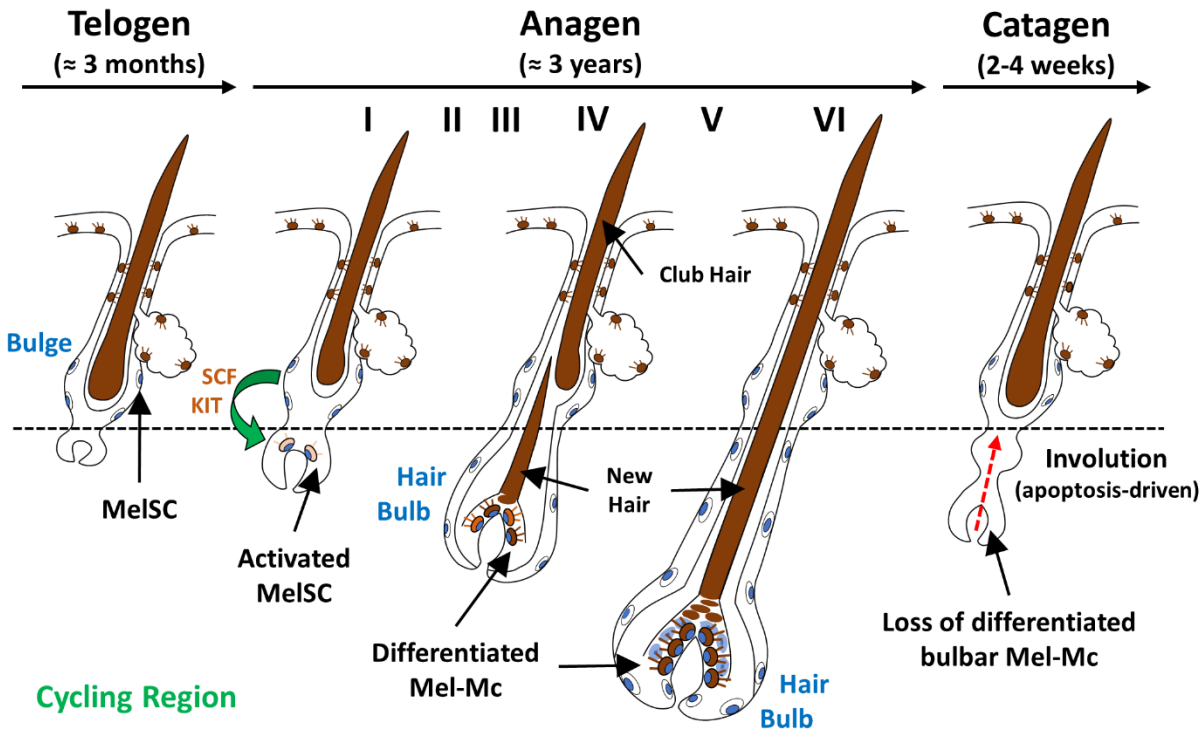


Figure I.2. Schematic representation of the fate of melanocytes during the anagen, catagen and telogen phases of the hair growth cycle. KIT: KIT proto-oncogene receptor tyrosine kinase; Mel-Mc: melanogenic melanocytes; MelSC: melanocyte stem cells; SCF: stem cell factor.

in the activity of phenylalanine hydroxylase (PAH) and GTP-cyclohydrolase I feedback regulatory protein (GFRP), setting the ideal conditions for the synthesis of L-tyrosine (L-Tyr, precursor of melanin). By anagen II, melanocytes are in a state of proliferation, that become quite significant at anagen III and remains until anagen VI (full anagen). In anagen II, tyrosinase activity and melanin also become readily detectable, increasingly rapidly through anagen III to V, reaching their highest levels at early anagen VI. During the progression of anagen III into VI, melanocytes develop more expansive Golgi and rough ER (needed for the melanosome biogenesis), increase the size and number of their melanosomes, and become highly dendritic. Moreover, melanocytes take up position in the hair bulb matrix and begin to transfer mature melanosomes into keratinocytes. At anagen VI, as proliferation ceases, bulbar melanocytes assume a highly differentiated status and become fully functional with respect to melanin synthesis. The activity of tyrosinase and melanin production remain steady during mid-to-late anagen VI. By the end of anagen VI, the earliest signs of imminent hair follicle regression are seen: L-Tyr availability declines, the expression and activity of tyrosinase and other melanogenesis-related proteins decreases rapidly, melanogenesis is attenuated, and melanocytes retract their dendrites [1,4,6,8,37,38,40,42,43].

During the transition from anagen to catagen, tyrosinase is no longer detectable in the hair bulb and the production of melanin is terminated [4,8,38,40]. As the catagen phase proceeds, an extensive apoptosis-driven involution process occurs, causing the loss of up to 70% of the hair follicle in a relatively brief period (2-4 weeks in human scalp) [4,8,39,44,45]. For some time, the hair bulb melanocytes were viewed as a self-perpetuating system capable of survive/avoid this process, being involved in the pigmentation of several hairs though multiple cycles of de-differentiation followed by re-differentiation. Today, although there is morphologic evidence for the survival of some of these melanocytes, it is known that most of them do not outlast the process. Thus, the current view suggests that melanocytes are recruited from the base of the permanent part of the hair follicles to populate the hair bulb in each new cycle. This region constitutes a reservoir of immature, slow cycling, self-maintaining MelSC that are fully competent to regenerate the pigmentary system of new hair bulbs at the beginning of each anagen phase [1,4,6,37,40,44,45]. Notch signalling, Wnt signalling pathway, PAX3, MITF and its downstream target BCL2 have all proved to play critical roles in the maintenance of these MelSC by promoting their survival [46–49].

Finally, telogen is a phase of relative proliferative quiescence and, in the human scalp, it lasts around 3 months. Despite that, it is characterized by intricated functionally relevant biochemical activity centred around the maintenance of several stem cell populations. In this context, it should be noted that telogen hair follicles present all precursors (MelSC) required for the development of a pigmentary system in the bulb of anagen follicles in the next cycle. These precursors are commonly small, have high nuclear cytoplasmic ratios, inactive cytoplasm with very few organelles, limited number of dendrites and do not synthesize melanin or express relevant melanogenesis-related proteins. By late telogen, the preparation of hair follicles for imminent regeneration (anagen development) is also characterized by intense biochemical activity. On the other hand, despite traditionally viewed as part of telogen, some authors defend that the active shedding of an old hair fibre (club hair) is a process that occurs as a distinct phase called exogen [4,8,50–53].

I.4. MOLECULAR AND CELLULAR BIOLOGY OF HAIR PIGMENTATION

Several critical steps must proceed in perfectly synchrony to achieve uniform and accurate pigmentation of hair shafts. These steps involve melanosomes biogenesis and the biochemical synthesis of melanin in follicular melanocytes as well as the transfer of melanin granules into surrounding keratinocytes. All these steps are under complex genetic control, with several genes encoding for a wide range of structural

proteins, enzymes, ion channels and transporters, receptors, growth factors, among others – Table I.1.

Table I.1. Most representative genes involved in the pigmentary process, with expression controlled by the transcription factor MITF. DHI: 5,6-dihydroxyindole; L-DOPA: L-3,4-dihydroxyphenylalanine

Symbol	Gene name	Function	Ref.
Melanosomes Biogenesis			
PMEL	Premelanosome protein	Amyloid fibril essential for melanosome biogenesis	[54]
MLANA	Protein melan-a	Regulation of PMEL expression	[54]
GPR143	G protein-coupled receptor 143	Regulation of melanosome maturation	[55]
Biochemical Synthesis of Melanin			
TYR	Tyrosinase	Hydroxylation of L-Tyrosine to L-DOPA Oxidative polymerization of DHI	[56–59]
TYRP1	Tyrosinase related protein 1	Stabilization of tyrosinase	[58,60]
DCT	Dopachrome tautomerase	Tautomerization of dopachrome	[60]
Regulation of Melanosomal pH			
SLC24A5	Solute carrier family 24, member 5	Na^+/H^+ exchanger	[61]
SLC45A2	Solute carrier family 45, member 2	K^+-dependent Na^+/Ca^{2+} exchanger	[62]
Melanosome Transport			
RAB27A	Ras-related protein RAB27A	Melanosome trafficking	[63]
Regulation of Melanogenesis			
MC1R	Melanocortin 1 receptor	Mediates melanocortin signalling	[64]
KIT	KIT proto-oncogene, receptor tyrosine kinase	Mediates stem cell factor signalling	[61]
EDNRB	Endothelin receptor type B	Mediates endothelin-1 signalling	[65]

MITF is the regulator of many genes associated with the pigmentary process; additionally, it also controls the expression of many other genes involved in differentiation, proliferation, and apoptosis [66–68]. MITF is a basic helix-loop-helix leucine zipper (bHLH-ZP) transcription factor that belongs to the MYC superfamily. At least 10 isoforms of MITF have been described in humans, with MITF-M being the specific isoform of the melanocyte lineage. As other bHLH transcription regulators, it recognizes the nucleotide consensus sequence CANNTG (E-box), with the association being maximal if this motif is 5'-flanked with T on either strand. At the N-terminus (NTAD), MITF exhibits the transcription activation domain, essential for interaction with transcriptional coactivators p300/CBP (CREB-binding protein). In turn, the transcription of *MITF* is controlled by a variety of regulators capable of binding to specific DNA sequences in its M promoter region: SOX9, SOX10, CREB (cAMP response element-binding protein), PAX3, LEF1 (lymphoid enhancer-binding factor 1), ZEB2 (zinc finger E-box binding protein 2), one cut domain 2 (ONECUT2) and MITF itself [61,69–75].

I.4.1 MELANOSOMES BIOGENESIS

Melanosomes are intracellular membrane-bound organelles, produced through the action of the Golgi and rough endoplasmic reticulum, inside which melanin synthesis takes place. These organelles are part of the secretory/endocytic pathway and, by sharing many common features with lysosomes [acidic luminal pH, presence of lysosomal-associated membrane proteins (LAMPs) in their outer membrane, among others], they were thought to be assembled via a similar process. However, despite the many common features, melanosomes also express an array of exclusive proteins (melanogenesis-related proteins, as well as certain LAMPs) and they present distinctive morphological characteristics (manifestation of an ellipsoid shape and internal structures composed of lamellae or regularly striated filaments). Therefore, it is presumed that the biochemical process of melanosome biogenesis forms a separate lineage [76–79].

The development and maturation of melanosomes proceeds through four stages, characterized by unique ultrastructural morphology – Figure I.3. These phases can be divided into two main steps: unpigmented and pigmented. The unpigmented step comprises the stages I and II during which the melanosome matrix is formed; at this point, melanosomes are referred to as immature or pre-melanosomes compartments. The pigmented phase comprises stages III and IV, in which the synthesis of melanin starts upon the formed matrix; at this point, melanosomes are denoted as mature/late compartments. Melanosomes are assembled in the perinuclear region, near the Golgi stacks, receiving all enzymatic and structural proteins required for melanogenesis [80,81].

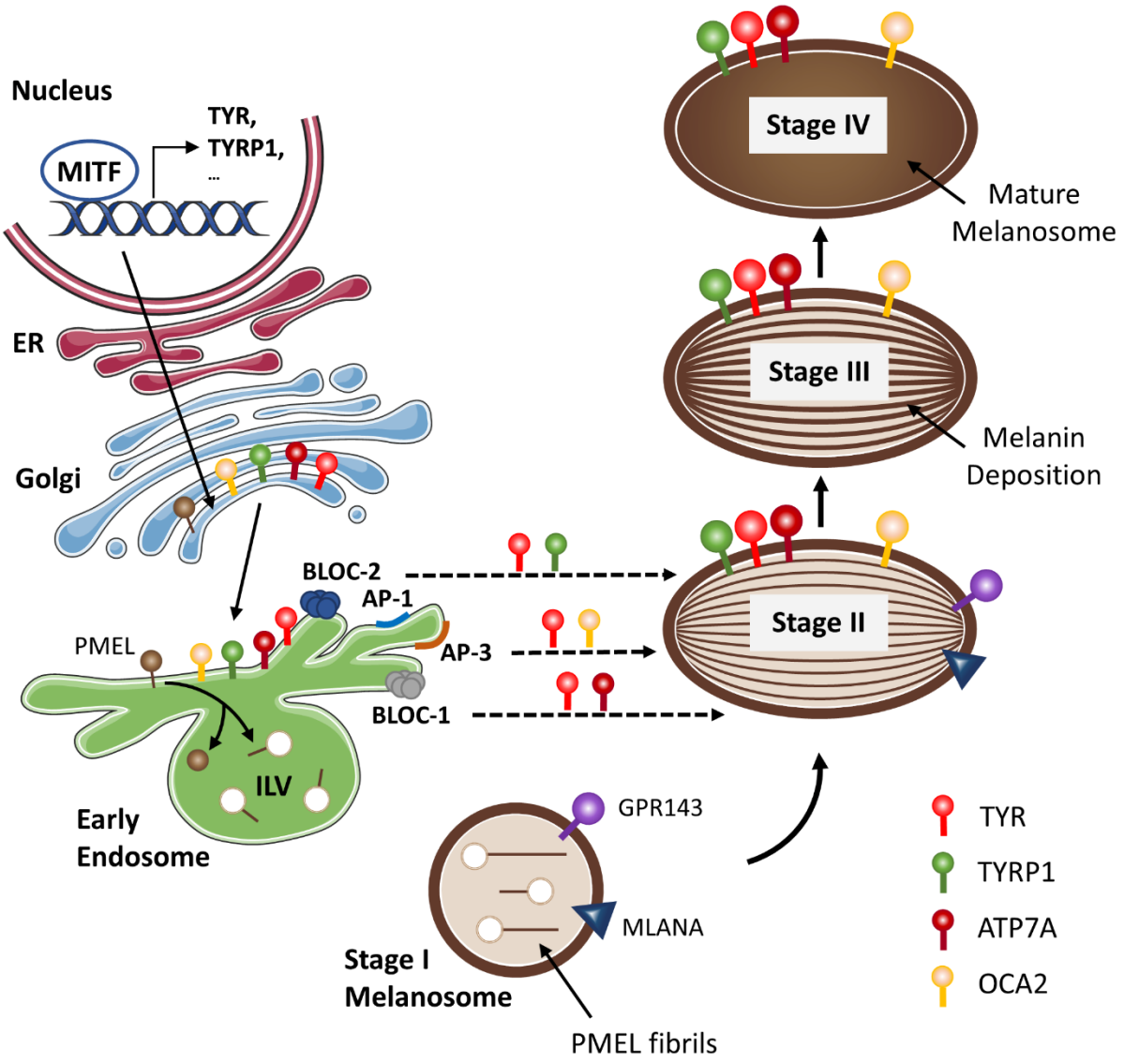


Figure I.3. Schematic representation of melanosomes biogenesis. AP-1/3: adaptor-related protein complex 1/3; ATP7A: ATPase copper transporting alpha; BLOC-1/2: biogenesis of lysosomal organelles complex 1/2; ER: endoplasmic reticulum; GPR143: G protein-coupled receptor 143; ILV: intralumenal vesicles; MITF: microphthalmia-associated transcription factor; MLANA: protein melan-a; OCA2: oculocutaneous albinism 2 protein; PMEL: premelanosome protein; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

In Stage I, immature melanosomes are spherical vacuolar domains of early endosomes that harbour intralumenal vesicles (ILVs) formed by invagination of the limiting membrane. The irregular arrays of amyloid fibrils that emanate from the ILVs make them distinct from early endosomes in other cells. Those fibrils are mainly composed of fragments of PMEL (premelanosome protein) [69,76,80–84]. In Stage II,

pre-melanosomes are characterized by a fully formed melanosome matrix. PMEL fibrils are organized into arrays of parallel sheets, transforming the spherical Stage I melanosomes into elongated, elliptical organelles. Although no active melanogenesis takes place in stage II, they already contain melanogenic proteins (TYR, TYRP1, among others). The formation of PMEL fibrils segregates the melanosomal from the endosomal pathways. Although the mechanism regulating the separation are unclear, it seems to involve GPR143 (G protein-coupled receptor 143) and MLANA (protein melan-a) [82–87]. In Stage III melanosomes, melanogenesis starts, with the pigment being regularly and uniformly synthesized on the fibrillar matrix. This causes the darkening and thickening of the matrix fibrils. By Stage IV, the melanosomes are saturated with melanin and the internal fibrillar structure becomes completely masked by the pigment; mature melanosomes are ready to be transferred to adjacent keratinocytes [76,78–80,85].

I.4.1.1 MAJOR PLAYERS IN MELANOSOMES BIOGENESIS

As stated above, the three major players in melanosomes development and maturation are PMEL, MLANA and GPR143; altogether, they form a complex in early stages melanosomes assuring their proper composition and structure.

I.4.1.1.1 PREMELANOSOME PROTEIN (PMEL)

PMEL (also known as PMEL17, gp100, SILV and ME20) is a type I transmembrane glycoprotein, composed of a short signal peptide, a transmembrane domain, a long luminal N-terminal domain, and a short cytoplasmic C-terminal domain. It is the major functional component of melanosomes, and the only melanosomal protein necessary for the formation of very stable, β-sheet rich oligomeric structures that act as scaffold upon which melanin is polymerized, condensed, and stored. PMEL fibrils takes part in the maintenance of melanosome integrity by preventing highly reactive (cytotoxic) melanin intermediates to freely diffuse within the organelle. The sequestering of melanin intermediates has also been shown to accelerate melanin synthesis and its condensation in PMEL fibrils may even optimize the transfer from melanocytes to keratinocytes. Despite its functional role, the amyloid nature of such fibrils is challenging for melanocytes since an incorrect formation and organization leads to toxicity. Thus, the processing, trafficking, sorting, and fibrillation of PMEL is highly regulated to avoid aggregation in the wrong compartments [83,85,87,88].

PMEL is synthesized in the ER and firstly modified there by removal of the signal peptide, the addition of

N-glycosylations and the formation of disulphide bonds. Then, PMEL is exported to the Golgi complex and further modified by the addition of *O*-glycosylations. PMEL also undergoes multiple proteolytic processing in the Golgi apparatus and post Golgi compartment by a proprotein convertase (PC), forming a large fibrillogenic Mα fragment which remains linked to the Mβ fragment by a disulphide bond [85,89–92]. From the trans-Golgi network (TGN), PMEL is targeted to endosomes and then to pre-melanosomes where it is cleaved by the β secretase BACE2 (β-site APP-cleaving enzyme 2); the adaptor-related protein complex 2 (AP-2) is required for the transport of PMEL from the TGN to endosomes and for melanosomal accumulation. The cleavage releases the Mα fragment associated to a portion of the Mβ fragment (MβN) and a C-terminal fragment (CTF). The latter is rapidly degraded in the lysosomes while the MβN fragments suffers another proteolytic processing, required for proper fibril formation. This processing is known to be mediated by ADAM17 (ADAM metallopeptidase domain 17), a disintegrin and metalloprotease, from a family of proteases known to be involved in ectodomain shedding and for playing a role in cellular processes as adhesion and migration; besides ADAM17, other proteins are also thought to be involved in the processing of MβN fragments [84,85,93–95]. Finally, fully processed PMEL fibrillogenic fragments are sorted to the ILVs that, allowing the loading and concentration of such fragments at their surface, provide a favourable environment for the nucleation of fibrils. Unlike most proteins, the sorting of PMEL to ILVs does not require ubiquitylation or the activity of the ESCRT (endosomal sorting complexes required for transport) machinery, being regulated by Apolipoprotein E (APOE) and tetraspanin CD63. Along with the maturation of PMEL fibrils into sheets, the ILVs disappear, probably by fusing with the membrane of melanosomes or degradation by lipases [81,85,96,97].

I.4.1.1.2 PROTEIN MELAN-A (MLANA)

MLANA (also known as melanoma antigen recognized by T cells 1 or MART-1) is an integral membrane protein. Besides melanosomes, it can be found in late endosomes, lysosomes, among others. MLANA forms a complex with PMEL, affecting its expression, trafficking, stability, and processing. MLANA also interacts biochemically with GPR143, providing stability to this receptor [81,86,98].

I.4.1.1.3 G PROTEIN-COUPLED RECEPTOR 143 (GPR143)

GPR143 (also known as oculocutaneous albinism 1 protein or OA1) is a pigment-cell specific transmembrane G protein-coupled receptor found in the membrane of melanosomes and other organelles like late endosomes and lysosomes [99,100]. This receptor acts as a biosensor of melanosome

maturation and provides regulation of their size and number by triggering the activation of a transcription cascade, involving MITF, that culminates in sustained PMEL expression [101]. It has also been proposed that GPR143 activity delays the delivery of PMEL-containing endosomes to the lysosomes in order to allow time for commitment with melanosome biogenesis [100]. Like many membrane GPCR, GPR143 might also couple to melanosomal ion channels and modulate ion gradients and membrane potential, important in fusion and fission events that regulate protein trafficking and organelle biogenesis [102].

I.4.1.2 PROTEIN TRAFFICKING TO MELANOSOMES

The maturation of melanosomes requires the delivery of several melanogenic proteins. Most of them are integral membrane glycoproteins, synthesized by ribosomes associated with the ER and become integrated in its membrane across which they translocate. In the ER, such proteins are N-glycosylated, folded, and assembled by enzymes and chaperones. Melanosomal proteins that are properly folded in the ER transit to the Golgi complex for functional modifications and from there to the endosomes; failure to be properly processed results in retro translocation of these melanosomal proteins in the ER and degradation by the proteasome [77,81]. The molecular intervenient on the transition of melanogenic proteins from the Golgi to endosomes are mainly unknown, but the interaction between GIPC (GAIP interacting protein COOH-terminus) and APPL (adaptor protein containing pleckstrin homology, phosphotyrosine binding domain, and leucine zipper motif) seems to be important, at least in the trafficking of TYRP1 [103,104].

Within endosomes, melanogenic proteins are usually loaded into vesicular carriers for delivery into melanosomes through a process involving the fusion of their limiting membranes. Two transport pathways involving multisubunit protein complexes are known to allow the trafficking of proteins from the endosomes to melanosomes – Figure I.3. One pathway is mediated by adaptor-related protein complexes, namely AP-1 and AP-3 [81]. The AP-1 complex is involved in the trafficking of TYR and TYRP1. The function of AP-1 requires the recruitment of microtubule motor protein KIF13A (kinesin family member 3A) to position endosomes near maturing melanosomes to facilitate the formation of transient connections between them for cargo transfer; two other molecular motor proteins, myosin VI (MYO6) and MYO1B were also shown to be part of the machinery that regulates the delivery of melanogenic enzymes to maturing melanosomes [81,105–108]. The AP-3 complex is required for the efficient delivery of TYR and OCA2 (oculocutaneous albinism 2 protein), a protein involved in the regulation of melanosomal pH and a major determinant of mammalian pigmentation; OCA2 itself have also been pointed out as being

implicated in the trafficking and processing of tyrosinase and related proteins [81,108–115]. The other transport pathway is mediated by biogenesis of lysosomal organelles complexes (BLOC). BLOC-1 allows the delivery TYRP1 and ATP7A to melanosomes; ATP7A is a copper transporting ATPase that supplies Cu^{2+} to the Cu^{2+}-dependent enzyme TYR, thus sustaining melanin synthesis The trafficking of TYRP1 by this pathway was shown to be dependent on the ESCRT-I complex. BLOC-2 is implicated in the delivery of TYR and TYRP1 to the melanosome [81,116–118].

SNAREs (Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor) are also reported to be involved in the delivery of proteins into the melanosomes due to their role in the membranes fusion process The interaction of melanosomal SNAREs syntaxin 3 and SNAP23 (synaptosome- associated protein 23) with SNARE VAMP7 (vesicle associated membrane protein 7) on TYRP1-containing vesicles regulates its trafficking to melanosomes. Additionally, the interaction of melanosomal VAMP7 with endosomal syntaxin 13 or VARP (VPS9-domain ankyrin repeated protein) is also accountable for proper delivery of TYR and TYRP1 to melanosomes [81,119,120].

The Rab family of small GTPases are also known to facilitate the trafficking of proteins to the melanosomes. By recruiting tethering/docking and fusion factors, as well as actin- and microtubule-based proteins, they establish molecular interaction between melanosomes and cargo vesicles and coordinate their positioning for cargo delivery. RABs that have been involved in the trafficking of melanogenic proteins, but also in other aspects of melanosomes biogenesis, are: RAB38/RAB32, RAB11, RAB9, RAB7, RAB33A, among others [81,121–128].

I.4.2 BIOCHEMICAL PATHWAY OF MELANOGENESIS

Melanogenesis *in vivo* produce mixtures of two chemically distinct types of melanins through a process often referred to as mixed melanogenesis. Eumelanin is a black-to-brown highly polymerized pigment while pheomelanin is reddish-brown to yellow and less polymerized. Pheomelanin is reported to be synthesized first and eumelanin is subsequently deposited on the preformed pheomelanin, giving melanin granules. In hair follicles, the synthesis of pheomelanin can be completely switched-off during eumelanogenesis, but pheomelanogenesis never occurs with total inhibition of eumelanin synthesis. The total amount of melanins produced is proportional to the availability of L-Tyr and the dopaquinone (DQ) formed, which in turn is proportional to the activity of tyrosinase. The ratio of eumelanin to pheomelanin is also determined by the activity of tyrosinase, but mainly influenced by the availability of cysteine [2,3,129–131].

The initiation of melanogenesis requires L-Tyr that can be directly transported from the extracellular space or synthesized inside melanocytes through hydroxylation of L-phenylalanine by PAH (EC 1.14.16.1). The activity of PAH depends on the essential cofactor/electron donor 6-BH_4, that melanocytes have full capacity for de novo synthesis and recycle – Figure I.4.

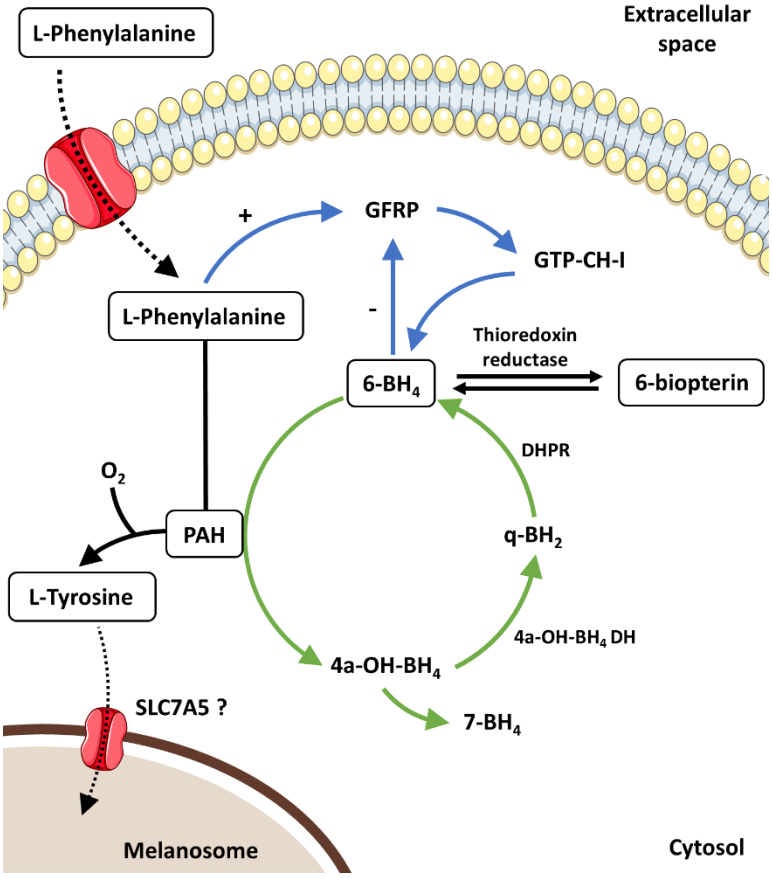


Figure I.4. Metabolism of L-phenylalanine to L-tyrosine via phenylalanine hydroxylase (PAH). The activity of PAH depends on the co-factor (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6-BH_4), which melanocytes have full capacity for de novo synthesis (blue arrows) and recycling (green arrows). 4a-OH-BH_4 DH: 4a-hydroxy-tetrahydrobiopterin dehydratase; 4a-OH-BH_4: 4a-hydroxy-tetrahydrobiopterin; 7-BH_4: (7R)-L-erythro-5,6,7,8-tetrahydrobiopterin; DHPR: dihydropteridine reductase; GFRP: GTP-cyclohydrolase I feedback regulatory protein; GTP-CH-I: GTP-cyclohydrolase I; q-BH_2: quinonoid dihydropterin; SLC7A5: solute carrier family 24, member 5.

The rate-limiting enzyme in de novo synthesis of 6-BH_4 is GTP-cyclohydrolase I (GTP-CH-I, EC 3.5.4.16). GTP-CH-I is stimulated by GFRP and, in turn, GFRP can be stimulated by L-phenylalanine or inhibited by

6B-H_4. [132–135]. The recycle of 6B-H_4 commences with the formation of L-Tyr from L-phenylalanine via PAH. This leads to the generation of 4a-hydroxy-tetrahydrobiopterin (4a-OH-BH_4) which through a non-enzymatically process can generate (7R)-L-erythro-5,6,7,8-tetrahydrobiopterin (7-BH_4). On the other hand, its enzymatic metabolization by 4a-hydroxy-tetrahydrobiopterin dehydratase (4a-OH-BH_4 DH, EC 4.2.1.96) originates quinonoid dihydropterin (q-BH_2) that it is reduced through the NADH-dependent dihydropteridine reductase (DHPR, EC 1.5.1.34) or reduced glutathione (GSH) to 6-BH_4. Additionally, thioredoxin reductase (TR, EC 1.8.1.9) can controls the redox status of 6-BH_4/6-biopterin, which has implications in the regulation of melanin production; reduced 6-BH_4 can bind to tyrosinase and inhibit the enzyme while oxidized 6-biopterin has no effect on it [134,136–138].

For the synthesis of melanin to occur, L-Tyr must be taken into melanosomes. SLC7A5 (solute carrier family 7, member 5) encodes for a member of the L-type aminoacids transporter family that it is specialized in the transport of histidine, tryptophan, tyrosine and neutral aminoacids. Although the inhibition of this transporter causes *in vitro* loss of pigmentation, its presence in melanosomes could not yet be validate and thus, the nature of tyrosine transporter inside melanosomes still remains elusive [139]. In melanosomes, melanogenesis begin with synthesis of L-3,4-dihydroxyphenylalanine (L-DOPA) from L-Tyr and subsequent oxidation to DQ – Figure I.5. The hydroxylation of L-Tyr to L-DOPA is catalysed by the multi-functional enzyme tyrosinase (EC 1.14.18.1), the most important enzyme in the melanogenic pathway. Alternative mechanisms for L-DOPA formation include the reduction of DQ back to L-DOPA or even direct hydroxylation of L-Tyr by tyrosine hydroxylase isoform I (TH I, EC 1.14.16.2) [8,130,140,141]. From here, the mixed melanogenesis proceeds, using the same precursor (DQ) for the synthesis of pheomelanin and eumelanin.

Pheomelanin is spontaneously produced from DQ if cysteine is present in concentrations above 1 μM – Figure I.5. The first step is the reductive addition of cysteine to DQ, giving rise to cysteinyldopa (CD) isomers 5-S-cysteinyldopa (5-S-CD) and 2-S-cysteinyldopa (2-S-CD) in a ratio of 5 to 1; an alternative route for the CD isomers formation is the conjugation of DQ with glutathione followed by the hydrolysis of the resultant glutathionyldopa by glutamyltranspeptidase. The second step is the redox exchange between CD isomers and DQ to produce CD-quinones (and DOPA), followed by their cyclization through dehydration to form ortho-quinonimine (QI). Then, QI is rearranged with or without decarboxylation to form 1,4-benzothiazine intermediates. The ring closure to yield such intermediates may involve a peroxidase/H_2O_2 reaction or TYR-catalysed oxidation; in this context, one enzyme indirectly affecting the production of pheomelanin is catalase (EC 1.11.1.6) that regulates hydrogen peroxide removal. The last

step involves the polymerization of benzothiazine intermediates to pheomelanin. The production of pheomelanins is preferred over the production of eumelanin in the presence of CD isomers concentrations above 10 μM [130,142–144].

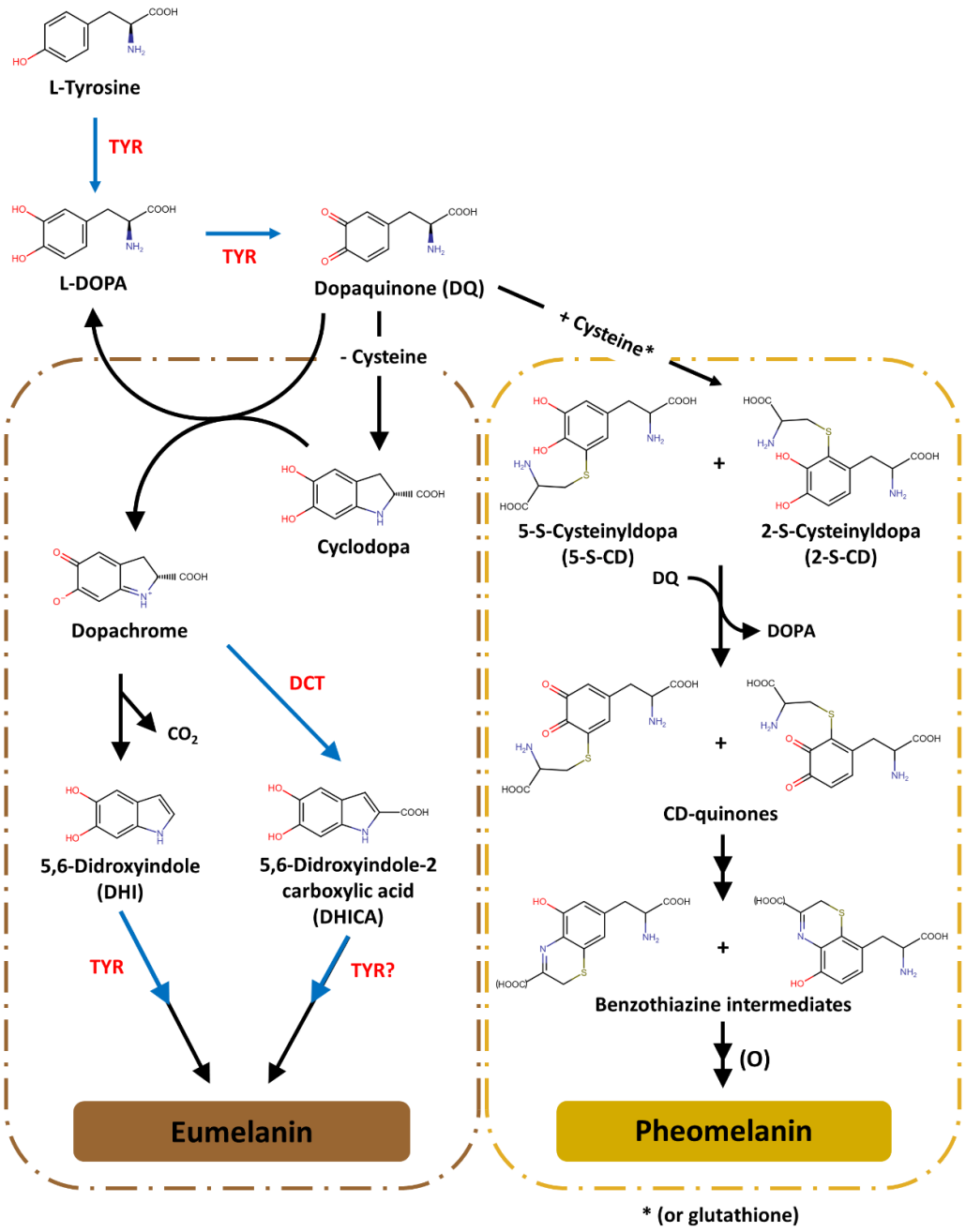


Figure I.5. Biosynthetic pathways involved in the production of eumelanin and pheomelanin.in human melanosomes. Spontaneous reactions are denoted as black arrows. Enzyme-catalysed reactions are signalized by blue arrows. CD: cysteinyldopa; L-DOPA: L-3,4-dihydroxyphenylalanine; TYR: tyrosinase; DCT: dopachrome tautomerase.

The production of eumelanin begins after most CD isomers and cysteine are depleted – Figure I.5. As a highly reactive ortho-quinone intermediate, DQ undergoes spontaneous intramolecular cyclization to give rise to cyclodopa. Then, cyclodopa rapidly undergoes a redox exchange with another DQ molecule to produce one molecule of dopachrome, a relatively stable intermediate, and one molecule of DOPA. In the absence of additional factors, dopachrome undergoes spontaneous decarboxylative rearrangement to form 5,6-dihydroxyindole (DHI) [145]. In the presence of DCT (EC 5.3.3.12), the tautomerization of dopachrome can also forms 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [146–148]. However, although DCT can be found in the human epidermis and follicular melanocytes of many mammalians, human bulbar melanocytes (at least those of eumelanic phenotypes) do not express this protein and human hairs contain low yield of DHICA [149,150]. Both dihydroxyindoles (DHI and DHICA) are further oxidized and assembled by cross-linking reactions into eumelanin polymers. Oxidative polymerization of DHI is catalysed directly by tyrosinase and indirectly by DQ. Oxidation of DHICA in mice is catalysed by TYRP1, but the human homologue does not act the same. Although its function is not completely clear, in humans, TYPR1 appears to ensure appropriate processing and stabilization of tyrosinase, to maintain melanosomal structure integrity and to function as tyrosine hydroxylase at low concentrations of substrate. Thus, tyrosinase seems to be responsible for the oxidation and incorporation of DHICA units into human eumelanins, along with peroxidase (EC 1.11.1.7) and PMEL [88,151–158].

I.4.2.1. MAJOR PLAYERS IN MELANINS SYNTHESIS

As already been exposed, the synthesis of melanins cannot occur without the activity of TYR and TRP1. In turn, the ratio of pheomelanin to eumelanin is determined by the availability of cysteine, which correlates with the activity of cystine transporters.

I.4.2.1.1. TYROSINASE (TYR)

Tyrosinase is a type 3 copper containing enzyme, involved in several steps of melanin synthesis. This membrane glycoprotein can be divided into three domains: a large N-terminal intra-melanosomal domain (that possesses a catalytic subdomain), a single transmembrane α-helix domain (anchoring tyrosinase in melanosome membrane) and a small C-terminal cytoplasmic tail. These three regions are conserved among TYR and TYR-related proteins and they follow quite similar processing and trafficking pathways. The maturation of tyrosinase includes a signal sequence cleavage, heavy glycosylation (N-glycosylations at Asn272, 319 and 353 are particularly important for proper maturation and stability), disulphide bond

formation and chaperone binding for proper folding. Tyrosinase first acquires enzymatic activity in the TGN, likely because this is where it first encounters its critical cofactor, the copper cation [81,159,160].

Tyrosinase catalyses the hydroxylation of L-Tyr to L-DOPA (tyrosine hydroxylase activity), the oxidation of DOPA to DQ (DOPA oxidase activity) and the oxidation DHI and DHICA into the eumelanin precursors indole-5,6-quinone and indole-5,6-quinone carboxylic acid, respectively. Tyrosinase is activated by its own substrate L-Tyr and co-factor L-DOPA; the latter accelerates the conversion of tyrosine into DQ by removing an initial lag period observed for tyrosine hydroxylation. On the contrary, DHI can inhibit its tyrosine hydroxylase activity and tyrosine can inhibit the conversion of DHI to indole-5,6-quinone. Tyrosinase exhibits optimal enzymatic activity at neutral pH and it is suppressed at pH bellow 5.8 [159–162].

I.4.2.1.2. TYROSINASE RELATED PROTEIN 1 (TYRP1)

Although TYRP1 has an undisputed role in melanin synthesis (mutations in the encoding gene causes a form of oculocutaneous albinism, OCA3), its catalytic activity remains unclear. From the several enzymatic activities attributed to the mouse homologue (tyrosine hydroxylase, L-DOPA oxidase, dopachrome tautomerase, DHICA oxidase), the human TYRP1 only appears to display tyrosine hydroxylase activity. However, it has been implicated in the maturation and stabilization of tyrosinase [160,163,164].

I.4.2.1.3. CYSTINE TRANSPORTERS

The synthesis of pheomelanin is dependent on the availability of cysteine, obtained through redox exchange of cystine with GSH. SLC7A11 is a cystine/glutamate exchanger, responsible for cystine transport into melanocytes and possibly into melanosomes; additionally, it also seems to regulate the transcription of several melanogenesis-related genes [165,166]. Cystinosin (CTNS) is a cystine/H^+ symporter located in melanosomes that controls the efflux of cystine; its ability to efflux protons along with cystine also render it a role in the control of melanosomal pH [167].

I.4.2.2. CONTROL OF MELANOSOMAL PH

During early stages of maturation (I and II), the melanosomal pH is low (around pH 4), which seems to favour the formation of PMEL fibrils [168]. However, in later stages, the activity of tyrosinase requires an increase in the pH. At weakly acidic pH (5.8-6.3), the production of pheomelanin is preferred because CD-quinone cyclization (bicyclic intermediate of pheomelanin) proceeds much faster than the cyclization

of DQ (first step of eumelanogenesis). At near neutral pH (6.8-7.4), eumelanin production is favoured.[141,169–171].

Since the pH of melanosomes is greatly influenced by their internal ionic equilibrium, it has been suggested that pH control can be achieved through the concerted action of a cascade of ion channels (allow ions to diffuse down their electrochemical gradient) and transporters (pumps and carriers undergoing conformational change to transport ions against electrochemical gradients) – Figure I.6 [3]. In turn, recently, it was proposed that soluble adenylyl cyclases (sAC) can modulate the melanosomal pH and pigmentation, likely by the regulation of such ion transporters through stimulation of EPAC (exchange proteins activated by cAMP) [172].

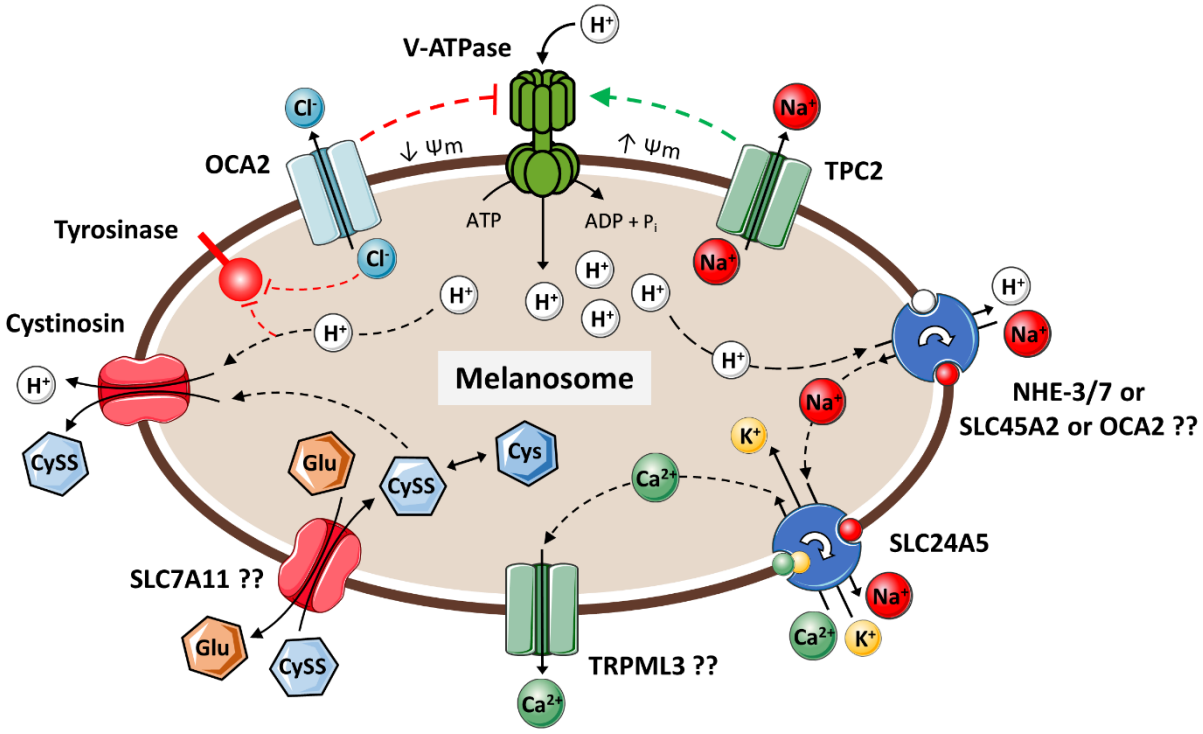


Figure I.6. Schematic representation of the cascade of ion channels and transporters with a role in melanogenesis. Cys: cysteine; CySS: cystine; Glu: glutamate; NHE: sodium hydrogen exchanger; OCA2: oculocutaneous albinism 2 protein; SLC(7A11/24A5/45A2): solute carrier family 7/24/45, member 11/5/2; TPC2: two pore segment channel 2; TRPML3: transient receptor potential cation channel, mucolipin subfamily, member 3; V-ATPase: vacuolar ATPase.

Vacuolar ATPases (V-ATPases) are known to be located in melanosomes, being a key component in

melanosome acidification [102,170,173,174]. To increase the pH, the H^+ protons taken up by V-ATPases are returned to the cytoplasm though sodium-proton exchangers. Melanocytes express cytoplasmic membrane sodium hydrogen exchangers (NHEs, namely NHE-3 and NHE-7) that appears to be involved in this process [175]. More important, OCA2 and SLC45A2 have been recognized as major players in regulating melanosomal pH and, as NHEs, they also have been proposed to act as sodium-proton exchangers [3].

OCA2 (also known as P protein) is a melanosomal membrane protein with a predicted structure consisting of 12 transmembrane domains [102,176,177]. Besides acting as a sodium-proton exchanger, OCA2 has been suggested to be an essential component of an anion channel, making melanosomes less acidic by allowing chloride ions to flow out of the melanosome and thus, modulating the membrane potential with consequent reduced vacuolar H^+-ATPase activity [178]. Interestingly, another study found that the mere expression of CLC7 (chloride voltage-gated channel 7) is implicated in the pigmentation [179]. The efflux of Cl^- ions by itself is also important in the context of melanogenesis because they were found to be inhibit human tyrosinase activity; in the same work, the Cl^- transporters SLC12A4 and SLC12A7 were found to be downregulated during TYR-mediated melanogenesis in HEK293 cells, providing further evidence of the Cl^--mediated negative regulation of melanogenesis [180]. The SCL45A2 (also known as membrane-associated transporter protein or MATP) is a melanosomal transporter, with predicted structure consisting of 12 transmembrane domains, sharing homology with sucrose/proton symporters. As OCA2, besides being involved in the regulation of pH and consequent tyrosinase activity, it has been implicated in tyrosinase trafficking and also in melanosome structure [102,173,181,182].

As the removal of H^+ from melanosomes implies the exchange with Na^+, the excess of these ions needs to be cycled out of the melanosomes. SLC24A5 (also known as sodium/potassium/calcium exchanger 5 or NCKX5) has been detected in melanosomes membranes, and its K^+-dependent Na^+/Ca^{2+} exchanger activity in association to the control of melanogenesis was already demonstrated [102,183,184] The exchanger activity of SLC24A5 also provides a link between cytosolic and melanosomal Ca^{2+}; melanosomes are enriched in Ca^{2+} because melanin has the ability to bind and chelate it, which has been implicated in the regulation of calcium homeostasis in melanocytes [185,186]. Additionally, transport of Ca^{2+} (and other ions) across the melanosomal membrane likely regulates its voltage, facilitating the fusion process between melanosomes and vesicles of protein transport or other organelles; in this context, melanosomes were found to form mitofusin 2-dependent contacts with mitochondria, which have implications in melanosome maturation and melanin synthesis [102,187]. It is important to mention that

the melanogenic effect of Ca^{2+} is not restricted to the actions upon the melanosomal membrane. Cytosolic Ca^{2+} is known to activate protein kinase C (PKC) that modulates the pigment levels in melanocytes [188–190]. Ca^{2+} is also required for the transport of L-phenylalanine into melanocytes (calmodulin-dependent Ca^{2+}-ATPase), and for its turnover into L-Tyr [67,191]. Recently, the small conductance Ca^{2+}-activated K^+ channels were also suggested to be functionally important in human pigmentation, although the mechanism of action needs further elucidation [192]. Finally, Ca^{2+} contributes to the process of melanosome transfer between melanocytes and keratinocytes [193]. In melanocytes, some ion channels at the plasma membrane have already been implicated in the Ca^{2+} influx and modulation of pigmentation: melanocyte-specific TRPM1 (transient receptor potential cation channel, subfamily M, member 1) [194–196], TRPML3 (transient receptor potential cation channel, mucolipin subfamily, member 3) [197], CRAC (calcium release activated channels) [198], among others [102].

The concerted neutralization of melanosomal pH downstream the activity of SLC24A5 was thought to be regulated by TPC2 (two pore segment channel 2) in virtue of the suspected activity in mediating the nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent efflux of Ca^{2+} [3,199]. However, TPC2 was later found to function as Na^+-selective channel [200]. Moreover, it was uncovered that TPC2 provides negative regulation of melanogenesis by increasing melanosomal membrane potential and acidity, possibly by providing a cation counterflux to enhance the H^+ transport by V-ATPases [201]. Thus, although TPC2 does not contribute to the efflux of Ca^{2+}, its activity seems to be part of the normal regulation of proton flux that maintain ion homeostasis and it may be important in melanosome acidification needed in early stages of melanosome maturation. In any case, other ions channels or transporters must exist in the melanosome membrane that counterbalance the influx of Ca^{2+}. Besides its location in plasma membrane, TRPML3 is also found in endosomal compartments and, if present in melanosomes, it may mediate this transport [102].

I.4.3 TRANSPORT AND TRANSFER OF MELANOSOMES

To ensure pigmentation of the hair shafts, melanocytes transfer mature melanosomes into neighbouring keratinocytes via dendritic projections. However, before being transferred to the keratinocytes, melanosomes need to be transported from their site of origin at the perinuclear area to the melanocyte periphery. The motility of melanosomes in melanocytes occurs along microtubules (composed of α:β-tubulin dimers) and actin filaments (composed of actin monomers) – Figure I.7 [202].

Microtubules act as tracks for the transport of melanosomes to the peripheral region. The movement

along microtubules is influenced by two classes of microtubule-associated motor proteins, the kinesins and the dyneins. A complex comprising RAB1A, SKIP/PLEKHM2 (pleckstrin homology domain-containing, family M, member 2) and kinesin-1 motor [composed of two KIF5B heavy chains and two kinesin light chain 2 (KLC2)] has been involved in anterograde transport of melanosomes. The Rab interacting lysosomal protein (RILP), the p150Glued subunit of the dynein-dynactin motor complex (also known as dynactin subunit 1 or DCTN1), melanoregulin and RAB36 are reported to be involved in the retrograde movement of melanosomes [69,203–208].

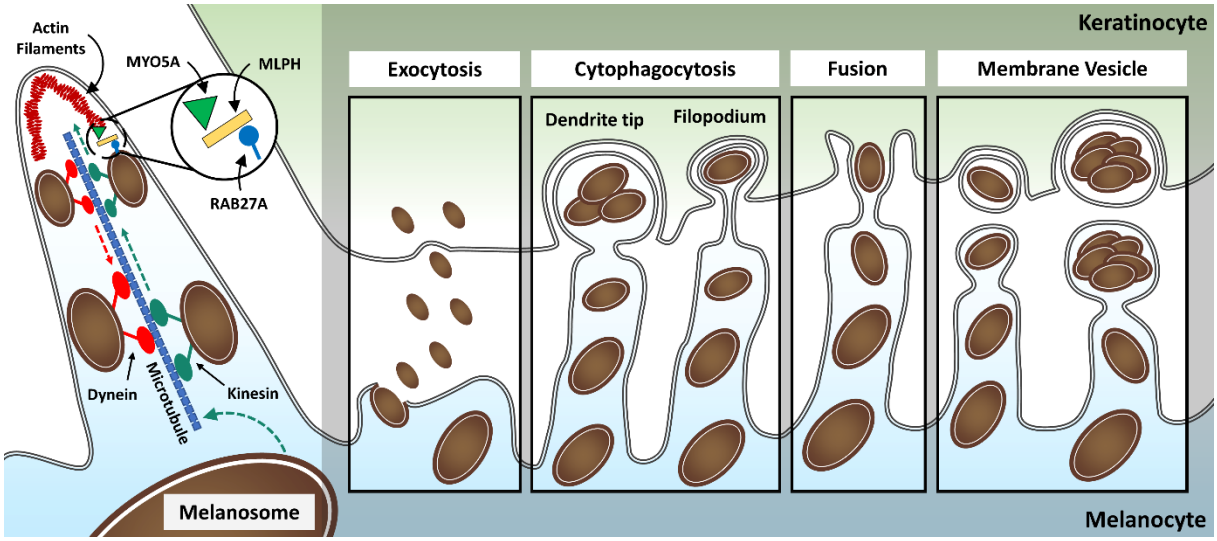


Figure I.7. Mechanisms of melanosome transport and transfer. Melanosomes bind to kinesin for moving along microtubules from the perinuclear region to the periphery of melanocytes. At the dendrites, the movement of melanosomes in the rich network of actin filament is regulated by the RAB27A-MLPH-MYO5A complex. Several models have been proposed regarding the transference of melanin from melanocytes to surrounding keratinocytes. MLPH: Melanophilin; MYO5A:myosin VA; RAB27A: Ras-related protein RAB27A.

At the periphery of melanocytes, the capture and movement of melanosomes in the rich network of actin filament of the dendrites is regulated by the RAB27A-MLPH-MYO5A complex [63,209–214]. RAB27A is responsible for linking synaptotagmin-like 2 (SYTL2) with phosphatidylserine, docking melanosomes at the plasma membrane [69,212,215–217] Melanophilin (MLPH, also known as SLAC2-A) is an effector of RAB27A and it functions as a linker between RAB27A and MYO5A; prohibitin (PHB) is necessary for the interaction between MLPH and RAB27A [211,218–220]. MYO5A is an ATP-dependent motor protein

that captures melanosomes in subcortical bundles at the periphery of dendrites and moves the melanosomes towards the plus end of actin filaments [210,221].

I.4.3.1. MODELS OF MELANIN TRANSFER

Once melanosomes are docked to the melanocyte membrane, melanin is transferred to keratinocytes. The exact mechanism is still unclear; several models are currently proposed for the transference of melanin from melanocytes to keratinocytes – Figure I.7 [222,223].

I.4.3.1.1. EXOCYTOSIS-MEDIATED TRANSFER

The exocytic-mediated transfer model states that melanosomal membrane fuses with the melanocyte membrane, resulting in the extracellular releasing of melanin. The pigment is posteriorly engulfed by keratinocytes through an endocytic process, probably phagocytosis. This theory is supported by electron microscopic observations of human skin and hair follicles depicting uncoated melanin (melanocore) in the intercellular space [224–226]. Further support is provided by the fact melanocytes express several SNAREs and Rab GTPases with well-known roles in the regulation of exocytosis, and some of them have even been implicated in the melanin transfer process [222,225]. Also, melanocytes are closely related to neural cells (by shared origin), which synaptic vesicles undergo exocytosis [222]. Finally, it was reported the requirement of the exocyst tethering complex in melanin transfer to keratinocytes [227].

I.4.3.1.2. CYTOPHAGOCYTOSIS-MEDIATED TRANSFER

The cytophagocytic-mediated transfer model proposes that melanin is transferred to keratinocytes by phagocytosis of the tip of a melanocyte dendrite or by the phagocytosis of filopodia that protrude from the dendrites. Regarding the first hypothesis, it was supported mainly by electron and time-lapse video microscopy studies from a time when less advanced techniques were used, with later optimized acquisition techniques failing to corroborate it [222]. The enclosure of filopodia by keratinocytes was suggested more recently as a filopodia-phagocytosis model for melanin transfer. This model proposes that MYO10 drives the formation and elongation of filopodia in melanocytes. Then, via integrins, melanocytes adhere to the membrane of keratinocytes and the MYO10-associcated motor force at the filopodia tips helps insert filopodia into the plasma membrane of keratinocytes, causing their taken up. Interestingly, in this context, filopodia were also shown to be involved in the melanosome transfer between keratinocytes [228].

I.4.3.1.3. MEMBRANE FUSION-MEDIATED TRANSFER

The fusion model assumes that melanocytes and keratinocytes fuse their membranes to connect their cytoplasms. This fusion appears to involve filopodia that extend from the dendrite of melanocytes and adhere to keratinocytes, resulting in the formation of conduits that admit unidirectional transport of melanosomes [229]. Although this kind of organelle transport exist in other cells, and transfer of melanosomes at filopodia locations have been observed, definitive proof of membrane fusion and melanosome transport across the channels formed has yet to be given [222].

I.4.3.1.4. MEMBRANE VESICLE-MEDIATED TRANSFER

The membrane vesicle-mediated transfer model reports that melanosome-containing vesicles are released into the extracellular space, adhere to keratinocytes and then, they are internalized by phagocytosis [222,223] It has also been hinted that multiple melanosomes can be concentrated at the filopodia for being released inside globules from various areas of the dendrites [230,231].

Although the exact mechanism (or combination of mechanisms) of melanin transfer remains unclear, phagocytosis (a process of cellular engulfment of particles with a diameter of more than 0.5 μM) is a mostly necessary step. The phagocytic nature of keratinocytes is widely known and, regarding melanin transfer, phagocytosis has been reported to be modulate mainly by the protease-activated receptor 2 (PAR2, also known as coagulation factor II receptor-like 1 or F2RL1) [232–237]. PAR2 is a seven-transmembrane G protein-coupled receptor, related to the thrombin receptors. The proteolytic cleavage at the extracellular N-terminal moiety exposes a new N-terminus that acts as a tethered ligand that binds the receptor leading to its activation. This activation induces the secretion of serine proteases by keratinocytes that are responsible in the first place for the cleavage of PAR2, thus creating a positive feedback loop [232,233]. Despite PAR2 performs the leading role in phagocytosis associated with melanin transfer, it is not expected to be the only receptor that modulates the process as suggested by the incomplete inhibition of melanin transfer upon treatment with serine protease inhibitors. Important to mention, the modulation of pigmentation by PAR2 goes beyond the direct activation of the phagocytic pathway in keratinocytes: PAR2 activation also increases melanocyte dendricity (upon stimulation of prostaglandins PGE2 and PGF2α release by keratinocytes) and the expression of SCF, a paracrine regulator of melanogenesis [238,239].

I.5. REGULATION OF FOLLICULAR MELANOGENESIS

Melanogenesis is under a complex control involving several positive and negative regulators/factors that act via autocrine, paracrine, and endocrine mechanisms – Figure I.8. Although much of the available data regarding the control of melanogenesis pertains to human epidermal or murine follicular melanocytes, it is thought that such regulators contribute in a similar way to human hair pigmentation; given that active follicular melanocytes are located beyond the reach of its direct stimulation, one notable exception is the UV radiation, the principle regulator of melanin synthesis in the epidermis [69,240].

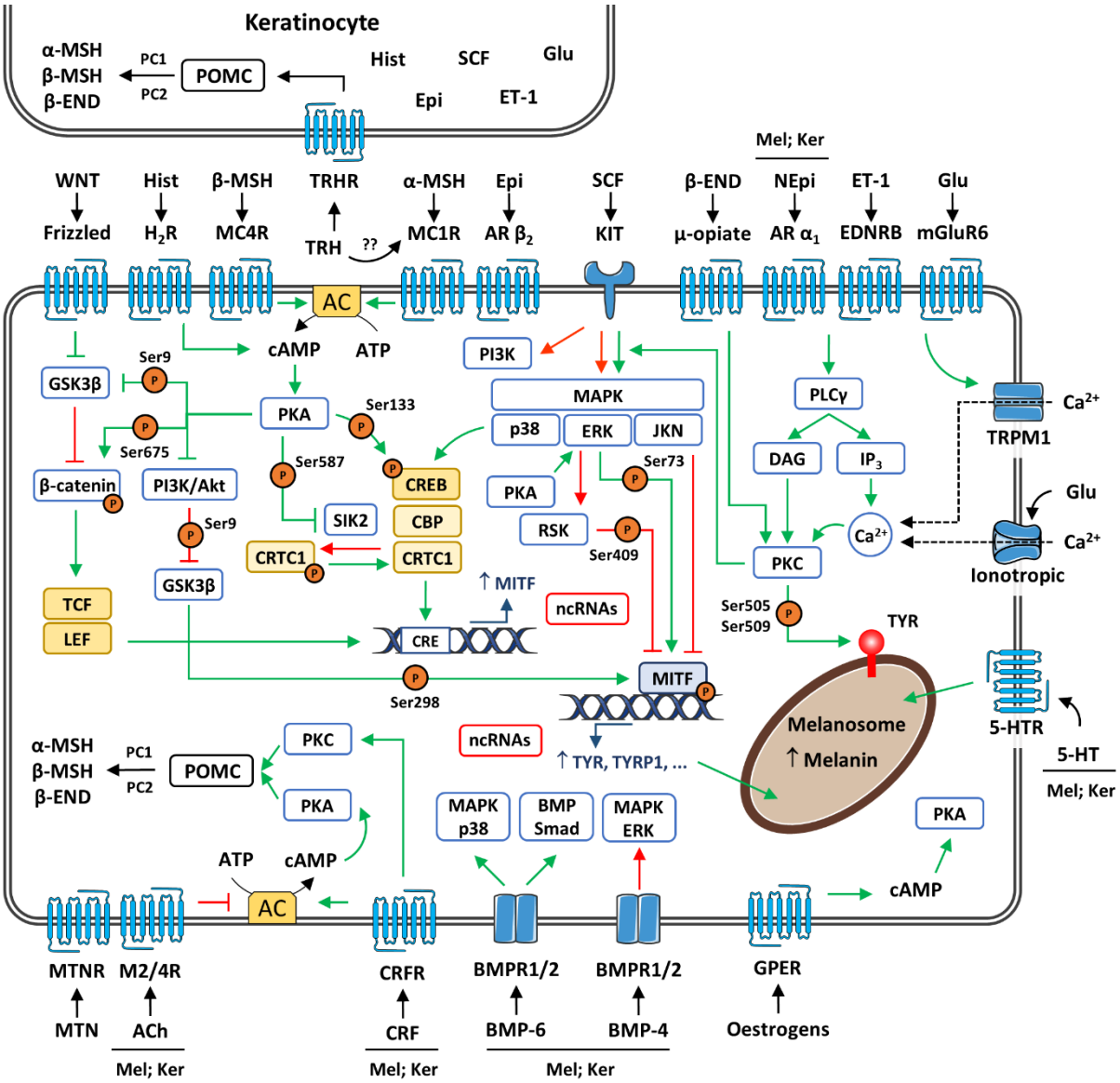


Figure I.8. Major molecular pathways involved in the regulation of melanin synthesis in melanocytes.

I.5.1. PRO-OPIOMELANOCORTIN (POMC)-DERIVED PEPTIDES

The hair follicles are local sources and targets for pro-opiomelanocortin (POMC)-derived peptides; although originally discovered in the anterior pituitary, these peptides are also expressed and secreted by melanocytes and keratinocytes [69,240–242]. POMC transcription and translation is hair cycle-dependent and increases significantly in the anagen phase. The proteolytic processing of POMC by PC1 produces the peptides adrenocorticotropin (ACTH) and β-Lipotropin (β-LPH), with the latter being further processed to β-endorphin (β-END) and β-melanocyte-stimulating hormone (β-MSH). PC2 cleaves the first 14 amino acids of the ACTH sequence to generate ACTH(1-14)OH, the precursor of α-MSH [243–245]. POMC-derived products are the main regulators of follicular melanogenesis, and even unprocessed POMC is reported to stimulate melanin production – Figure I.8A. Additionally, POMC-derived peptides also influence the hair growth [242,246–250].

ACTH, α-MSH and β-MSH share an essential core peptidic sequence of four amino acids (His-Phe-Arg-Trp) that allows the binding to melanocortin receptors. While the latter acts on melanocortin 4 receptor (MC4R), ACTH and α-MSH bind to MC1R, a G protein-coupled membrane receptor abundantly expressed in melanocytes of hair follicles. Nonetheless, activation of both receptors leads to the stimulation of adenylyl cyclase (AC), an enzyme that catalyses the conversion of ATP to cAMP (cyclic adenosine monophosphate) [69,240,251–254]. Then, cAMP binds to the regulatory subunit of PKA (protein kinase A), allowing the catalytic subunit to be liberated and activated; termination of this cellular signalling is provided by phosphodiesterases (PDEs), which hydrolyse free and PKA-bounded cAMP, leading to reassociation of the two subunits [255]. Activated PKA is translocated to the nucleus where it induces the phosphorylation of CREB at Ser133. Phosphorylated CREB activates the expression of specific genes containing the consensus CRE (cAMP responsive element) sequences in their promoters, as *MITF*; PKA also phosphorylates the nuclear transcriptional coactivator CBP that interacts with CREB family proteins for PKA-dependent gene expression. Consequently, the increased transcription of *MITF* upregulates the expression of several genes involved in synthesis of melanin, but also in the regulation of melanogenesis (as *MC1R*), melanocyte proliferation and dendrite formation [69,240,252,256,257]. Besides direct phosphorylation of CREB, PKA also increases the transcription of *MITF* by inhibiting (via phosphorylation at Ser587) salt-inducible kinase 2 (SIK2) activity. SIK2 promotes the phosphorylation of CREB-regulated transcription coactivator 1 (CRTC1), preventing its translocation from the cytoplasm to the nucleus, an essential step for CREB-mediated gene expression. CRTC1 and SIK-2 have been shown to be fundamental determinants of melanogenic program in mice [258,259].

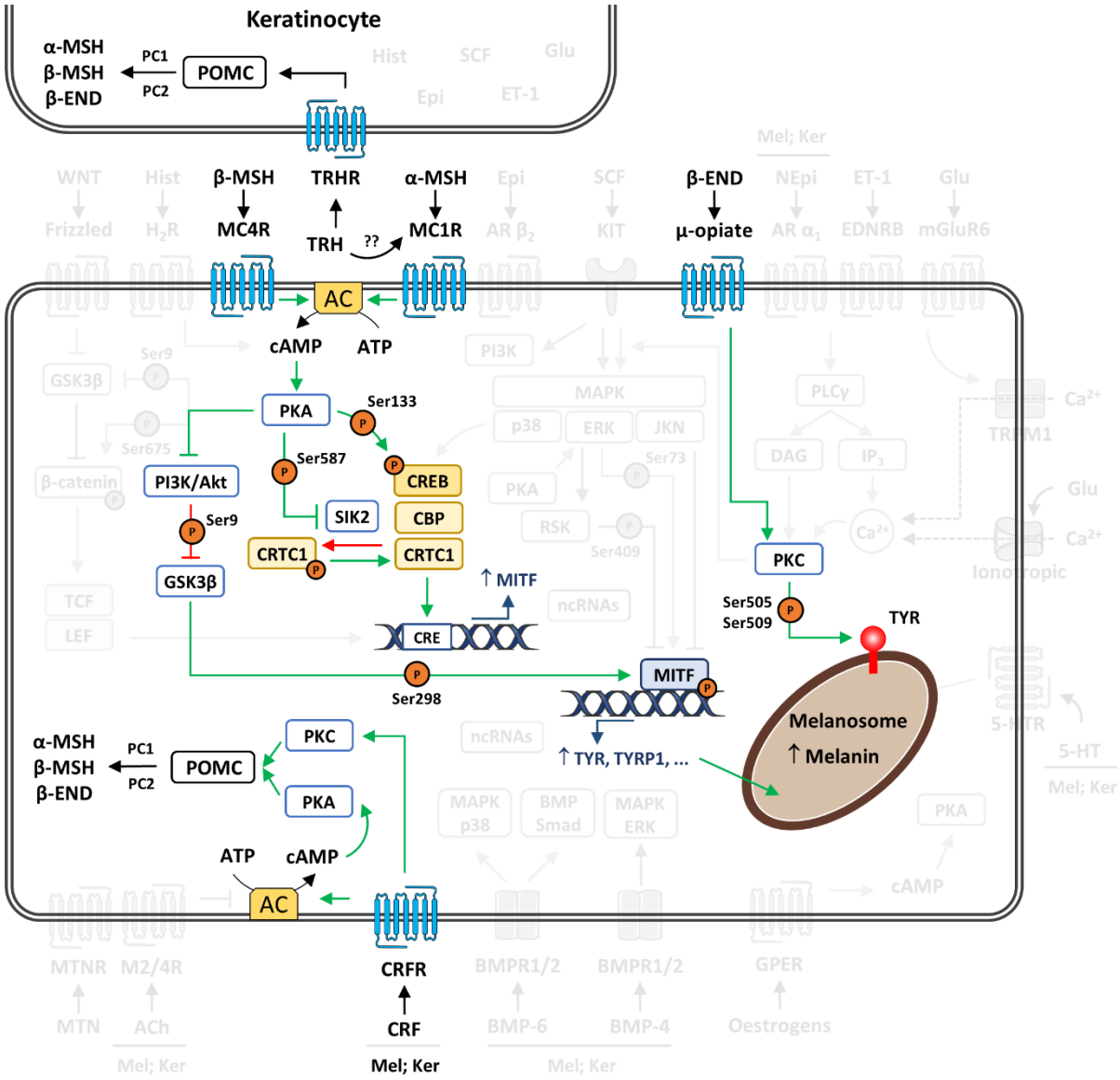


Figure I.8A. Highlight of molecular pathways involved in the regulation of melanin synthesis by pro-opiomelanocortin (POMC)- derived peptides. α/β-MSH: α/β-melanocyte-stimulating hormone; β-END: β-endorphin; AC: adenylyl cyclase; Akt: serine/threonine kinase; cAMP: cyclic adenosine monophosphate; CBP: CREB-binding protein; CREB: cAMP response element-binding protein; CRE: cAMP responsive element; CRF(R): corticotropin-releasing factor (receptor); CRTC1: CREB-regulated transcription coactivator 1; GSK3β: glycogen synthase kinase 3β; Ker: keratinocytes; MC1/4R: melanocortin 1/4 receptor; Mel: melanocytes; MITF: microphthalmia-associated transcription factor; PC1/2: proprotein convertase 1/2; PI3K: phosphatidylinositol 3-kinase; PKA/C: protein kinase A/C; SIK2: salt-inducible kinase 2; TRH(R): thyrotropin-releasing hormone (receptor); TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

The peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and PGC-1β are other transcriptional regulators that are modulated by the cAMP/PKA pathway. α-MSH signalling increase the expression of PGC-1α and stabilize both PGC-1α and PGC-1β proteins, probably through phosphorylation mediated by PKA, with consequent stimulation of MITF, tyrosinase expression and synthesis of melanin [260]. PGC-1α and PGC-1β coactivate many transcription factors, including the nuclear hormone peroxisome proliferator-activated receptor γ (PPARγ), that also has shown to be involved in the α-MSH-induced melanogenesis; other PPARs subtypes are reported to be expressed in melanocytes and PPARα, but not PPARβ/δ, is known to contribute to the control of melanogenesis [261–264].

PKA also inhibits the activity of phosphatidylinositol 3-kinase (PI3K) and one of its key effectors, the serine/threonine kinase Akt; the activation of Akt depends on the phosphorylation of its Thr308 and Ser473 residues upon binding to the PI3K phospholipid products. Inactive Akt is incapable of phosphorylate the glycogen synthase kinase 3β (GSK3β) at Ser9 which promotes its inactivation. Thus, GSK3β can phosphorylate MITF at Ser298, which facilitates its binding to the *TYR* promotor and stimulates melanogenesis. Curiously, PKA can promote the degradation of GSK3β (phosphorylation at Ser9) in a cross talk with the Wnt/β-catenin signalling pathway, addressed below (Section I.5.2 and Figure I.8B) [265–268]. The inhibition of PI3K/Akt pathway by PKA not only avoids the degradation of GSK3β, but also prevents the activation of serine/threonine kinase p70S6K1, and mammalian target of rapamycin (mTOR) along with its complex (mTORC1). Both factors have been implicated in the negative regulation of melanogenesis and the cAMP-induced melanogenesis has shown to be, at least in part, mediated by their inhibition [269–274].

The remaining POMC-derived peptide, β-END, has shown to positively regulate melanogenesis, proliferation and dendricity in melanocytes of the epidermis and hair follicles by binding to its μ-opiate receptor. Contrarily to other POMC-derived peptides, β-END is not expected to promote its melanogenic modulatory effect through the cAMP/PKA signalling pathway; in fact, signalling through μ-opiate down-regulates the level of cAMP. A PKC-dependent pathway (addressed bellow) have been suggested as a downstream mechanism for stimulation of melanogenesis by β-END [69,240,246,275].

I.5.1.1. CORTICOTROPIN-RELEASING FACTOR (CRF)

Corticotropin-releasing factor (CRF, also known as corticotropin-releasing hormone or CRH) is the most proximal element of the hypothalamic-pituitary-adrenal axis (HPA). The binding of CRF to its receptors mediates the POMC expression and subsequent production of the derived peptides. Both skin and hair

follicles contain an equivalent to the HPA axis, with keratinocytes and melanocytes expressing CRF and the corresponding G protein-coupled corticotropin-releasing factor receptors (CRFR); isoforms of the CRF1R subtype are expressed in the epidermis while isoforms of the CRF2R subtype are expressed in the hair follicle. CRF can induce the synthesis of POMC through signalling involving PKA and PKC – Figure I.8A [69,240,276,277].

I.5.1.2. THYROTROPIN-RELEASING HORMONE (TRH)

Thyrotropin-releasing hormone (TRH) is the most proximal regulatory element of the hypothalamic-pituitary-thyroid axis (HPT), controlling the production of thyroid hormone. TRH is recognized as a potent stimulator of POMC expression, with human skin and hair follicles being reported to transcribe it along with its receptor (TRHR). The expression of TRH stimulates growth, melanin synthesis and melanocyte dendricity in hair follicles; in the epidermis, TRH does not appear to affect melanogenesis. In hair follicles, the expression of TRHR is confined to the inner root sheath, being absent in the follicular pigmentary unit. This suggests that THR stimulate the production of POMC in keratinocytes, with the derived peptides posteriorly acting on melanocortin receptors of melanocytes – Figure I.8A. Alternatively, TRH have also been proposed to bind directly to MC1R, which could explain the reported cases of normal hair pigmentation in the absence of melanocortin synthesis [278,279]. Besides TRH, the related thyroid hormones have also shown to directly affect human hair pigmentation [280].

I.5.2. WNT PROTEINS

The Wingless-type MMTV integration site family (Wnt) consist of cysteine-rich lipoglycoproteins members that act on G protein-coupled frizzled (FZD) receptors. The binding to FDZ receptors activates an intracellular cascade of events involving (canonical form) or not (noncanonical form) the transcription regulator β-catenin [69].

The non-canonical pathways are diverse but poorly characterized; melanin suppression by WNT5 is known to be dependent on noncanonical Wnt/Ror2 pathway [281]. In the canonical pathway, the binding of WNTs to FZD receptors and low density lipoprotein-related protein (LRP) 5/6 coreceptor inhibits the constitutive degradation of β-catenin by GSK3β, promoting its accumulation; further stability of β-catenin is provided by PKA through phosphorylation of the Ser675 – Figure I.8B [268,282]. β-catenin is then translocated into the nucleus, where it interacts with T-cell factor (TCF)/LEF transcription factors to upregulate the transcription of *MITF*. β-catenin also interacts directly with the MITF (phosphorylating the

Ser298 residue) and increase binding of MITF to the M box of the *TYR* promotor [69,283,284].

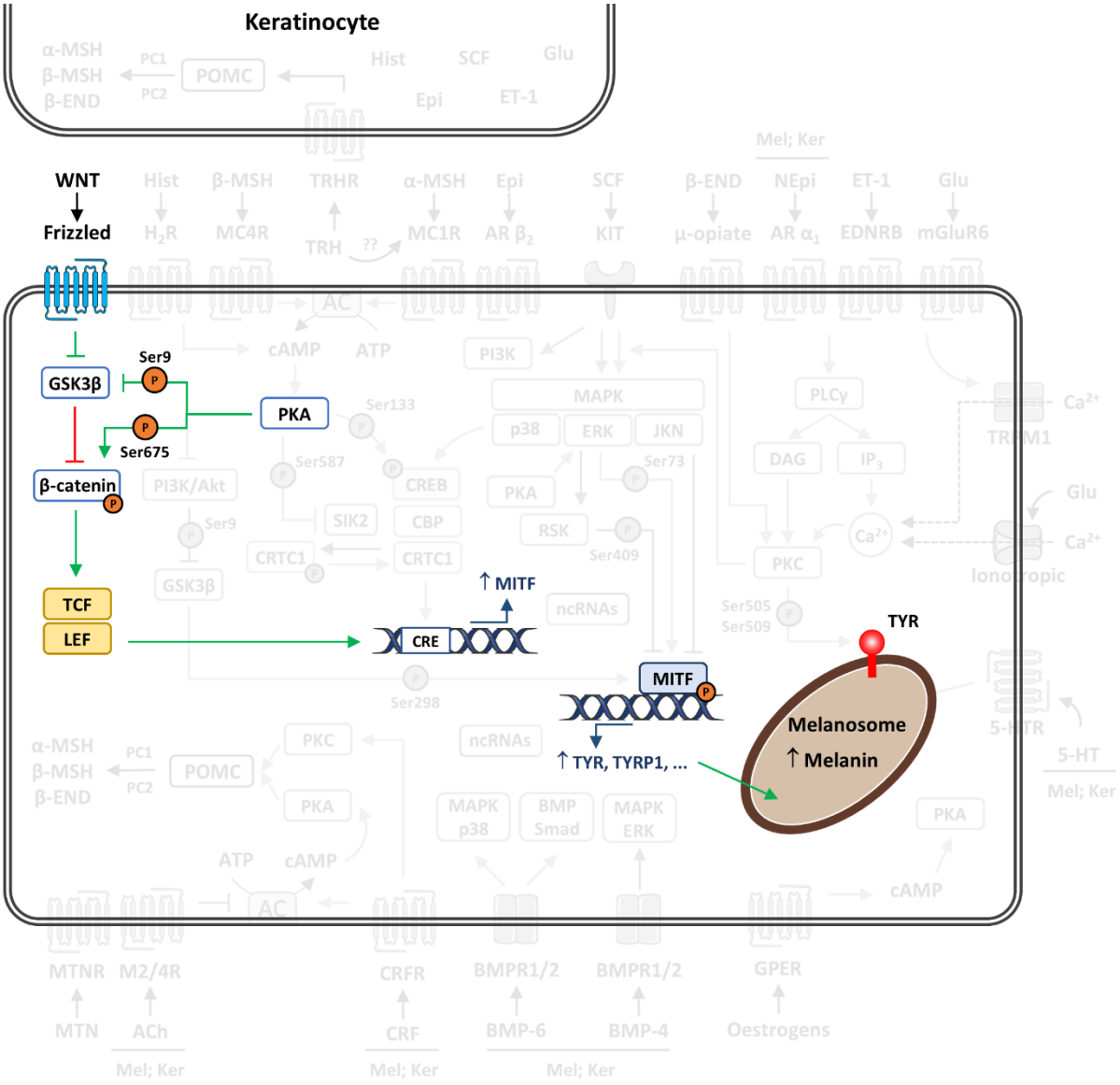


Figure I.8B. Highlight of molecular pathways involved in the regulation of melanin synthesis by WNT proteins. GSK3β: glycogen synthase kinase 3β; LEF: lymphoid enhancer-binding factor; MITF: microphthalmia-associated transcription factor; PKA: protein kinase A; TCF: T-cell factor; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

In the absence of WNTs, β-catenin is phosphorylated (Ser33, Ser37, Ser45 and Thr41) by a multiprotein complex containing axin, adenomatous polyposis coli (APC), casein kinase Iα (CKIα) and GSK3β; these phosphorylations lead to ubiquitination and proteasomal degradation [268,285,286]. The activation of

the Wnt/β-catenin signalling pathway by WNT1, WNT3a, WNT7A/B or WNT10B have been implicated in the upregulation of melanogenesis, melanocyte differentiation and proliferation and hair follicle regeneration [287–292]. Many other ligands have also shown to modulate the Wnt signalling pathway, and consequent production of melanin, by interacting with WNT proteins: Dickkopf 1 (DDK1) [293,294] WNT inhibitory factor 1 (WIF1) [295] and secreted frizzled-related protein 2 (SFRP2) [296].

I.5.3. STEM CELL FACTOR (SCF)

SCF is a growth factor secreted by keratinocytes and fibroblasts. SCF is the specific ligand of KIT, which is expressed in various types of cells including melanocytes. The binding of SCF to the extracellular cellular domain of KIT prompts its dimerization, activation of intrinsic tyrosine kinase activity and autophosphorylation. Then, KIT can phosphorylate various substrates that lead to the activation of the mitogen-activated protein kinases (MAPK) signalling pathways and modulation of melanocyte proliferation, differentiation and melanogenesis. Of note, SCF/KIT signalling can also activate PI3K, which effect on melanogenesis was discussed before (Section I.5.1 and Figure I.8A) [69,240,297–300].

There are three well-characterized subfamilies of the MAPK superfamily known to have crucial roles in melanin synthesis: the extracellular signal-regulated kinases (ERK, mainly 1/2), the c-Jun N-terminal kinases (JNKs, mainly 1/2, also known as stress-activated protein kinases or SAPKs) and the p38-MAPK – Figure I.8C [69,301,302]. Activation of ERK, which can additionally be achieved through cAMP/PKA or PKC-mediated signalling, is known to cause phosphorylation of MITF at Ser73, allowing the recruitment of the transcriptional coactivator CBP/P300 of CREB and consequent transcription of melanogenic enzymes. However, such phosphorylation, along with another one at Ser409 induced by RSK (p90 ribosomal S6 kinase, ERK downstream activation), also leads to MITF destabilization via increased ubiquitination, followed by proteasome-dependent degradation; in due course, the production of melanin is decreased [69,71,303–309]. Several lipid second messenger have shown to modulate the production of melanin, mainly through the ERK signalling pathway: sphingosylphosphorylcholine [272,310–313], ceramide [314–316], sphingosine-1-phosphate [317,318] and lysophosphatidic acid [319]. Activation of JNK has also been reported to inhibit melanogenesis via degradation of MITF. In addition, the antimelanogenic effect of JNK was linked to the blockage of nuclear translocation of CRTC3 (CREB-regulated transcription coactivator 3) and further impairment of the transcriptional activity of CREB [320]. Activation of p38-MAPK induces phosphorylation of CREB, via downstream MSK1 (mitogen- and stress-activated protein kinase 1), activating MITF that promotes the transcription of melanogenesis-related genes and the increase in melanin synthesis. However, there are also some evidence demonstrating the

involvement of p38-MAPK in the inhibition of melanin production by proteasome-dependent degradation of tyrosinase and other related proteins [300,321–323].

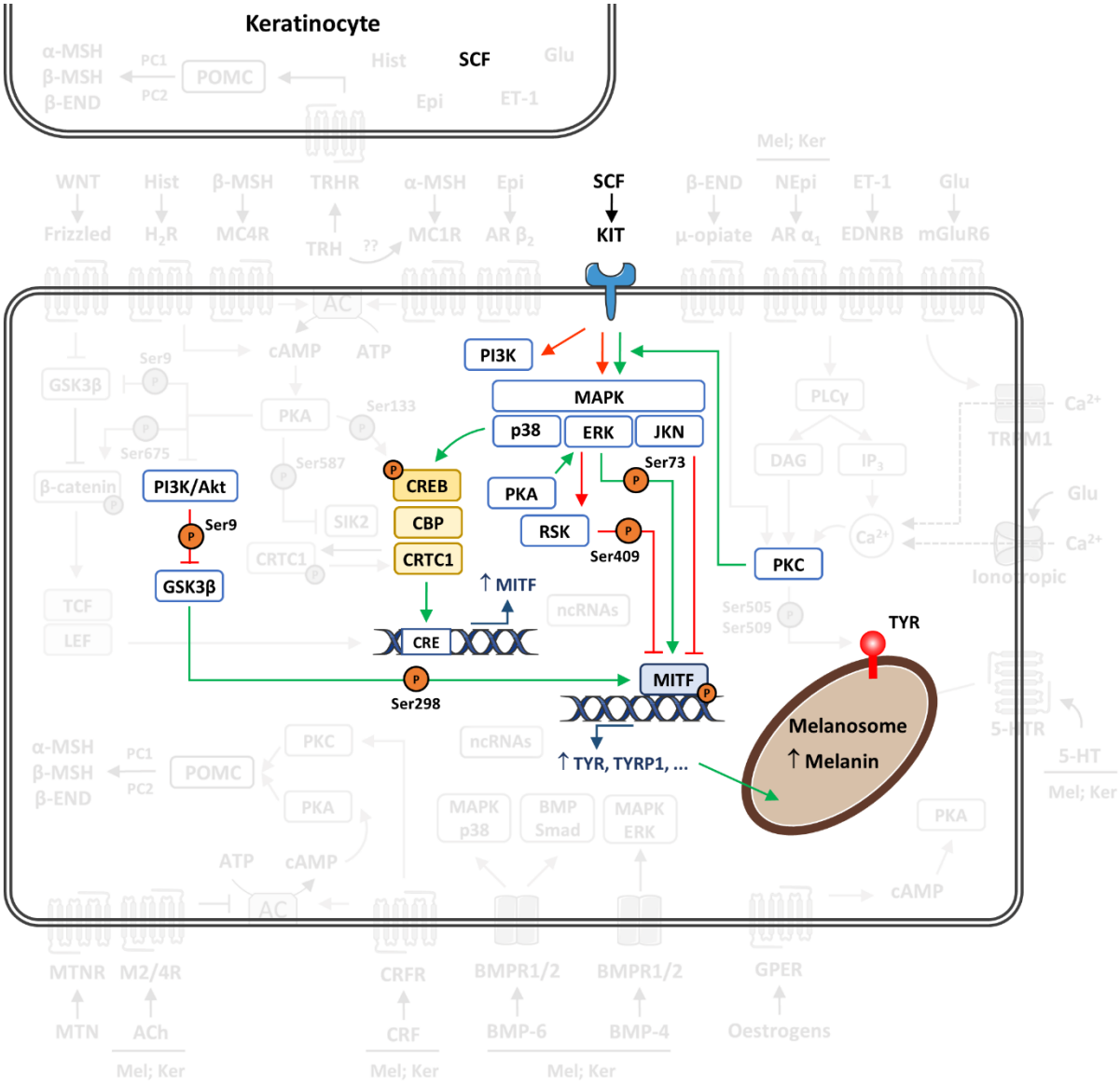


Figure I.8C. Highlight of molecular pathways involved in the regulation of melanin synthesis by stem cell factor (SCF). CREB: cAMP response element-binding protein; ERK: extracellular signal-regulated kinases; JNK: c-Jun N-terminal kinases; KIT: KIT proto-oncogene, receptor tyrosine kinase; PI3K: phosphatidylinositol 3-kinase; PKA/C: protein kinase A/C; MAPK: mitogen-activated protein kinases; MITF: microphthalmia-associated transcription factor; RSK: p90 ribosomal S6 kinase; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

I.5.4. ENDOTHELIN 1 (ET-1)

ETs are peptides present in a broad range of tissues, including the skin. The three known ETs isopeptides (ET-1,2 and 3) are obtained through cleavage of preproendothelin by prohormone convertases. ET-1 is synthesized and secreted by keratinocytes, acting on G protein-coupled receptor EDNRB of melanocytes with ensuing stimulation of proliferation, melanogenesis and dendricity – Figure I.8D [69,240,324–328].

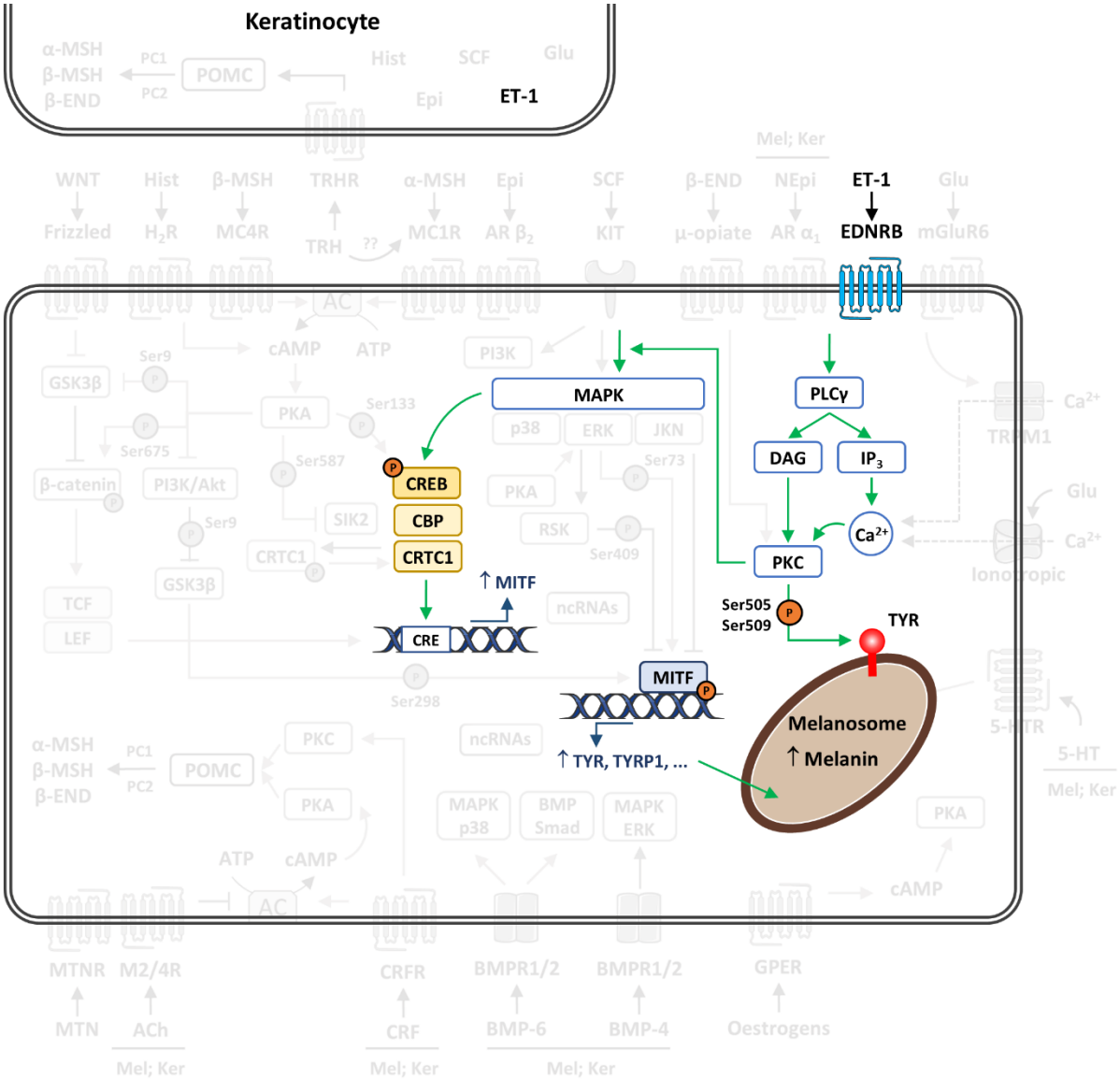


Figure I.8D. Highlight of molecular pathways involved in the regulation of melanin synthesis by endothelin 1 (ET-1). CREB: cAMP response element-binding protein; DAG: diacylglycerol; EDNRB: endothelin receptor type B; IP_3: inositol–triphosphate; MAPK: mitogen-activated protein kinases; MITF: microphthalmia-associated transcription factor; PKC: protein kinase C; PLCγ: phospholipase Cγ; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

Upon binding of ET-1 to EDNRB, phospholipase Cγ (PLCγ) is activated, increasing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), with further generation of inositol–triphosphate (IP_3) and diacylglycerol (DAG). IP_3 raises the intracellular concentration of Ca^{2+} and DAG induces the activation of Ca^{2+}/phospholipid-dependent PKC. Subsequently, PKC activates the MAPK signalling pathway that culminates in the phosphorylation of CREB and induction of *MITF* transcription [69,198,240,324,329]. PKC is also known to be translocated from the cytosol to the melanosomal membrane (upon interacting with RACK1, Receptor for Activated C-Kinase 1), promoting the phosphorylation of Ser505 and Ser509 on the cytoplasmic domain of tyrosinase; such phosphorylation increases the activity of the enzyme and induces the formation of a complex with TYRP1 that further enhances melanin synthesis [188–190,330].

I.5.5. NEUROTRANSMITTERS

Acetylcholine (ACh) is synthesized and degraded by keratinocytes and melanocytes; the degradation process is controlled through the action of acetylcholinesterases (AChE). ACh interacts with G protein-coupled muscarinic receptors, with all five subtypes (M1R-M5R) being detected in human melanocytes. The activation of M1R, M3R and M5R stimulates phosphoinositide metabolism whereas M2R and M4R hinder the activity of AC – Figure I.8E. Both events are known to inhibit the synthesis of melanogenesis, and the control of melanogenesis by ACh have been proposed by several researchers [240,331–334].

Glutamate is supplied to melanocytes by adjacent keratinocytes. Activation of G protein-coupled metabotropic glutamate receptor 6 (mGluR6) increases melanin production via the activation of TRPM1 calcium channel – Figure I.8E. Glutamatergic signalling through ionotropic glutamate receptors [glutamate receptor 2 (GluR2, AMPA receptor), glutamate receptor 4 (GluR4, AMPA receptor) and NMDA receptors 2A and 2C (NMDAR2A, NMDAR2C)] is implicated in the transcriptional regulation of *MITF* – Figure I.8E [69,196,335].

Histamine generation has been demonstrated in keratinocytes, being present in several skin compartments including hair follicles. Although melanocytes express several histamine receptors, the increased production of melanin induced by histamine melanogenic appears to be related to the stimulation of G protein-coupled H_2 receptor, which cause cAMP accumulation and subsequent PKA activation – Figure I.8E [240,336–338]. Despite that, a depigmenting effect of histamine have also been demonstrated [339,340].

Norepinephrine and epinephrine act on G protein-coupled α_1 and $\beta_2$adrenergic receptors of melanocytes. Autocrine and paracrine (keratinocytes) norepinephrine is the preferred ligand for the α_1 adrenoreceptor,

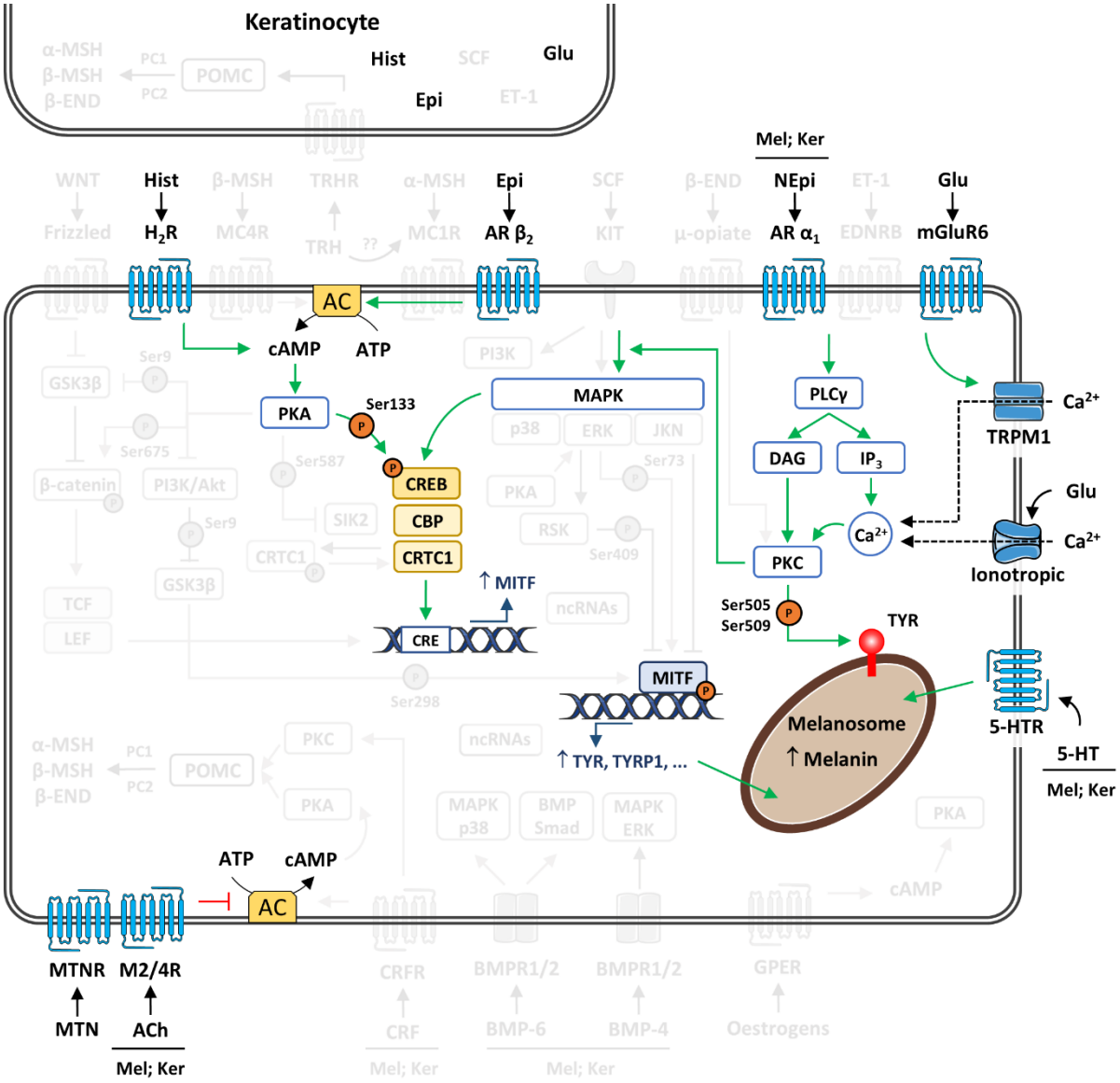


Figure I.8E. Highlight of molecular pathways involved in the regulation of melanin synthesis by neurotransmitters. 5-HT(R): serotonin (receptor); AC: adenylyl cyclase; Ach: acetylcholine; AR: adrenergic receptor; cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; DAG: diacylglycerol; (N)Epi: (nor)epinephrine; H_2R: histamine H_2 receptor; Hist: histamine; IP_3: inositol–triphosphate; Ker: keratinocytes; PKA/C: protein kinase A/C; PLCγ: phospholipase Cγ; MAPK: mitogen-activated protein kinases; Mel: melanocytes; M2/4R: muscarinic 2/4 receptor. m(Glu)R6: metabotropic (glutamate) receptor 6; MITF: microphthalmia-associated transcription factor; MTN(R): melatonin (receptor); TRPM1: transient receptor potential cation channel, subfamily M, member 1; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

stimulating melanogenesis in a PKC-dependent pathway – Figure I.8E. Epinephrine (produced by keratinocytes) preferentially target the β_2 adrenergic receptors, stimulating melanin through AC and downstream cAMP/PKA signalling pathway – Figure I.8E [67,69,78,240,341].

Serotonin (5-hydroxytryptamine, 5-HT) is produced and metabolized by melanocytes and keratinocytes. The actions of 5-HT are mediated by its interaction with G protein-coupled receptors (5-HTR), that are widely detected in melanocytes. 5-HT was shown to positively modulate *in vitro* (cell culture and hair follicles) and *in vivo* (mice) production of melanin through interaction with 5-HT_{1A}R, 5-HT_{1B}R and 5-HT_{2A}R – Figure I.8E [240,342,343]. However, recently, it was found that interaction between neurokinin 1 receptor (NK1R) and 5-HT_{1A}R prevents the synthesis of melanin induced by the activity of the latter [344]. NK1R is activated by Substance P (SP), an undecapeptide belonging to the tachykinin family, causing a decrease in melanin production by inhibiting the expression of MITF and p-p38-MAPK, and increasing p-p70S6K1 [344–346]. Moreover, SP can also inhibit the expression of 5-HT_{2A}R and neutralize the pro-melanogenic effect of 5-HT [347]. Paradoxically, NK1R and SP have been reported to positively regulate melanogenesis through Wnt/β-catenin signalling pathway and increased secretion of ET-1 [293,348]. Melatonin (MTN), which it is obtained from 5-HT, is also reported to decrease the production of melanin. The effects of MTN are mediated by binding to nuclear or membrane-bound G protein-coupled MTN receptors (MTNR1A and MTNR1B, both found on melanocytes). The activation of membrane bound melatonin receptors is known to inhibit AC – Figure I.8E [240,349,350].

I.5.6. BONE MORPHOGENETIC PROTEINS

BMPs are the largest family of secreted signalling molecules of the transforming growth factor (TGF)-β superfamily. They are powerful regulators of skin development, controlling epidermal homeostasis, hair follicle growth and skin/hair pigmentation. BMP signalling is influenced by the BMP type, presence of antagonists, development stage of the target tissue, and target cell receptors. Keratinocytes and melanocytes express BMP4 and BMP6, their receptors (serine/threonine kinase receptors type I and II, BMPR1 and BMPR2) and the respective antagonists, sclerostin and noggin, all together affecting the production of melanin – Figure I.8F [240,351–354]. BMP6 has been shown to stimulate melanogenesis by upregulating tyrosinase expression and activity; additionally, it also stimulates the formation of filopodia and MYO10 expression, associated with increased melanosome transfer from melanocytes to keratinocytes. The effects of BMP6 are achieved through activation of the canonical BMP/Smad pathway, and non-canonical p38-MAPK and PI3K pathways; the canonical BMP/Smad pathway involves

phosphorylation of cytosolic transcription regulators (Smad proteins) which are translocated to the nucleus where they can alter gene expression [240,351]. On the contrary, BMP4 downregulates melanogenesis by targeting MITF to proteasome-mediated degradation, via the MAPK/ERK pathway [69,351,353–355]. TGF-β1, another member of the TGF-β superfamily, also provides negative regulation of melanogenesis via the MAPK/ERK pathway [356–360].

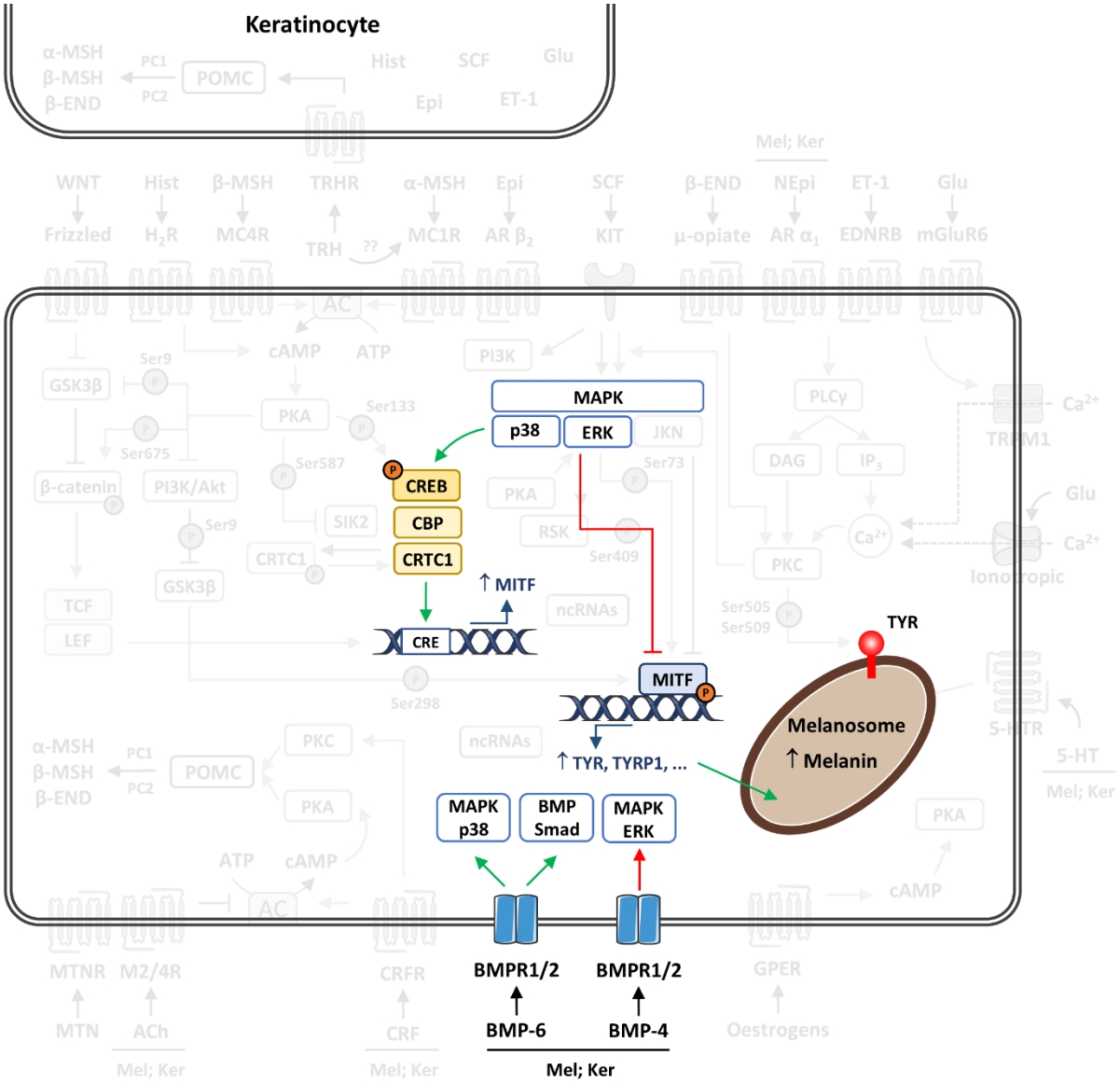


Figure I.8F. Highlight of molecular pathways involved in the regulation of melanin synthesis by bone morphogenetic proteins (BMP). BMPR1/2: bone morphogenetic proteins receptors 1/2; CREB: cAMP response element-binding protein; ERK: extracellular signal-regulated kinases; Ker: keratinocytes; MAPK: mitogen-activated protein kinases; Mel: melanocytes; MITF: microphthalmia-associated transcription factor; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

I.5.7. OESTROGENS

Oestrogens have significant effects on many aspects of skin physiology including hair growth and pigmentation. The binding of oestrogens to their receptors upregulates MITF, TYR and consequently, melanin contents, with the involvement of the PKA pathway – Figure I.8G [240,329,361,362].

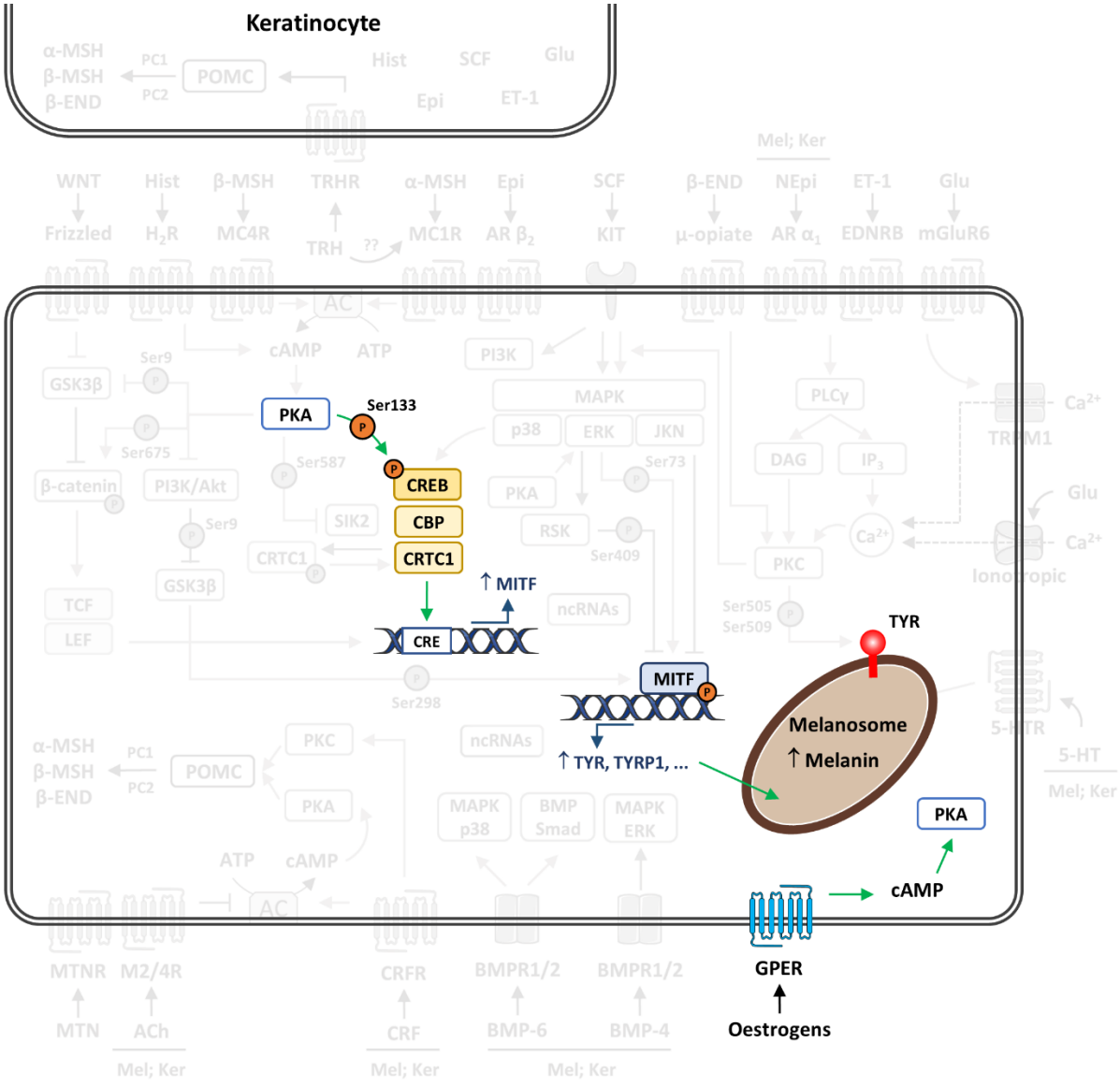


Figure I.8G. Highlight of molecular pathways involved in the regulation of melanin synthesis by oestrogens. cAMP: cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; GPER: G protein-coupled oestrogen receptor; MITF: microphthalmia-associated transcription factor; PKA: protein kinase A; TYR: tyrosinase; TYRP1: tyrosinase related protein 1.

Classically, the oestrogen action was viewed as mediated by two nuclear oestrogen receptors: ERα and ERβ; keratinocytes and melanocytes express both receptors, with the latter being preferentially expressed in the hair follicles [240,329]. More recently, a G protein-coupled oestrogen receptor (GPER) have also been reported. The activation of this receptor also enhances melanogenesis via the cAMP/PKA signalling pathway – Figure I.8G [363,364].

I.5.8. CELL ADHESION MOLECULES

P-cadherin, which regulates cell-cell recognition and signalling, is involved in hair follicle morphogenesis, hair fibre production and follicular regression; additionally, it was found to be a regulator of pigmentation. The silencing of P-cadherin in organ cultures of melanogenically active human scalp hair follicles significantly reduced melanogenesis, possibly via GSK3β-mediated Wnt signalling. Interestingly, the pigmentary role of P-cadherin seems to be exclusive of the hair follicles as epidermal pigmentation was unaffected by its knockdown in organ-cultured skin [365]. The cadherin desmoglein 1 (DSG1), that regulates keratinocyte-specific cell-cell adhesion, was also found to regulate keratinocyte-melanocyte paracrine crosstalk and inhibit the production of melanin [366]. Neuregulins are a group of peptide growth factors that mediate cell-cell interactions in several organ systems through tyrosine kinase receptors of the ErbB family. Neuregulin 1 (NRG1), secreted by fibroblasts, was described to increase the pigmentation of melanocytes in tissue culture and in an artificial skin model. [367]. Laminin-332, derived from keratinocytes, not only plays a critical role in adhesion-related cell functions in melanocytes but it also regulates melanogenesis by controlling the uptake of tyrosine [368].

I.5.9. NON-CODING RNAS

Non-coding RNAs (ncRNAs), particularly microRNA (miRNAs) and the long non-coding RNAs (lncRNAs), are emerging as new regulators of the pigmentation process. miRNAs are about 20-25 nucleotides in length that, by targeting specific mRNAs, cause their degradation or inhibit their translation into proteins [69]. Several miRNAs have showed to interfere with pigmentation by targeting MITF: miR-25, miR-137, miR-141-3p, miR-197, miR-200a-3p miR-218, miR328, miR-574-5p, miR-634, miR-675, miR-720, miR-766, miR-1225-3p, miR-1308 and miR-3196 [369–376]. Many miRNAs target other genes than MITF: miR-21a-5p (SOX5), miR-27a-3p (WNT3A), miR-125b (SH3BP4, SRC homology 3 domain-binding protein 4), miR-137 (KIT), miRNA-143-5p (M5A), miR-145 (SOX9, TYR, TRP1, MYO5A, RAB27A, FSCN1, but also MITF), miR203 (KIF5b, and possibly CREB1), miR-211 (TGFBR2, TGFβ receptor 2) and miR-434 (TYR)

[373,377,386,387,378–385]. Many regulators of pigmentation, with unknown targets, have also been disclosed: miR-9, miR-21, miR-28, miR-130b, miR-139-5p, miR-155, miR-182, miR-193, miR-206, miR-221, miR-222, miR330-5p, miR-335, miR-365 and miR-455 [384,388–390]. Of note, miR-230, miR330-5p, miR-675 miR-3196 are not produced by melanocytes, being delivered from keratinocytes via exosomes [373,374,389]. LncRNAs are greater than 200bp in length and regulate diverse biological functions and also some microRNAs [69]. Some lncRNAs (CD1C-2:1, H19, TCONS_00049140, UCA1) have already been shown to have an important role in melanogenesis, although their role in not yet fully described [391–393].

I.5.10. OTHER REGULATORS

Zinc α-2-glycoprotein is produced by keratinocytes and it is proposed that it may play a part in the negative regulation of melanin production [394]. An undisclosed member of the heat shock proteins (HSP)70 family have been shown to suppress melanin production *in vitro* and *in vivo* through direct interaction with MITF; HSP are constitutively expressed in the skin and confer protection against stressors [395]. Conversely, HSP70-1A expression in dark skin melanocytes was found to be higher compared to those of light skin phenotypes, contributing to skin colour diversity [396]. Ligands of the purinergic receptor type 2 X7 (P2X7) [397], odorant receptor 51E2 [398], olfactory receptor OR2A4/7 [399], aryl hydrocarbon receptor (AHR) [400] and Type I cannabinoid receptor (CB1) [401] are also expect to contribute to the regulation of melanogenesis; the expression of these receptors by human melanocytes have been proved, and their stimulation increased the production of melanin.

I.6. DIVERSITY OF HUMAN HAIR NATURAL COLOUR

The diversity in human hair colour arises mostly from the quantity and ratio of eumelanin and pheomelanin produced. Mostly eumelanin is the default hair pigmentation, being predominant in more than 90% of the human population. Eumelanic phenotypes range from black, dark brown, medium brown, light brown to blond. All these phenotypes contain small, nearly constant, amounts of pheomelanin (0.85-0.99 μg melanin/mg of hair), with the varying contents of eumelanin being accountable for the visual differences in hair colour. Dark brown hair reportedly contains eumelanin at 66% (14.6 μg/mg) the level of black hair (22.2 μg/mg), medium brown at 47% (10.4 μg/mg), light brown at 40% (8.7 μg/mg) and blond hair at 23% (4.7 μg/mg). Red hair is the only phenotype that contains comparable amounts of

eumelanin and pheomelanin: 3.8 and 4.7 μg/mg, respectively [2,3,402]. The activity of TYR (which, as discussed before, is greatly affected by melanosomal pH) and cysteine content of melanosomes have been proposed to play critical roles in determining the amount and ratio of the two human hair pigments. Those are influenced by several polymorphisms in a wide range of pigmentary genes.

The red hair colour phenotype, characterized by abnormal high pheomelanin contents, is caused mostly by polymorphisms in *MC1R*, the master regulator of pigment-type switching. The most penetrant *MC1R* polymorphisms are R142H, R151C, R160W and D294H [2,403–408]. Such polymorphisms cause low levels of MC1R signalling, leading to downregulation of various pigmentary genes: *TYR*, *TYRP1*, *OCA2*, *SLC24A5* and *SLC45A2*; *CTNS* is also expected to be downregulated, leaving high levels of cystine (and consequently, cysteine) inside melanosomes. Consequently, the melanosomes are cysteine-rich, acidic and with low tyrosinase activity, leading to the production of low to medium levels of pheomelanin and eumelanin [2,3,409,410]. Polymorphisms in *ASIP* also show strong association with red hair [2,3,403,404,411]. *ASIP* encodes for the agouti signalling protein, an antagonist of MC1R, that competes with α-MSH in binding to the receptor [252,256,257,412–415]. By impacting on the acidification of melanosomes, polymorphisms in *SLC45A2*, *SLC24A5* and *OCA2* may additionally contribute to the red hair phenotype [2,3]. Furthermore, polymorphism in cystine/glutamate exchanger *SLC7A11* have also been proposed to be important for pheomelanin production [403].

The blond phenotype is characterized by high levels of MC1R signalling, reduced activities of ion transporters, and full activity of CTNS. These suppressed, but pro-eumelanogenic conditions make melanosomes acidic and cysteine deficient, leading to the production of trace amounts of pheomelanin and low levels of eumelanin [3]. No single gene has been reported as the main responsible for this phenotype, with polymorphisms in a number of genes as *ASIP*, *KITLG* (encoding for SCF), *MC1R*, *TPCN2* (encoding for TPC2) and *TYRP1* being associated with blond hair and found to differentiate it from brown hair [2,403,423,424,411,416–422].

The dark hair phenotypes (brown to black) are characterized by high levels of MC1R signalling and full activities of ion transporters and CTNS. Under these conditions, melanosomes become neutral and cysteine deficient, leading to the production of trace amounts of pheomelanin and high levels of eumelanin [3]. Several studies have confirmed an association between dark hair and polymorphisms in genes such as *SLC24A5*, *SLC45A2*, *HERC2* (HECT and RLD domain containing E3 ubiquitin protein ligase 2), and *IRF4* (interferon regulatory factor 4) [2,403,423,425,426].

I.7. AGE-INDUCED HAIR GREYING

The greying of hair (canities) is one of the most obvious and common signs of aging, occurring to a varying degree in all individuals, regardless of gender or race. A rule of thumb for hair greying is that by 50 years of age, 50% of people have 50% grey hair. The term grey hair widely refers to an admixture of pigmented and non-pigmented (white) hairs, although sometimes a single hair fibre can show a progressive dilution from black, through grey to white over several hair cycles or within the anagen phase of a single cycle. Hair greying usually appears first at the temples, spreading to the vertex and then to the remainder of the scalp, affecting the occiput last. At its simplest, the pigment loss in greying hair follicles is due to a marked reduction in melanogenically active melanocytes in the hair bulb. Although it has not yet been completely understood why melanocytes are lost with aging, some mechanisms have been proposed. – Figure I.9 [1,5,6,8,36,427,428].

The traditional view proposes that depletion of hair follicle bulbar melanocytes correlates with oxidative stress. The melanogenic activity of bulbar melanocytes is likely to generate large amounts of reactive oxygen species (ROS), via the oxidation of tyrosine and DOPA to melanin [429]. Although melanocytes possess an efficient antioxidant system, it appears to become impaired with age causing the accumulation of ROS that may generate significant oxidative stress in both melanocytes and anagen hair bulb epithelium. In this context, melanogenic bulbar melanocytes are best suited to assume a postmitotic, terminally differentiated (pre)senescence status to prevent cell malignant transformation [1,36,427,430–433]. The involvement of ROS in the onset of canities have been supported by several observations. Some melanosomes from grey hair bulbs were identified within auto-phagolysosomes, suggesting that they are defective and perhaps leaking reactive metabolites. Melanosomes in greying and white hair bulbs are highly vacuolated, a common cellular response to increased oxidative stress. Moreover, the common mitochondrial DNA deletion, also a marker of oxidative stress, occurs more prominently in grey hair follicles compared to pigmented ones [1,5,36,433].

A depletion of the hair follicle MelSC reservoir has additionally been associated with the onset of greying. In average, a scalp hair follicle experience 7-15 seedings of melanocytes over the grey-free lifespan. It is possible that canities may reflect an exhaustion of MelSC reservoir seeding potential; in fact, there is some experimental evidence suggesting that this potential is limited [1,36,434]. A defective, age-related, maintenance of MelSC, probably due to an imbalance between the antiapoptotic protein BCL2, MITF (its transcriptional regulator) and other factors, may also be accountable for the depletion of the MelSC reservoir [1,49,427,433,435–437]. Although many studies have shown the absence of MelSC from white

hair follicles, there are reports of hair greying reversibility. In this context, the greying seems to occur due to some defect in the MelSC activation or migration to the hair bulb at the beginning of a new hair cycle [5,36,427].

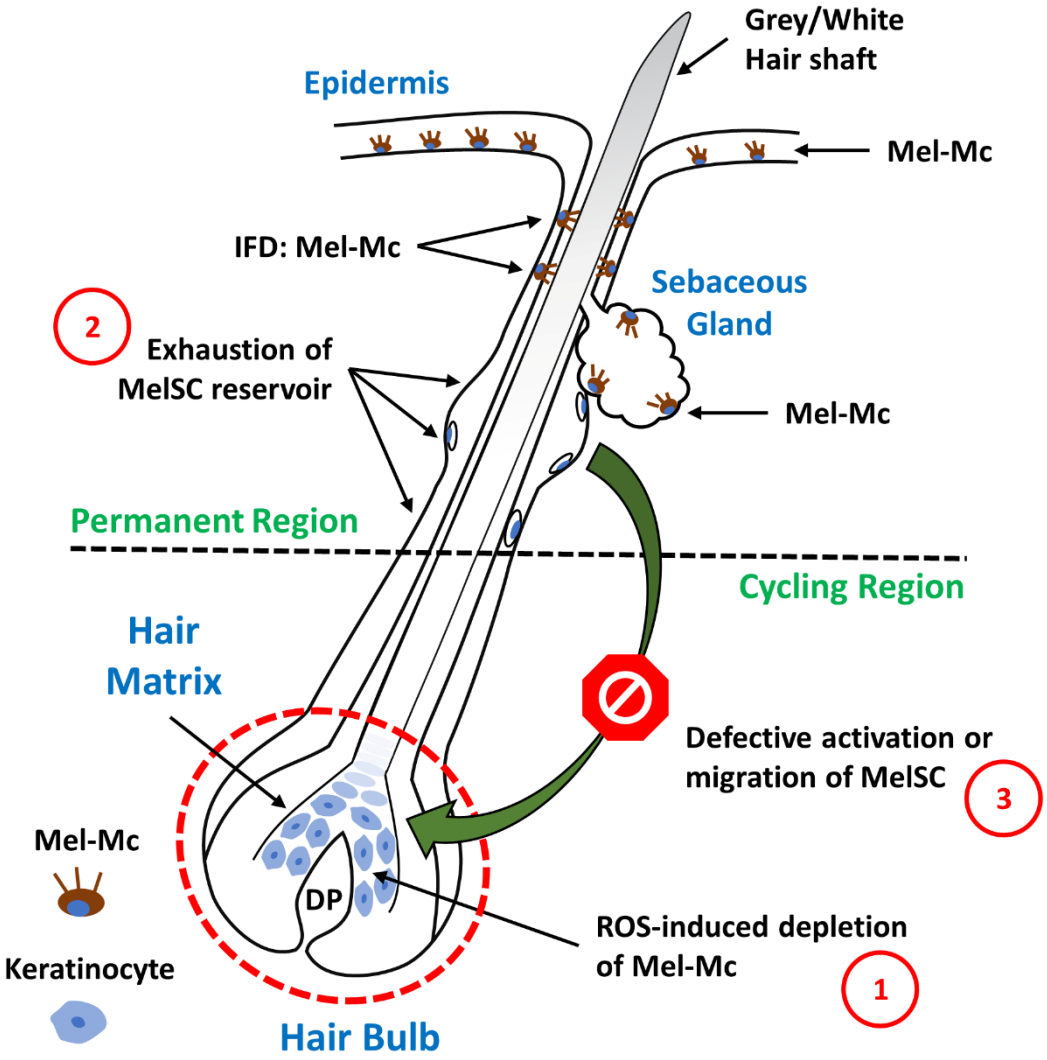


Figure I.9. Aged hair follicle, presenting grey/white hair .The age-related pigment loss in hair fibres may be caused may be caused by 1) depletion of melanotic melanocytes (Mel-Mc) in the hair matrix, 2) exhaustion of the melanocyte stem cell (MelSC) reservoir and 3) MelSC defective activation or migration during the onset of anagen phase of the hair growth cycle.

I.8. MODIFICATION OF THE HAIR FIBRE COLOUR

Throughout human history, people have changed the hair colour to segregate the social status. Nowadays,

regardless of economical and education background, millions of individuals worldwide commonly dye the hair to enhance youth and beauty and to follow fashion trends. Because hair colouration became popular among the general population, hair colouring products now represent one of the most rapidly growing beauty and personal care markets [438,439]. Regarding the year 2019, the global hair colour market was valued at approximately 22.2 billion USD, and it is expected to generate revenue of around 37.4 billion USD by 2026 (Zion Market Research, NY, USA).

Hair dyeing systems can be divided into oxidative and non-oxidative. Additionally, according to the colour durability, they are classified into temporary, semi-permanent, and permanent. Temporary non-oxidative dyes only deposit on the hair surface, leaving the fibre after the first washing. The semi-permanent non-oxidative dyes also interacts predominantly with the cuticle (the most external part of the hair strand), with a small penetration of dyes into the hair cortex occurring; this kind of colouration resists a few washes. Temporary and semi-permanent products do not require chemical reactions to impart colour as they rely on van der Waals forces for adhesion of direct dyes to the hair fibre.

Regarding permanent oxidative products, hair colouration happens upon reaction between colourless precursors (developer and coupler) in the presence of an oxidizing agent (H_2O_2), and under alkaline conditions (ammonia). The combination of oxidizing and alkaline agents causes swelling of the hair cuticle, facilitating the diffusion of the small precursors into the fibre and the bleaching of the natural melanin pigments. Then, the colourless precursors undergo oxidation to form large, coloured molecules that become trapped inside the fibre cortex. The permanent oxidative hair colour products provide the greater efficacy of dyeing and are the most used, representing about 80% of the hair colouring market [438–440].

Due to the destructive nature of the permanent dye products, the hair fibre structure suffers cumulative damages that lead to split ends, dry and dull hair [441]. More important, those products have been identified as the source of various adverse health effects as chemical and allergic reactions that many times result in acute or mild dermatitis with consequent hair loss [439,442–445]. Even more alarming, it is the fact that some studies raised the possibility that long-term usage of permanent dyes can ensue serious and systemic side-effects as the increased risk of developing certain cancers [439,446,447]. Despite the inconvenience and liability, in the absence of other ways, many people continue to dye the hair for cosmetic purposes. Thus, development of safer ways for hair colour modification is more than ever a pertinent issue.

Changes in hair colour have been reported as a side-effect of many drugs used in the treatment of several

diseases – Table I.2. Those changes are either as hair lightening or darkening, and even repigmentation of grey/white hair have been reported. Since such alterations are mostly transient, with the hair returning to its original colour after drug withdrawal, these findings raise the attractive possibility of using known, safe, and already approved drugs in hair colour modification as an alternative or in addition to the conventional methods. In fact, as showed here, the process of hair pigmentation offers countless druggable targets for modification of the colour phenotype. However, the cosmetic feasibility of a drug-based approach for modification of the hair colour has yet to be proved.

Table I.2. Drugs reported to change the colour of hair as a side effect of medical treatments

Drug	Reason for use	Hair change	Ref.
α-Interferon	Melanoma	Lightening	[448]
Acitretin	Psoriasis	Repigmentation	[449,450]
Atezolizumab	Lung cancer	Repigmentation	[451]
Chloroquine	Malaria prophylaxis	Lightening	[452]
	Dermatomyositis		[453]
Cisplatin	Metastatic germ cell neoplasm of the testis	Darkening	[454]
		Lightening	
Cyclosporin	Psoriasis	Darkening	[455]
Dabrafenib	Metastatic melanoma	Repigmentation	[456]
Dasatinib	Chronic myeloid leukaemia	Lightening	[457]
Defibrotide	Deep venous thrombosis	Darkening	[458]
Erlotinib	Lung adenocarcinoma	Repigmentation	[459]
Etretinate	Psoriasis	Lightening	[460]
		Repigmentation	[461]
	Pityriasis rubra pilaris		[462]
Hydroxychloroquine	Discoid lupus erythematosus	Lightening	[463]
Imatinib mesylate	Gastrointestinal stromal tumour	Lightening	[464]
	Chronic myeloid leukaemia		[465]
L-Thyroxine	Hypothyroidism	Repigmentation	[466]

Table I.2. (Continued)

Drug	Reason for use	Hair change	Ref.
Latanoprost	Open-angle glaucoma	Repigmentation	[467]
Lenalidomide	Multiple myeloma	Repigmentation	[468]
Levodopa	Parkinson's disease	Repigmentation	[469]
Nivolumab	Lung cancer	Repigmentation	[451]
***para*-aminobenzoic acid**	Lymphoblastoma cutis	Darkening	[470]
	Dermatomyositis		
	Dermatitis herpetiformis		
	Scaly erythroderma		
	Scleroderma		
Pazopanib	Hürthle cell carcinoma	Lightening	[471]
	Myofibroblastic sarcoma		[472]
Pembrolizumab	Lung cancer	Repigmentation	[451]
Prednisone	Bullous pemphigoid	Repigmentation	[473]
Sunitinib malate	Gastrointestinal stromal tumour	Lightening	[474]
Tamoxifen	Breast cancer	Repigmentation	[475]
Thalidomide	Multiple myeloma	Repigmentation	[476]
Triptorelin	Precocious puberty	Greying	[477]
Valproic acid	Seizures	Lightening	[478]
Vemurafenib	Metastatic melanoma	Repigmentation	[456]
Verapamil	Hypertension	Repigmentation	[479]

I.9. CONCLUDING REMARKS

Being one of the most distinguishing human features, hair colour has been the subject of extensive studies over the last decades. Here, an overview of the major findings is provided. The identification and comprehension of the events underlying the pigmentation of hair is an important starting point for the development of innovative hair cosmetics to change colour from inside out.

CHAPTER II

FLUORESCENT QUANTIFICATION OF MELANIN

Chapter II*

Fluorescent quantification of melanin

ABSTRACT

Melanin quantification is reportedly performed by absorption spectroscopy, commonly at 405 nm. Here, we propose the implementation of fluorescence spectroscopy for melanin assessment. In a typical *in vitro* assay to assess melanin production in response to an external stimulus, absorption spectroscopy clearly overvalues melanin content. This method is also incapable of distinguish non-melanotic/amelanotic control cells from those that are actually capable of performing melanogenesis. Therefore, fluorescence spectroscopy is the best method for melanin quantification as it proved to be highly specific and accurate, detecting even small variations in the synthesis of melanin. This method can also be applied to the quantification of melanin in more complex biological matrices like zebrafish embryos and human hair.

*** This chapter is based on the following scientific paper:**

Bruno Fernandes, Teresa Matamá, Diana Guimarães, Andreia Gomes and Artur Cavaco-Paulo. Fluorescent quantification of melanin. *Pigment Cell Melanoma Res.* 2016, 29(6): 707-712.

II.1 INTRODUCTION

The *in vitro* measurement of melanin is the basis of many studies involving differentiation of pigmented malignant melanoma cells, the protective role of compounds against UV light and the development of treatments for pigmentation disorders such as vitiligo [480]. Also, in the cosmetic field, the development of new skin whitening agents created the necessity to accurately measure melanin in order to evaluate its significance [481].

There are several methods reported in the literature to assess the amount of melanin in biological samples. Electron spin resonance spectrometry (ESR) allows the measurement of electron spin resonance signals based on free radicals derived from melanin; this technique is highly specific, but it lacks sensitivity. Regarding photoacoustic spectroscopy, it has the advantage of being non-destructive and efficiently applicable to solid, gel and solution samples [481]. High performance liquid chromatography (HPLC) is also used in the quantification of melanin, particularly when it is necessary to distinguish between eumelanin and pheomelanin. This method is highly sensitive and accurate, allowing the quantification of different types of melanin present in samples, through the detection of their specific degradation products [482]. As these techniques are not easily performed in most laboratories (they require very specific equipment and expertise), absorption spectroscopy is the most extensively used method to quantify melanin [481]. Following solubilization of melanin pigments from cells or tissue samples in hot strong alkali (Soluene-350 or 1 M NaOH), total amount of melanin can be estimated spectrophotometrically by analysing absorbance and comparing the data obtained with a standard curve of synthetic melanin or melanin isolated from *Sepia officinalis* [483].

Although it is the most popular method for melanin quantification, absorption spectroscopy presents major drawbacks and does not always provide the sensitivity and specificity required [483]. Thus, the need for a reliable, simple, and economical method for melanin quantification is still a pertinent issue. Many years ago, Rosenthal *et al.* proposed the use of fluorescence spectroscopy to quantify melanin in cell cultures and tumours [480]. Melanins do not fluoresce but, once they are subjected to oxidative conditions (heating in alkaline hydrogen peroxide solution), they acquire fluorescence. Sachs was the first to report that pigments of various origins become fluorescent after oxidation with hydrogen peroxide. Recently, other authors confirmed that degradation of melanin is accompanied by the development of strong fluorescence [484]. The method originally proposed by Rosenthal *et al.* has the potential to overcome the disadvantages of using absorption spectroscopy; according to the authors, soluble products are formed and the fluorescent signal of oxidized melanin is not affected by proteinaceous or lipid

contamination [480]. However, its application to melanin quantification in biological samples is still scarcely reported.

We revisited the fluorimetric method in order to optimize the oxidative conditions for accurate melanin quantification by fluorescence spectroscopy in melanoma cells. Subsequently, we compared the two methods regarding their limits of detection, linearity, specificity, reproducibility, and applicability in melanin quantification to demonstrate the advantages of using fluorescence spectroscopy. Two of the most frequent melanin standards (synthetic and *Sepia* melanin) and different pigmented and non-pigmented human cell lines were used in this comparison. The fluorimetric method was also successfully applied to the quantification of melanin in more complex pigmented samples: zebrafish embryos and human hair.

II.2 MATERIAL AND METHODS

II.2.1. MELANINS, CHEMICALS AND BIOLOGICAL SAMPLES

Synthetic melanin, melanin from *Sepia officinalis*, kojic acid (KA), sepiapterin, guanine, tetrahydrobiopterin (BH_4), hydrogen peroxide solution (50 wt. % in H_2O, stabilized), sodium hydroxide (NaOH) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Forskolin (Fsk) was a product of Abcam (Cambridge, UK).

BJ-5ta (human foreskin fibroblasts) and A-375 (amelanotic human skin malignant melanoma) cell lines were purchased from ATCC (American Type Culture Collection, Virginia, USA). The human skin keratinocytes (NCTC2544 cell line) were purchased from Instituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna (Brescia, Italy). SK-Mel-1 and SK-Mel-23 cell lines (derived from pigmented human skin melanomas) were kindly provided by Doctor Sofia Magina (Centro de Investigação Médica, Faculdade Medicina do Porto, Portugal) and Doctor Francisco X. Real (Epithelial Carcinogenesis Group, Centro Nacional de Investigaciones Oncológicas, Spain), respectively. All cell lines were cultured in 75 cm^2 culture flasks containing Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS Superior, Biochrom GmbH, Berlin, Germany) and 1% (v/v) of antibiotic/antimycotic solution (Sigma-Aldrich®, St. Louis, MO, USA). The flasks were kept at 37 °C in a humidified atmosphere containing 5% CO_2. At every 2-3 days, cell culture medium was refreshed.

Adult wild-type zebrafish (*Danio rerio* Hamilton, 1822) were obtained from Interdisciplinary Centre of

Marine and Environmental Research (CIIMAR) and maintained at 26 ± 1 °C on a photoperiod of 12 h light/12 h dark. The animals were fed *ad libitum* twice a day with commercial flakes (Tropic and Tetra mix) and supplemented with live brine shrimp eggs (*Artemia* sp.). Before the breeding, males and females were isolated with a ratio of 3:5 within a 30 L aquarium under the same photoperiod, but at a temperature of 30 ± 1 °C. The next day, breeding fishes were removed 1.5 h after the beginning of the light period, the eggs collected and cleaned.

Black, dark-brown, medium blonde and light blonde human hair samples were provided by International Hair Importers & Products Inc. (Glendale, NY, USA). Reddish-brown, red, and white hair samples were collected from different volunteers. Prior to melanin quantification, all hair samples were subjected to a washing procedure, extensive rinsing and air-dried overnight.

II.2.2. MELANIN QUANTIFICATION BY ABSORPTION AND FLUORESCENCE SPECTROSCOPY

Stock standard solutions of synthetic and *Sepia* melanin were prepared in the aqueous solvent 1 M NaOH containing 10% (v/v) of DMSO, at a concentration of 200 µg/mL.

The quantification of melanin by absorption spectroscopy was performed at 405 nm, according to the literature [485–487]. The protocol for the quantification of melanin by fluorescence spectroscopy was optimized regarding the amount of hydrogen peroxide solution used and time for melanin oxidation. Melanin standard solutions were treated with different volumes of 50% (w/w) aqueous solution of hydrogen peroxide and the volume of solutions was adjusted with the solvent (final volume: 1 mL) to normalize the melanin concentration at 100 µg/mL. The solutions were vortexed, and the oxidation was allowed to proceed at 25 °C. The fluorescence of oxidized melanins was monitored up to 8 h. Based on the work published by Kayatz *et al.*, the excitation and emission wavelengths were set at 470 nm and 550 nm, respectively [484]. Using the optimized conditions, the fluorimetric method was validated with respect to linearity, limits of quantification and detection. The same validation was performed for the spectrophotometric method for comparison purposes.

BJ-5ta, NCTC2544, A-375, SK-Mel-1 and SK-Mel-23 cells were counted and pelleted in order to have a final concentration of $5x10^6$ cells/mL in 1 M NaOH containing 10% (v/v) DMSO. After incubation at 80 °C for 1 h, the lysates were clarified (3000g for 5 min) and the absorbance measured. The conditions for the complete oxidation of cell lysates with hydrogen peroxide were also optimized, following the protocol used for melanin standard solutions. Both methods were compared again regarding linearity, limits of quantification and detection of melanin with respect to the concentration of cells used. Both methods

were also compared in terms of their specificity. Different amounts of synthetic or *Sepia* melanin were added to A-375 (amelanotic) cells lysates ($1.25x10^6$ cells/mL). The samples were analysed, using the two methods described above, to check the influence of the matrix (presence of cell debris as proteins or lipids) in melanin quantification.

Zebrafish embryos (ten) were collected at different time points: 8, 24, 48, 80, 120 hours post fertilization (hpf). This analysis was performed in compliance with the guidelines on the protection of experimental animals by the Council of Europe, Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (120 hpf), which corresponds to the end of the embryonic development, therefore no license is required. After being pelleted and suspended in 200 µL of 1 M aqueous NaOH containing 10% (v/v) DMSO, samples were incubated at 80 °C for 1 h with orbital shaking. Aqueous hydrogen peroxide solution was added at the final concentration of 30% (v/v) and the mixtures incubated at 25 °C for 4 h. After centrifugation to remove debris, the fluorescence was measured and melanin content calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) of *Sepia* melanin standards. To assess the interference of sepiapterin, guanine and BH_4 in the quantification of melanin, different amounts of these compounds were added to *Sepia* melanin solutions of variable concentrations. The solutions were oxidized (using the same conditions used for the embryos) and their fluorescence measured.

Hair samples were weighted and digested in 2.5 M NaOH (25 °C for 24 h, with orbital shaking); then, the mixtures were diluted to obtain a solution of 5 mg of hair/mL in 1 M NaOH containing 10% (v/v) DMSO. After incubation of the samples at 80 °C for 1 h, the conditions for complete oxidation of hair lysates were optimized and the limits of melanin detection determined (regarding the amount of hair to be used). Melanin content of hair samples was calculated with a standard curve, generated by the fluorescence (after oxidation) of *Sepia* melanin standards. The results were normalized by amount of hair in each sample.

All the fluorescence and absorbance measurements were performed using a SynergyMx multiwall plate reader spectrophotometer (Bio-Tek® Instruments Inc., VT, USA).

II.2.3. *IN VITRO* MODULATION OF MELANOGENESIS

BJ-5ta, NCTC2544, A-375, SK-Mel-1 and SK-Mel-23 cells were seeded on 6-well plates at a density of $3.5x10^5$ cells/well. The cells were treated with 20 µM of Fsk (melanogenesis inducer), 2 mM of KA (melanogenesis inhibitor), 0.2% (v/v) ethanol (vehicle control) or without any particular supplement

(negative control) for 72 h. At the end of the treatment, cells were collected by trypsin treatment, pelleted, and suspended in 500 µL of 1 M aqueous NaOH containing 10% (v/v) DMSO. After incubation at 80 °C for 1 h, the lysates were clarified (3000g for 5 min) and the absorbance measured. Then, aqueous hydrogen peroxide solution was added to the samples at the final concentration of 30% (v/v). The mixtures were incubated at 25 °C for 4 h, the resulting colourless to pale yellow solutions were centrifuged to remove cell debris and their fluorescence was measured. Melanin content was calculated by interpolating the results with standard curves, generated by the absorbance or fluorescence (after oxidation) of synthetic or *Sepia* melanin standards. The results were normalized by protein levels in each sample. Protein content in the samples was determined using the *DC* Protein kit assay (Bio-Rad, CA, USA) with bovine serum albumin (BSA) as the protein standard.

II.2.4. STATISTICAL ANALYSIS

All the results are expressed as mean value ± standard deviation (SD) of five independent experiments, unless stated otherwise. Statistical analysis was performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA).

II.3 RESULTS AND DISCUSSION

Melanin production is the obvious main function of melanocytes and its quantification is required for fundamental biology studies as well as for more applied research involving these cells. Figure II.1 summarizes the protocol that we propose to quantify melanin by fluorescence spectroscopy in *in vitro* cell culture assays. This protocol results from several optimization experiments where the fluorescence and absorption spectroscopy methods were extensively compared regarding their limits of detection, linearity, specificity, and reproducibility (Supplementary Material, Section II.5.1).

Regarding the parameters of sensitivity and linearity, fluorescence spectroscopy has an equal or slightly better performance than the traditional method – Table II.1. However, as shown in the following section (also Supplementary Material, Section II.5.1), the differences between the two methods become evident during the quantification of melanin in the presence of complex matrices like cell lysates.

II.3.1 MELANIN QUANTIFICATION IN *IN VITRO* CULTURED MELANOMA CELLS

We have compared the performance of fluorescence and absorbance-based methodologies in a typical *in*

In vitro assay

1 - Collect 155000 to 2500000 pigmented and control cells (non-melanotic) by centrifugation (1000*g*, 10 min).

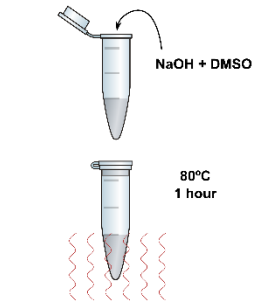
Cell collection and washing

2 - Discard the medium, wash the pellets with 0.5mL of 1X PBS and collect the cells by centrifugation (1000*g*, 10min).

Melanin solubilization

3 - Discard the supernatant and ressuspend the cell pellets in 0.5mL of 1M NaOH containing 10% (v/v) of DMSO; samples can be stored at -20 or -80ºC at this step. Prepare the standard curve of *Sepia* melanin in parallel.

4 - Incubate samples and standards 1h at 80ºC, in a water bath or dry incubator; vortex once in a while.

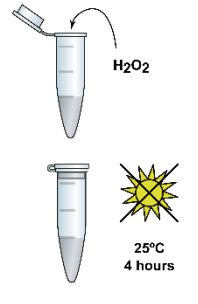


Melanin solution clarification

5 - Centrifuge samples and standards at 3000*g* for 5min. Transfer 0.3mL to a new micro-tube and use the remaining supernatant to quantify total protein with a compatible kit.

Melanin oxidation

6 - Add hydrogen peroxide solution (50%,w/v) to samples and standards at a final concentration of 30% (v/v); incubate 4h at room temperature in the dark.



Fluorescence measurement

7 - Centrifuge at 3000*g* for 5min and measure fluorescence using the excitation and emission wavelengths as 470nm and 550nm, respectively.

Figure II.1. Schematic overview of the proposed protocol for melanin quantification by fluorescence spectroscopy in *in vitro* assays.

Table II.1. Comparison of the fluorescence and absorbance-based methodologies for melanin quantification, regarding sensitivity and linearity

	Detection Limits				Range of Linearity for Melanin Standards (μg/mL)	
	Melanins (μg/mL)		Cell Lines (cells/mL)			
	Synthetic	*Sepia*	SK-Mel-1	SK-Mel-23	Synthetic	*Sepia*
Fluorescence	0.098	1.563	310000	310000	1 – 100	5 – 100
Absorbance	0.391	1.563	310000	630000	1 – 100	5 – 100

vitro assay to determine melanin production. After incubation of several cell lines with a well-known inducer (Fsk) and inhibitor (KA) of melanogenesis, their effects on melanin synthesis were assessed by both methods – Figure II.2. The control cell lines were all non-melanin producers: BJ-5ta (human foreskin fibroblasts), NCTC2544 (human skin keratinocytes) and A-375 (amelanotic human skin malignant melanoma). The test cell lines were SK-Mel-1 and SK-Mel-23, established from metastatic sites of pigmented human skin melanomas [488,489]. The amount of melanin was normalized to total amount of protein in cell lysates, which is a frequently used alternative to counting cells for each experimental condition. The melanin contents presented in Figure II.2 were calculated by interpolating the results with standard curves, generated either by the fluorescence or absorbance of *Sepia* melanin. Melanin contents were also calculated with standard curves of synthetic melanin (Supplementary Material, Figure II.S8).

At first glance, the absorbance-based method overvalues the melanin concentration when compared to the fluorescence-based method. Another key outcome from the results shown in Figure II.2 is that, only by fluorescence spectroscopy, all control cell lines indicate no significant melanin production. With absorption spectroscopy, measurable amounts of melanin (which are melanocyte specific) are erroneously detected and, according to Figure II.2B, there are no differences between the melanin produced by pigmented SK-MEL-1 cells and A-375 or NCTC2544 control cells. This result proves the high specificity of the fluorescence-based method as it erased the interference of other cell components from the detection/quantification of melanin.

The effects of the incubation of melanoma cells with an inductor or inhibitor of melanogenesis were also striking. After 72 hours of incubation, as expected, the treatment with Fsk significantly increased the intracellular level of melanin in SK-Mel-23 cells when compared to cells treated with 0.2% (v/v) EtOH, the

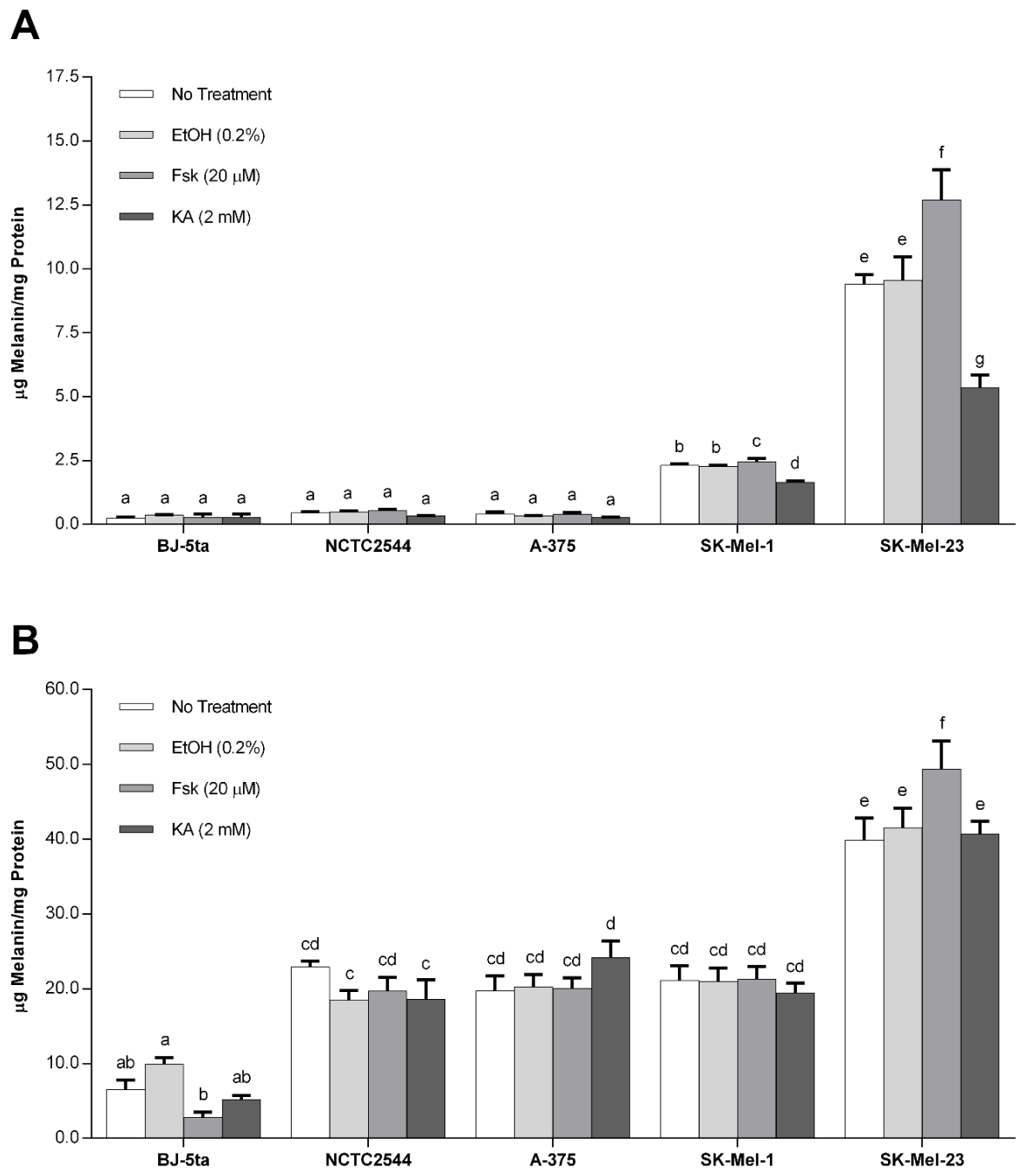


Figure II.2. Effect of forskolin (Fsk) and kojic acid (KA) on intracellular melanin content of non-melanotic (BJ-5ta, NCTC2544), amelanotic (A-375, melanoma cells with no visible pigmentation), or melanized cells (SK-Mel-1 and SK-Mel-23, pigmented melanoma cells), after 72 h of treatment. Melanin content was calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) (A) or absorbance (B) of *Sepia* melanin solutions of known concentration, prepared in 1 M NaOH containing 10% (v/v) of DMSO. The results were normalized by total protein levels in each sample. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. EtOH: ethanol.

vehicle control. However, the pronounced depigmentation effect of KA (assessed by visual inspection) was only perceptible after oxidation of cell lysates and quantification of melanin by fluorescence. Concomitantly, the effect of the treatments on the melanin content of SK-Mel-1 was only detectable by fluorescence. Contrarily to SK-Mel-23 cells, visual inspection of these cells does not provide any insight regarding the effect of the compounds on melanogenesis. Nevertheless, the sensitivity of the method is so powerful that even small variations in the synthesis of melanogenesis can be detected and accurately quantified by fluorescence spectroscopy. Using absorption spectroscopy, these differences were neglected. On the one hand, according to the calibration curves generated (Supplementary Material, Figure II.S3), absorbance-based quantification is much less sensitive to small variations of melanin. On the other hand, the contribution of non-melanotic material to absorbance background signal is so pronounced that melanin quantifications are overvalued, and some variations can be masked. The contribution of non-melanotic material to melanin quantification is also clear by the analysis of the results for other cell lines. As mentioned before, NCTC2544 and A-375 cells have melanin contents similar to SK-Mel-1 despite of being unable to produce melanin.

II.3.2 MELANIN QUANTIFICATION IN ZEBRAFISH EMBRYOS AND HUMAN HAIR

In order to test the applicability of the proposed methodology on biological samples more complex than *in vitro* cultured cells, fluorescence spectroscopy was used to quantify melanin in zebrafish embryos and human hair – Figure II.3. The protocol presented in Figure II.1 (for cultured cells) was slightly adjusted in order to be applied to more complex samples, mainly in terms of NaOH concentration and incubation time during tissue disruption and melanin solubilization.

Zebrafish Danio rerio is a powerful model system to study genetic mechanisms of vertebrate development, including the earliest events of pigment cells biology [490]. More recently, zebrafish was also established as an *in vivo* model to evaluate the activity of melanogenic regulatory compounds, making the quantification of melanin in those *in vivo* models of the highest importance [491,492]. Using fluorescence spectroscopy, a melanin production profile during zebrafish embryonic development was successfully drawn up to 120 hpf – Figure II.3A. For each measurement, only ten embryos were used (Supplementary. Material, Section II.5.1.6). According to the literature, much higher number of embryos (at least 100 embryos/mL of NaOH) are commonly used to perform melanin quantification by absorbance spectroscopy [491,493]. As expected, until 24 hpf, the embryos did not present quantifiable pigmentation (non-melanogenic control embryos). From 48 to 120 hpf, there is a steady increase in melanin

production; the amount of melanin shows significant statistical difference in embryos collected at the three chosen time points. Also important to mention is that other pigments produced by zebrafish do not interfere in the quantification of melanin by this method (Supplementary Material, Figure II.S6).

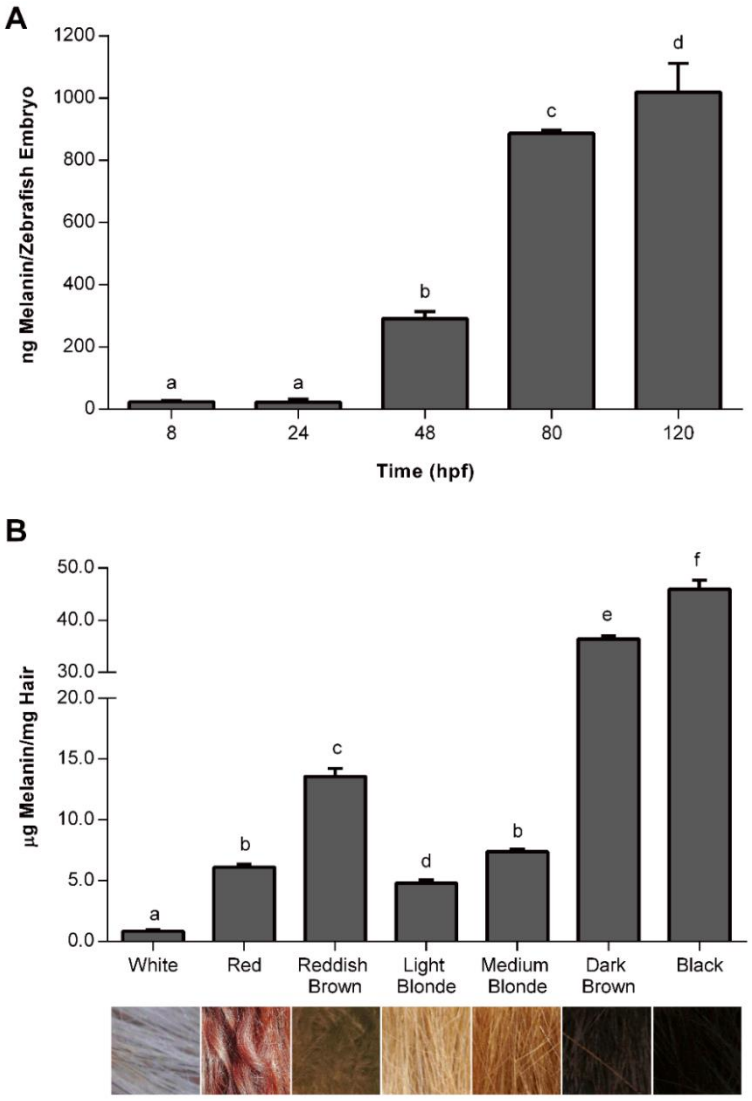


Figure II.3. Quantification of melanin in biological samples by fluorescence spectroscopy. (A) Developmental changes in the accumulation of melanin in zebrafish embryos. (B) Contents of total melanin in human hair samples of various colours (visual phenotypes). Melanin contents were calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) of *Sepia* melanin solutions of known concentration, prepared in 1 M NaOH containing 10% (v/v) of DMSO. The results were normalized by number of embryos (A) or amount of hair (B) in each sample. Data were analysed by one-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. hpf: hours post fertilization.

Fluorescence spectroscopy was also used to determine total melanin contents in hair samples of different colours – Figure II.3B. Using hair concentrations above the detection limit (Supplementary Material, Section II.5.1.6), the measured melanin contents are according to the expected and shows an excellent correlation with the visual phenotypes: black hair has the highest amount of melanin followed by dark-brown, reddish brown, medium blonde and light blonde hairs. The total melanin content in red hair (described as containing similar amounts of eumelanin and pheomelanin) is similar to that of medium blonde hair and comparable to what was reported by others, using HPLC [402]. HPLC is highly sensitive and, contrarily to absorbance and fluorescence spectroscopy, it also allows the analysis of melanins composition through the specific detection and quantification of the different degradation products of eumelanin and pheomelanin. According to the literature, H_2O_2 is efficient in the alkaline oxidation of both eumelanin and pheomelanin [402]. However, it is still unknown if their oxidation products exhibit similar spectroscopic properties, being properly accounted for the quantification of total melanin by the fluorimetric method here proposed. Although the results obtained offer some insight regarding the usefulness of fluorescence spectroscopy in the quantification of samples containing pheomelanin, more studies are needed to understand the contribution of each type of melanin to the fluorescent signal. Therefore, the fluorescence-based method should be used in essentially eumelanic systems.

II.4 FINAL REMARKS

The quantification of melanin in cell cultures by fluorescence spectroscopy was successfully validated. The protocol proposed is easy to perform and highly reproducible. Compared to the traditional widespread methodology based on absorbance, fluorescence measurement of melanin oxidation products is more sensitive and accurate in the quantification of melanin in biological samples. However, in the absence of non-melanogenic components, absorption spectroscopy is also expected to perform well but the methods employed in the extraction of melanin are exhaustive and time consuming. Melanin oxidation followed by the fluorescence measurement do not require the previous isolation of melanin from samples. This work supports fluorescence spectroscopy as the best choice for routine total melanin quantification in complex biological matrices.

II.5 SUPPLEMENTARY MATERIAL

II.5.1 OPTIMIZATION OF THE FLUORESCENCE-BASED PROTOCOL

II.5.1.1. MELANIN OXIDATION

The conditions for complete oxidation of melanins, amount of hydrogen peroxide solution used and time of reaction, were optimized using the two most frequent melanin standards: synthetic and *Sepia* melanins – Figure II.S1. Being a natural melanin, *Sepia* melanin resembles more closely the composition of melanin in biological tissues and mimics its fluorescent and optical properties. Synthetic melanin was also tested because, being cheaper than *Sepia* melanin, it is widely used as a standard for melanin quantification though its adequacy is very controversial.

For both melanins, low fluorescent signals were detected in the samples before the oxidation with hydrogen peroxide. This autofluorescence is probably caused by some degradation/air oxidation of melanins (in an uncontrolled fashion way) due to the highly alkaline media and heat treatment used for its dissolution [494,495]. Then, the fluorescence intensity increases with increasing concentrations of hydrogen peroxide for up to 20% (v/v); synthetic melanin requires two hours to be completely oxidized while *Sepia* melanin requires four hours. After complete oxidation, fluorescence intensities of both solutions are stable for at least four hours (eight hours was the maximum reaction time tested by us).

Unexpectedly, fluorescence intensity of synthetic melanin was found to be almost three times higher when compared to *Sepia* melanin. The differences in composition among these two pigments can be responsible for the differences found. Natural melanins (as *Sepia* melanin) are composed of a chromophoric fraction covalently linked to proteins that act as scaffolding matrix for melanin deposition or as enzymes catalysing various steps of melanogenesis [496]. By acid hydrolysis analysis, *Sepia* melanin from Sigma-Aldrich® was found to contain protein contents as high as 40%. On the other hand, the protein content of synthetic melanins is negligible [150,497]. Since only the indole residues of melanin are responsible for the fluorescence detected after oxidation, this can explain the results obtained [498]. Also, synthetic melanins, composed mainly of 5,6-dihydroxyindole units (DHI), are usually prepared by oxidizing tyrosine with hydrogen peroxide. *In vivo* melanogenesis occurs in the presence of tyrosinase and natural melanins are heteropolymers of DHI and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) in a ratio of approximately 1:1 [494]. The differences in the structural composition of these melanins can affect the oxidation process and the type/amount of degradation products, which can also account for the results obtained.

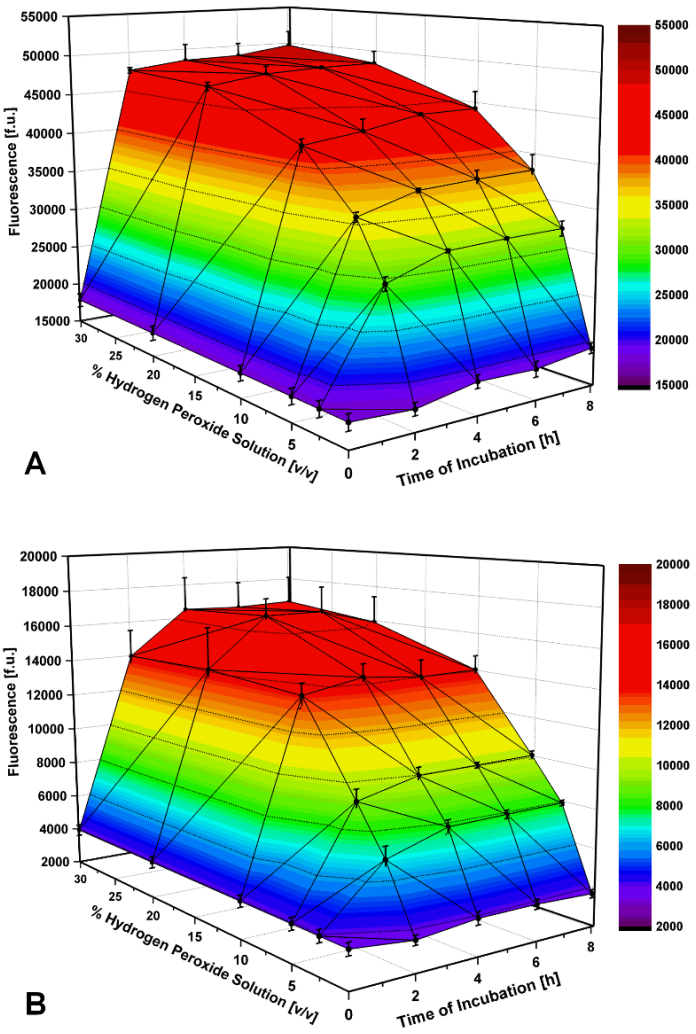


Figure II.S1. Time response study of the fluorescence of synthetic (A) and *Sepia* (B) melanin solutions, oxidized at 25 °C with different amounts of hydrogen peroxide ($\lambda_{excitation}$ = 470 nm; $\lambda_{emission}$ = 550 nm). Melanin standard solutions were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. After addition of hydrogen peroxide, the volume of samples was adjusted to normalize the melanin concentration at 100 µg/mL. The data was analysed by two-way ANOVA, followed by *post hoc* Tukey's test.

II.5.1.2. LIMITS OF DETECTION

The limit of detection for a method is commonly defined as the lowest analyte concentration likely to be reliably distinguished from the blank sample (solution containing no analyte) [499]. We measured the absorbance and the fluorescence of solutions containing different amounts of synthetic or *Sepia* melanins and compared the values with the blank sample – Figure II.S2. The graphical representation is categorical due to a data visibility purpose only. Regarding synthetic melanin, Figure II.S2A, even the lowest concentration tested (0.098 µg/mL) provides a fluorescent signal significantly different from that obtained

for the blank. On the other hand, measuring its absorbance signal, melanin can only be reliably detected at concentrations above 0.391 μg/mL. Using this melanin standard, fluorescence spectroscopy proved to be at least four times more sensitive than the traditional absorption spectroscopy. For *Sepia* melanin, Figure II.S2B, the sensitivity of both methods was set at 1.563 μg/mL.

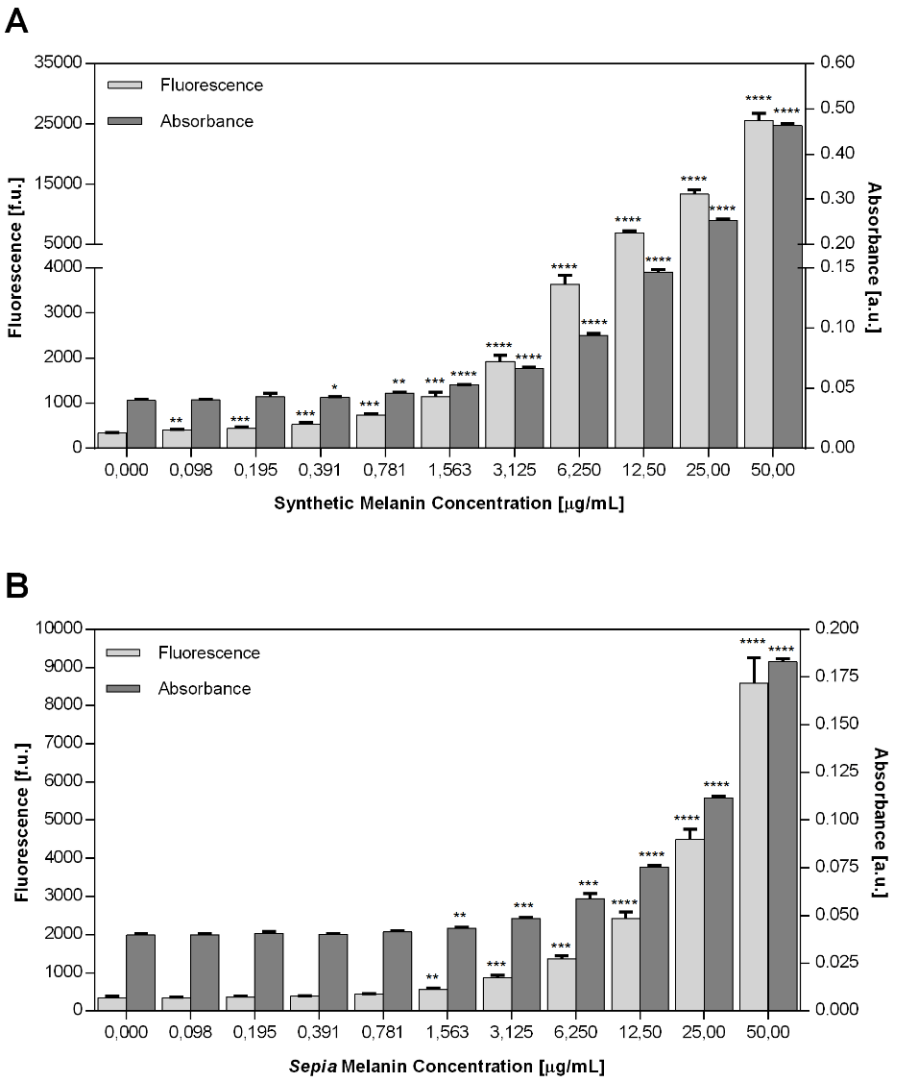


Figure II.S2. Fluorescence and absorbance values of solutions containing different concentrations of synthetic (A) and *Sepia* (B) melanins. Solutions were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO, by two-fold serial dilutions. Absorbance of solutions was measured at 405 nm. Fluorescence intensity was measured ($\lambda_{excitation}$= 470 nm; $\lambda_{emission}$ = 550 nm) after incubation of melanin solutions with 20% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by one-way ANOVA, followed by *post hoc* Dunnett's test. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 or ****p ≤ 0.0001, compared to the respective blank sample (0 μg/mL).

II.5.1.3. LINEARITY

We studied the correlation between the quantity of pigment and signal intensity (absorbance or fluorescence) in the concentration range 0.391 – 100 µg/mL for synthetic melanin and 1.563 – 100 µg/mL for *Sepia* melanin – Figure II.S3.

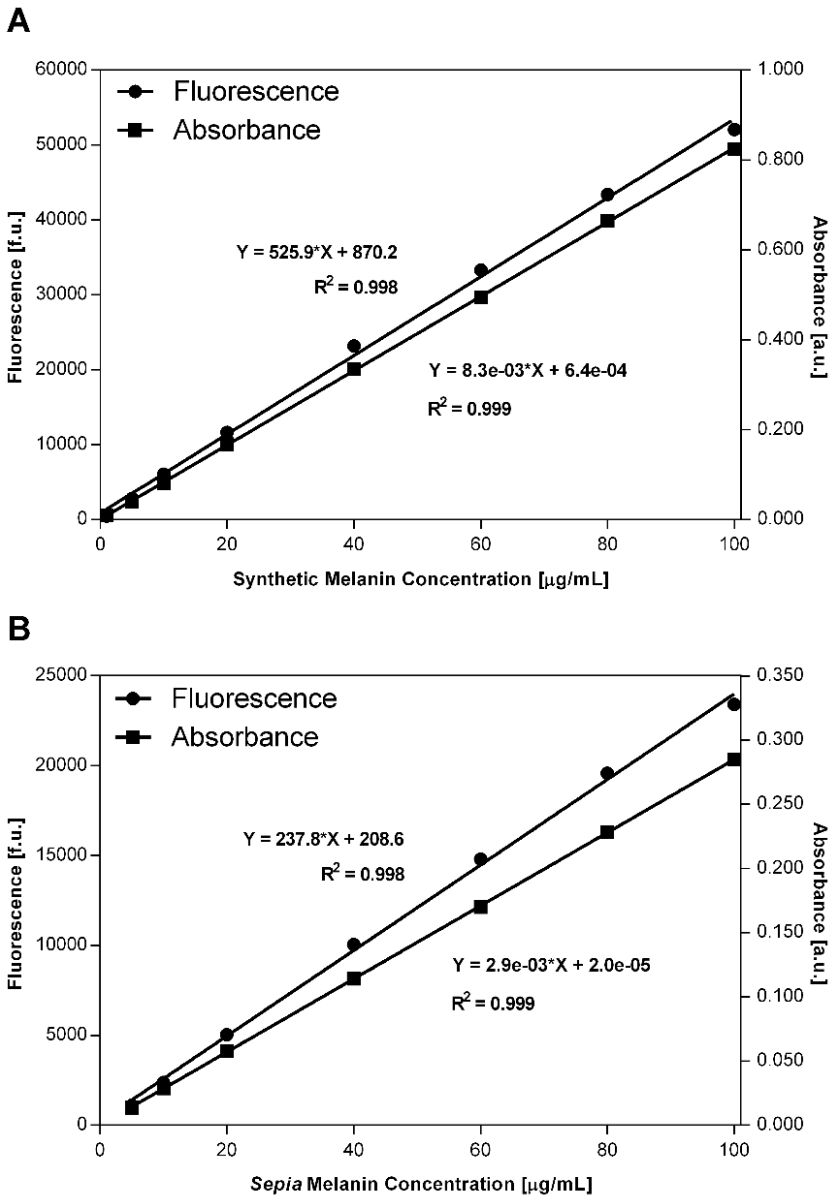


Figure II.S3. Plots of standard curves of synthetic (A) and *Sepia* (B) melanins obtained by fluorescence and absorption spectroscopy. Solutions were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO, by two-fold serial dilutions. Absorbance of solutions was measured at 405 nm. Fluorescence intensity was measured ($\lambda_{excitation}$= 470 nm; $\lambda_{emission}$= 550 nm) after incubation of melanin solutions with 20% (v/v) hydrogen peroxide solution for 4 h.

As shown in Figure II.S3, plots of the fluorescence and absorbance intensities against melanin concentration indicate good linearity in the range of 1 – 100 μg/mL for synthetic melanin (Figure II.S3A) and 5 – 100 μg/mL for *Sepia* melanin (Figure II.S3B). The correlation coefficients (R^2) obtained by florescence spectroscopy are insignificantly lower than the ones obtained by absorption spectroscopy. This is caused by the apparent loss of linearity near the highest standard for the fluorimetric method that do not compromise the goodness-of-fit of linear regression for this method; run tests confirmed that deviations from linearity for both methods are not significant. Moreover, statistically, the slopes obtained by both methods are considered extremely different ($p \leq 0.0001$). As fluorimetric method presents the higher values, this method is expected to be more sensitive to small changes in melanin concentration.

II.5.1.4. OXIDATION OF CELL LYSATES

Given the complexity of biological samples, we have repeated the optimization process to guarantee the complete oxidation of cell lysates. The best conditions were defined as 30% (v/v) hydrogen peroxide and 4 h of reaction (data not shown). Using these conditions, we have determined the detection limits for both methods in terms of cells number – Figure II.S4.

The minimal concentration of pigmented melanocytes (SK-Mel-1 and SK-Mel-23) to produce fluorescent signals significantly different from the blank sample (no cells) and from the lysates of non-melanotic cells (BJ-5ta and NCTC2544) and amelanotic melanoma cells (A-375) is 310000 cells/mL – Figure II.S4A. Regarding to absorbance measurement, the limit of detection increases to 630000 cells/mL when SK-Mel-23 cells are used – Figure II.S4B. This decrease in sensitivity is cell-type dependent, therefore it should be verified for all the cell lines to be used.

II.5.1.5. SELECTIVITY AND REPRODUCIBILITY

Using cell concentrations above the limits of detection for melanin, we have studied the selectivity (degree to which a method can quantify the analyte accurately in the presence of interferences) and reproducibility (agreement between independent test results obtained under stipulated conditions) of both methods. To evaluate the interference of other cellular components present in the lysates, we added known amounts of synthetic and *Sepia* melanins (above the detection limit previously determined) to A-375 cell lysates and analysed the samples by both methodologies – Table II.S1 and Table II.S2. A-375 cells lysates were selected as these cells do not produce melanin, but the respective lysates can reflect the proteinaceous or lipidic composition of melanized melanocytes.

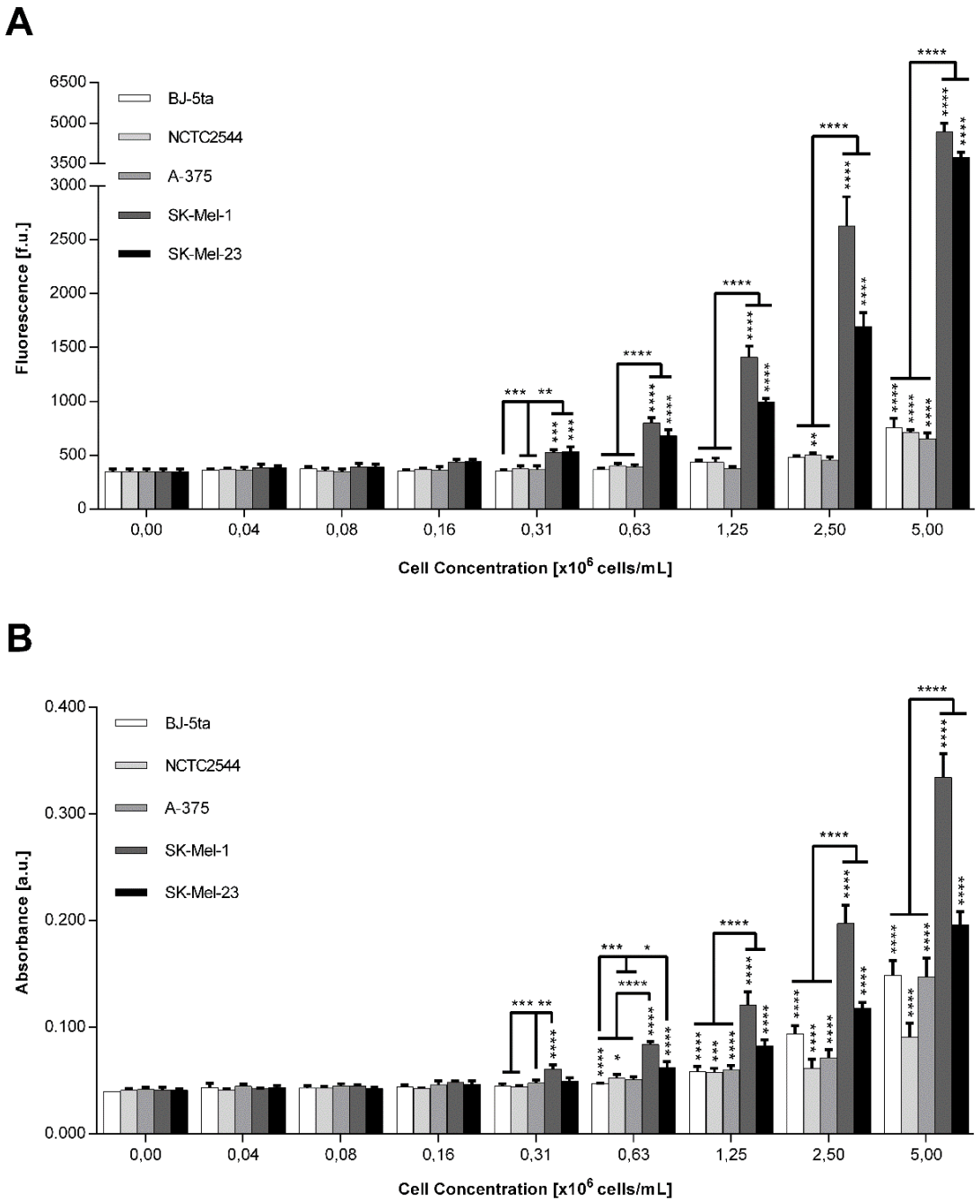


Figure II.S4. Fluorescence (A) and absorbance (B) intensities of lysates containing different amounts of non-melanotic (BJ-5ta, NCTC2544), amelanotic (A-375, melanocytes with no visible pigmentation) or melanized (SK-Mel-1 and SK-Mel-23) cells grown in culture. Cell lysates were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation of cell lysates with 30% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by two-way ANOVA, followed by *post hoc* Dunnett's test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ or ****$p \leq 0.0001$, when melanocytes concentrations were compared to the respective blank sample (0 cells/mL) or against the same concentration of non-melanotic and amelanotic cells.

Table II.S1. Quantification of different amounts of synthetic melanin in A-375 cell lysates, by fluorescence and absorbance-based methods. Cell lysates were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation of cell lysates with 30% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to the respective hypothetical value. Coefficient of variation (CV) was determined on samples assayed based on 5 independent experiments

Theoretical Melanin (µg/mL)	Fluorescence			Absorbance		
	Melanin (µg/mL)	Relative Error (%)	CV (%)	Melanin (µg/mL)	Relative Error (%)	CV (%)
100	97.5 ± 3.2	2.5	3.32	100.3 ± 1.6	0.3	1.58
80	79.0 ± 1.0	1.3	1.32	81.0 ± 2.3	1.3	2.81
60	59.5 ± 1.1	0.8	1.88	65.6 ± 0.6*	9.3	1.04
40	40.0 ± 2.0	0.0	5.06	47.7 ± 1.3*	19.3	2.70
20	22.9 ± 0.4*	14.5	1.69	24.5 ± 0.7*	22.5	2.79
10	12.0 ± 0.5*	20.0	3.90	15.2 ± 0.8*	52.0	5.06
5	6.2 ± 0.6*	24.0	9.05	10.3 ± 0.8*	106.0	8.09

As shown in Table II.S2, and considering the statistical analysis performed, the presence of cell contaminants does not affect the quantification of *Sepia* melanin by fluorescence. On the other hand, absorption spectroscopy is much more susceptible to the interference of non-melanotic material and measures higher amounts of *Sepia* melanin than the expected for all the tested concentrations. Amounts of synthetic melanin higher than expected are also measurable in cell lysates by fluorescence (concentrations below 20 µg/mL) and by absorbance (concentration below 60 µg/mL) but the employment of fluorescence is less affected by the presence of other cell components; the values obtained are closer to the theoretical ones, as shown by the relative errors – Table II.S1. In order to avoid the interference of the lipidic components from the cells, the measurement of absorbance could have been performed at 490 nm instead of 405 nm. However, after exhaustive revision of the literature, we concluded that absorption wavelengths closer to 400 nm (particularly 405 nm) are the most common choice for melanin quantification in biological samples. This is probably because the use of higher

wavelengths decreases the sensitivity of method, which was confirmed by us prior to this study. Thus, compared to the absorbance-based protocol mentioned in this paper, the quantification of melanin by fluorescence is more selective and the absolute errors were much lower.

For both methods, regardless the standard melanin used, the reproducibility of the assays can be considered good to excellent as the day-to-day variations accounted for less than 10% (according to the coefficient of variation obtained) – Table II.S1 and Table II.S2.

Table II.S2. Quantification of different amounts of *Sepia* melanin in A-375 cell lysates, by fluorescence and absorbance-based methods. Cell lysates were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation of cell lysates with 30% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to the respective hypothetical value. Coefficient of variation (CV) was determined based on five independent experiments

Theoretical Melanin (µg/mL)	Fluorescence			Absorbance		
	Melanin (µg/mL)	Relative Error (%)	CV (%)	Melanin (µg/mL)	Relative Error (%)	CV (%)
100	98.1 ± 2.0	1.9	1.99	115.3 ± 2.5*	15.3	2.17
80	79.4 ± 3.5	0.8	4.38	93.7 ± 1.9*	17.1	1.99
60	57.9 ± 4.0	3.5	6.90	71.0 ± 4.7*	18.3	6.60
40	41.9 ± 1.6	4.8	3.72	54.0 ± 4.6*	35.0	8.50
20	20.0 ± 1.0	0.0	5.01	36.1 ± 2.9*	80.5	8.17
10	10.9 ± 0.7	9.0	6.72	26.9 ± 2.4*	169.0	9.00
5	5.2 ± 0.2	4.0	4.33	21.5 ± 1.1*	330.0	5.12

II.5.1.6. OXIDATION OF ZEBRAFISH EMBRYOS AND HUMAN HAIR

Regarding zebrafish embryos, the melanin detection limit in terms of number of embryos required was determined. To obtain fluorescent signals significantly different from the blank sample (no embryos) and from the lysates of amelanotic embryos, 10 embryos/mL must be used – Figure II.S5. We considered as amelanotic embryos, those collected at 8 and 24 hpf. According to the literature, melanophores (melanin-

containing dark cells, analogues to melanocytes in higher vertebrates) only appear in zebrafish embryos at approximately 24 hpf; at 25 hpf, they begin to display visible pigmentation [500,501].

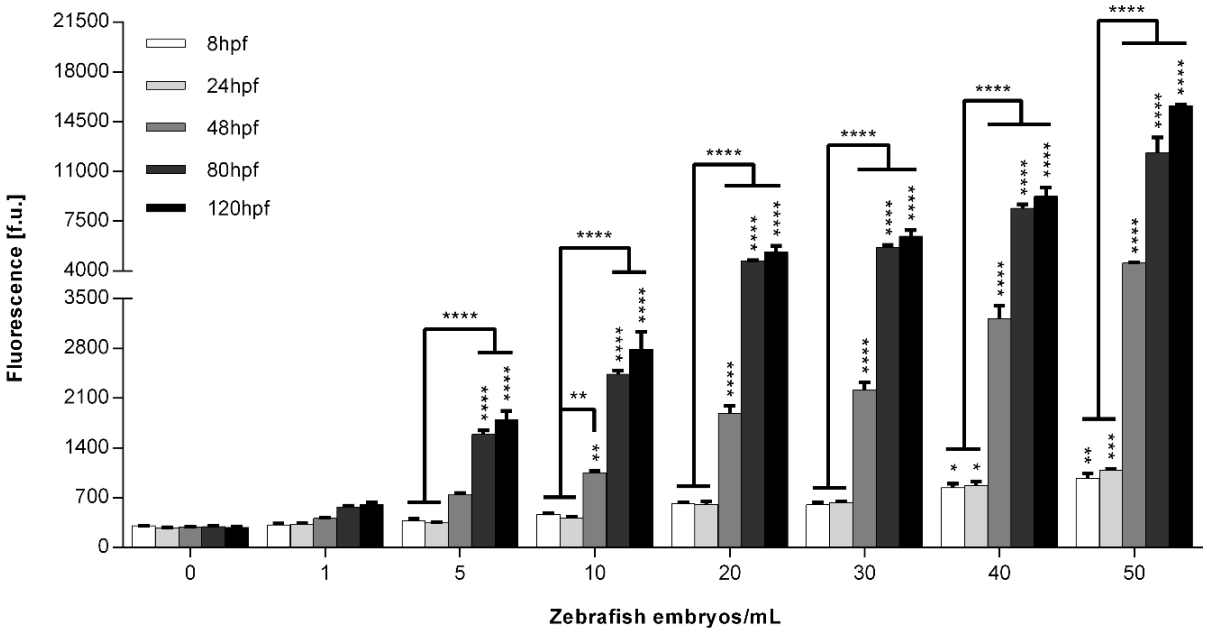


Figure II.S5. Fluorescence intensities of lysates containing different amounts of zebrafish embryos at specific time points of development (8 – 120 hours post fertilization). The lysates were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation of lysates with 30% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by two-way ANOVA, followed by *post hoc* Dunnett's test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ or ****$p \leq 0.0001$, when samples concentrations were compared to the respective blank sample (0 embryos/mL) or against the same concentration of amelanotic samples (8 and 24 hpf).

Zebrafish possess other kinds of chromophores than melanophores: xanthophores (that accumulate sepiapterin, a pteridine that fluoresces in green after illumination with blue light) and iridophores (that synthesize guanine-rich light-reflective platelets) [502,503]. In this work, we evaluated their interference with fluorescence signals generated by solutions containing different concentrations of melanin – Figure II.S6.

In a range of concentrations spanning those found in zebrafish embryos during embryonic development, neither the presence of sepiapterin or guanine interfere in the quantification of melanin by fluorescence spectroscopy. This is also true for the presence of BH_4 – a highly important/expressed pteridine in

zebrafish embryos, used as a precursor for tyrosine formation and cofactor for several enzymes – tested under the same conditions [502,504].

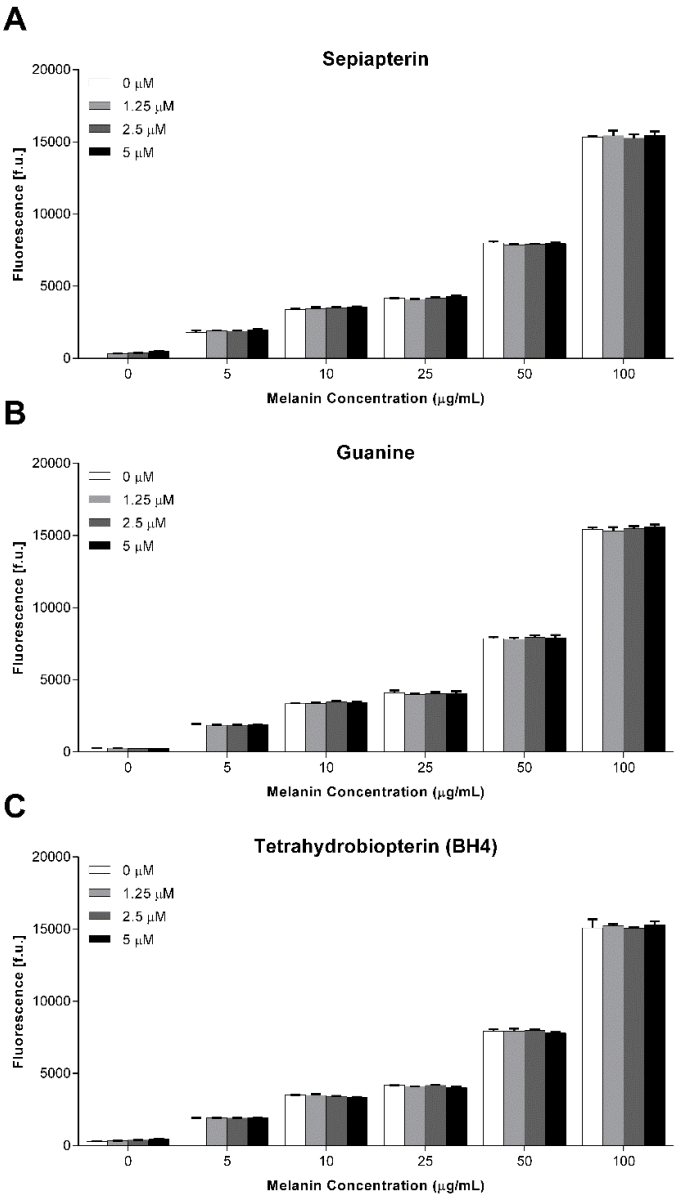


Figure II.S6. Fluorescence intensities of *Sepia* melanin-containing solutions in the presence of variable amounts (0 – 5 μM) of sepiapterin (A), guanine (B) or tetrahydrobiopterin (BH_4) (C). Solutions were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation with 30% (v/v) hydrogen peroxide solution for 4 h. Data was analysed by two-way ANOVA, followed by *post hoc* Dunnett's test.

In order to accurately quantify melanin in human hair by fluorescence spectroscopy, we first set the

conditions for its complete oxidation; this optimization process was performed using heavily pigmented African hair (5 mg/mL). Although human hair and the other biological samples used in this work have quite different compositions, the same oxidative conditions can be applied – 30% (v/v) hydrogen peroxide and 4 h of reaction (data not shown). Thereafter, regarding the amount of hair to be used, the limits of melanin detection were determined. To produce fluorescent signals significantly different from the blank (no hair) and amelanotic samples (white hair), oxidation must be performed in lysates containing a minimal concentration of 0.5 mg of hair/mL – Figure II.S7.

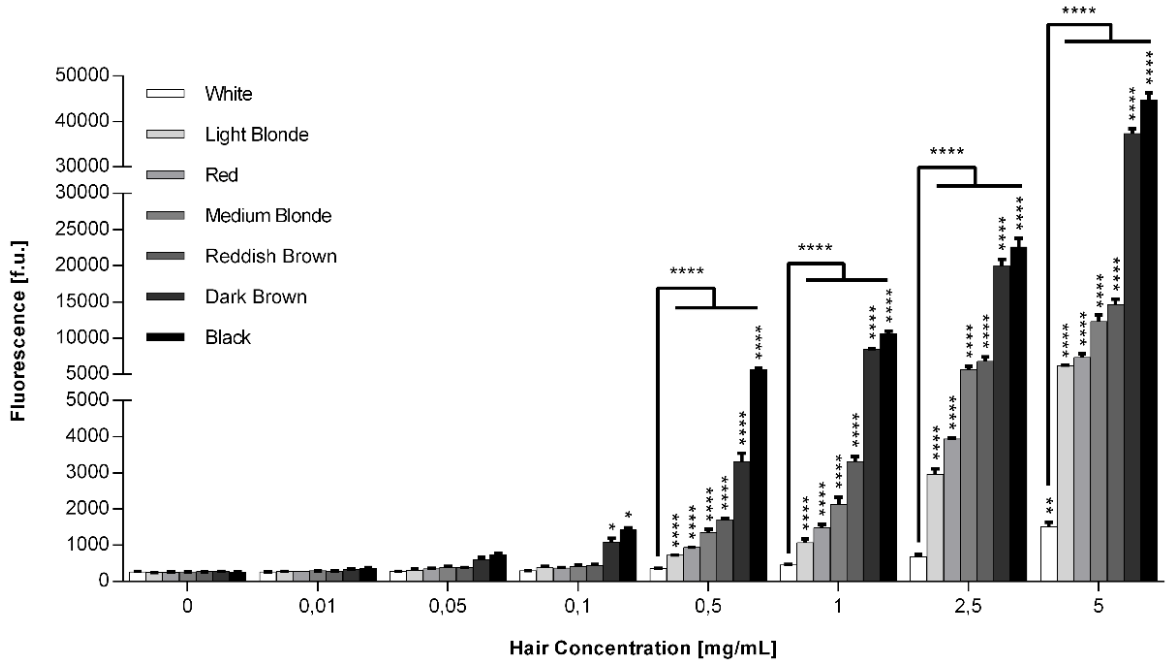


Figure II.S7. Fluorescence intensities of lysates containing different amounts of human hair of variable colours. The lysates were prepared in 1 M aqueous NaOH containing 10% (v/v) of DMSO. Fluorescence intensity was measured after incubation of lysates with 30% (v/v) hydrogen peroxide solution for 4 h. Data were analysed by two-way ANOVA, followed by *post hoc* Dunnett's test. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ or ****$p \leq 0.0001$, when samples concentrations were compared to the respective blank sample (0 mg of hair/mL) or against the same concentration of amelanotic samples (white hair).

II.5.2 FIGURE II.S8

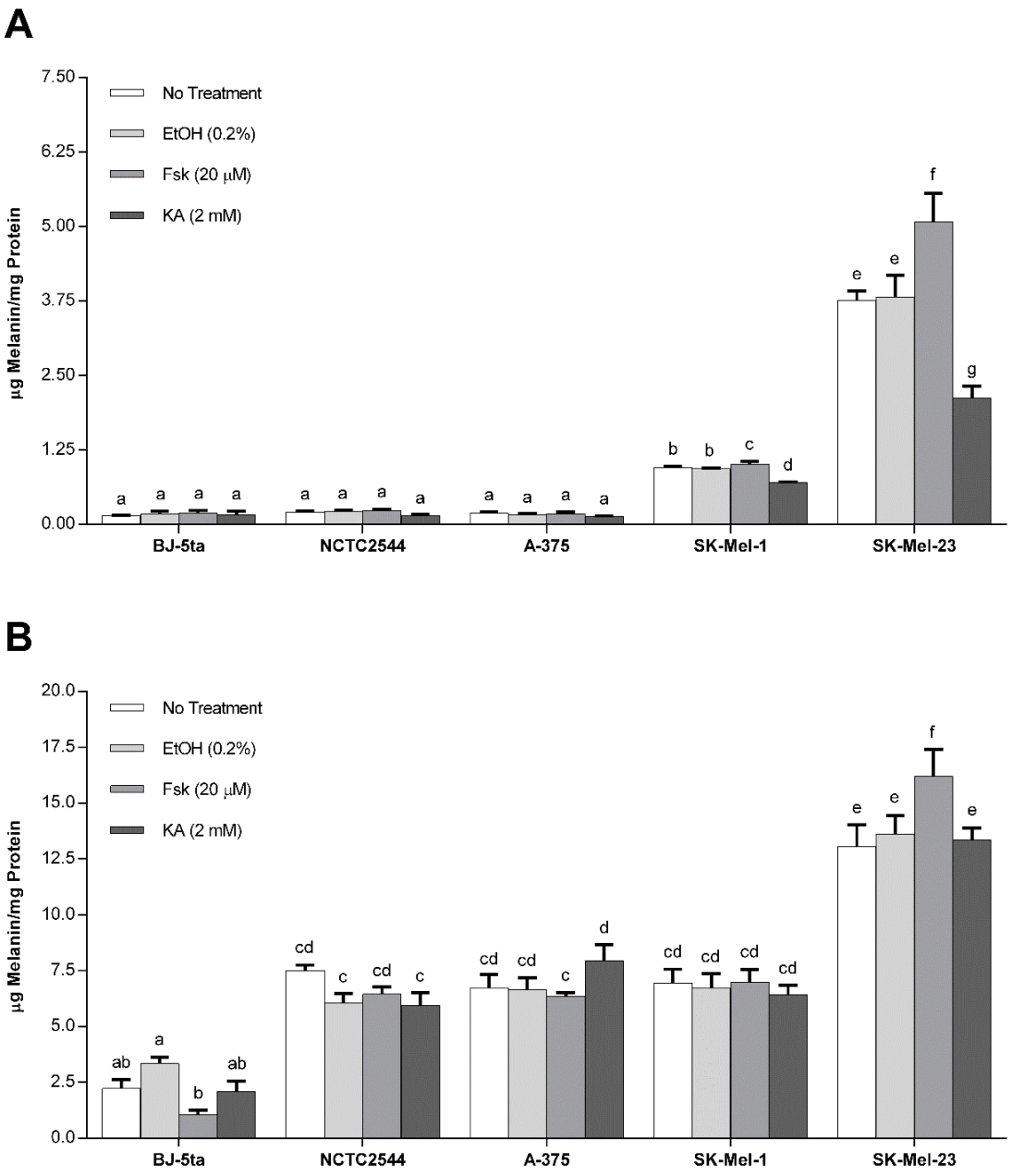


Figure II.S8. Effect of forskolin (Fsk) and kojic acid (KA) on intracellular melanin content of non-melanotic (BJ-5ta, NCTC2544), amelanotic (A-375, melanoma cells with no visible pigmentation) or melanized cells (SK-Mel-1 and SK-Mel-23, pigmented melanoma cells), after 72 h of treatment. Melanin content was calculated by interpolating the results with standard curves, generated by the fluorescence (after oxidation) (A) or absorbance (B) of synthetic melanin solutions of known concentration, prepared in 1 M NaOH containing 10% (v/v) of DMSO. The results were normalized by total protein levels in each sample. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

CHAPTER III

DISCOVERY OF NEW MELANOGENESIS MODULATORS FOR HAIR COLOUR MODIFICATION: AN *IN VITRO* TO *IN VIVO* STUDY

Chapter III*

Discovery of new melanogenesis modulators for hair colour modification: an *in vitro* to *in vivo* study

ABSTRACT

The colour of hair is mostly defined by its content in melanins, a class of natural pigments synthesized by melanocytes in the follicle, through a highly regulated biochemical process known as melanogenesis. In theory, the darkening or lightening of hair could be achieved by the manipulation of follicular melanogenesis. The aim of the work described in this chapter was to find molecular candidates for follicular melanogenesis modulation via topical delivery. By screening a collection of 1200 generic drugs with a long history of human usage, twenty-three compounds showed to significantly alter the melanin content of *in vitro* cultured SK-Mel-23 cells. The top four inducers and inhibitors of melanogenesis were further validated regarding their melanogenic effect, and their cytotoxicity and dose-response activity was studied. Taking into consideration the data generated, and the already known toxicological profile in humans, three compounds were chosen for further *in vivo* testing on human volunteers: Compound A+ was chosen as an agent of hair darkening while Compound B- and Compound C- were chosen as agents of hair lightening. The results obtained in a clinical study with intervention of cosmetics support the idea of changing hair colour by topical treatment of the scalp with these new cosmeceutical ingredients. In the future, this approach is expected to serve as a safer and less fibre damaging alternative to the current cosmetic procedures employed in hair colouration.

*** This chapter is based on the following scientific paper:**

Bruno Fernandes, Cristiana Costa, Teresa Matamá, Andreia C. Gomes and Artur Cavaco-Paulo. To be submitted.

III.1. INTRODUCTION

Hair colour has always had an enormous social and cosmetic impact, as exemplified by the ancient trend of dying the hair to appear more attractive. Currently, the concern with hair colour (along with length, shape, and amount) is higher than ever as society focuses more and more on beauty and youthfulness [438,439]. Although hair dyes are used by millions of individuals worldwide, they have the potential to induce chemical and allergic reactions that can result in acute or chronic mild dermatitis with consequent hair loss [439,442–445]. Some studies have also raised the possibility that long-term usage of permanent hair dyes may be associated with an increased risk of developing certain cancers [439,446,447]. Also, the colouration procedures cause split, dry, and dull hair due to cumulative damages on fibre structure [441]. Therefore, the development of safer ways for hair colour modification is a very pertinent issue.

The therapeutic usage of many drugs has shown different side effects on hair colour, texture, and shape. Drug-induced changes in hair colour are either as lightening or darkening (including repigmentation of grey/white hair) [448–479]; in most cases, the hair returned to its original colour after drug withdrawal. These findings raised the attractive possibility of using known, safe, and already approved drugs as an alternative or in addition to conventional cosmetic methods of colouration. A few patents have claimed the topical usage of some drugs with melanogenic properties to promote a change in the natural colour of hair fibres [505,506]. However, the lack of unequivocal *in vivo* cosmetic relevance for those compounds still makes the proof of a follicular-based approach for hair colour modulation a need in the art; this is the main goal of the work presented in this chapter.

In this study, Prestwich Chemical Library® (an off-patent collection of small drugs, mostly approved by FDA, EMA and other agencies) was screened regarding the effect on *in vitro* melanin production. Then, the melanogenic activity of the top hit inducers and inhibitors was confirmed, and the changes is the activity of tyrosinase (rate-limiting enzyme of melanogenesis) was investigated as a preliminary study of the mechanisms of action involved in their melanogenic effects. Finally, a clinical study with intervention of cosmetics (RNEC No.: 92938) was conducted to validate the *in vivo* potential of selected drugs as cosmeceutical ingredients for the follicular modulation (darkening or lightening) of hair colour.

III.2. RESULTS AND DISCUSSION

The goal of this work was to identify novel agents for hair colour modulation in well characterized drugs, already in use for other applications. Drug repurposing is an increasingly employed strategy for

researchers in industry and academia: general pharmacology and toxicology are already established as they are the structure, chemical properties and biological functions of most of the repurposed drugs, sparing the cost and time needed to develop new products and facilitating further analysis of the novel functionality. For cosmetic applications, this approach became particularly relevant since animal testing was prohibited in the U.E.; today, one of the most reliable ways to assure that newly developed cosmetic products present *in vivo* low risk is to formulate them using compounds with already known toxicological profile.

III.2.1. SCREENING OF MELANOGENESIS MODULATORS

In this work, Prestwick Chemical Library® was screened to identify new modulators of melanogenesis among 1200 small molecules. Several aspects were considering for choosing this library. First, most of the compounds in the library have a long history of human usage, with their toxicological profiles being well-characterized. Second, the original patents of compounds have expired, allowing industrial protection of repurposing. Finally, some of the most represented targets of this library have direct implication in melanogenesis: adrenergic receptors, histamine receptors, oestrogen receptors, phosphodiesterases (PDEs), serotonin receptors (5-HTR), cholinesterases, among others. Notedly, high throughput screenings (HTS) of melanogenesis modulators are not widely explored, and they usually target tyrosinase activity instead of the direct evaluation of cellular melanin contents. This is supposed to be happen because, until the development of the fluorescence-based approach documented in this thesis (Chapter II), the existing methods for quantification of melanin were mostly impracticable at large scale assays compared to the ones employed in the assessment of tyrosinase activity [507]. The major problem with screening melanogenesis modulators based on their actions on tyrosinase is the reported existence of several compounds that change the pigmentation status of melanocytes without affecting the catalytic activity of the enzyme. In this sense, for more highly inclusive screenings, a direct quantification of melanin must be favoured.

The screening of Prestwick Chemical Library®, regarding the ability to alter cellular levels of melanin was performed on SK-Mel-23 cells. Average melanin production obtained with each compound is shown in Figure III.1, as a percentage of vehicle control (cells treated with 1% DMSO). The method used for the identification of hits has involved selecting a standard deviation (σ) threshold relative to the whole assay mean (μ). The cut-off for hit melanogenesis inducers was set at $\mu + 3\sigma$, a commonly used threshold in high throughput screenings corresponding to a false positive error rate of 0.00135. The cut-off for

melanogenesis inhibitors was adjusted to $\mu - 2\sigma$ due to a very low incidence of hits when using a more conservative approach [508–510].

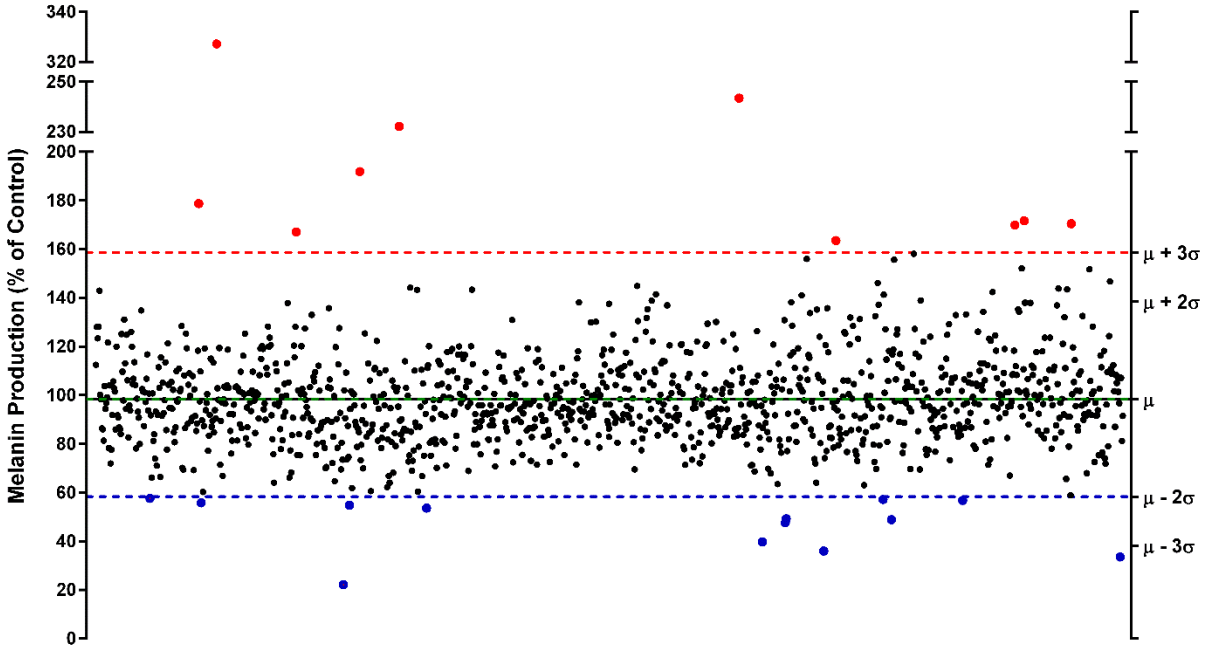


Figure III.1. High Throughput Screening (HTS) of Prestwick Chemical Library® regarding the *in vitro* modulation of melanin. SK-Mel-23 cells were treated with compounds for 72 h and then, melanin quantification was performed using a fluorescence-based method. The melanin contents were normalized by total protein levels in each sample and expressed as a percentage of the vehicle control, 1% (v/v) DMSO. HTS-Corrector software (version 2.0) was used to examine HTS data and hit selection. The cut-off for hit melanogenesis inducers and inhibitors was set at $\mu + 3\sigma$ and $\mu - 2\sigma$, respectively. μ: whole mean assay. σ: standard deviation.

About 2% of the tested compounds were identified as hit modulators of melanin synthesis, which is higher than the typical rate of < 0.1% reported for many screenings of large libraries of small molecules [511]. The rate obtained is presumably related to the fact that the most representative cell targets of the drugs included in this library have direct implications on melanogenesis. From the 23 targeted hits, 10 increase the cellular level of melanin (hit inducers) and 13 decrease the cellular level of melanin (hit inhibitors) – Supplementary Material, Table III.S1. To the best of our knowledge, apart from protriptyline hydrochloride and trimipramine maleate salt, the melanogenic effects presented by those drugs are reported here for the first time. The top four hit inducers and inhibitors were selected for further studies – Table III.1.

Table III.1. Top hit compounds and their effect on intracellular melanin content of SK-Mel-23

	Compound	Melanin Content (% of Control ± SD)		
		Screening	Validation	t-test
Inducers	Compound A+	327.2 ± 11.48	198.5 ± 13.93	t(2) = 10.08 p = 0.0097
	Compound B+	243.5 ± 7.686	169.8 ± 15.49	t(2) = 6.031 p = 0.0264
	Compound C+	232.2 ± 33.22	218.8 ± 2.263	t(2) = 0.5696 p = 0.6264
	Compound D+	191.9 ± 4.158	202.1 ± 34.44	t(2) = 0.4134 p = 0.7194
Inhibitors	Compound A-	22.22 ± 7.757	71.65 ± 2.899	t(2) = 8.442 p = 0.0137
	Compound B-	33.56 ± 16.82	78.00 ± 4.243	t(2) = 3.623 p = 0.0685
	Compound C-	35.98 ± 14.94	31.10 ± 6.930	t(2) = 0.4186 p = 0.7162
	Compound D-	39.75 ± 13.15	64.60 ± 9.758	t(2) = 2.146 p = 0.0825

III.2.1.1. VALIDATION OF SELECTED HIT COMPOUNDS

The validation of the melanogenic effect was first attempted using re-supplies of selected hits from sources other than Prestwick Chemical; the only exception was Compound C+, due its low commercial availability – Table III.1. The melanin contents of SK-Mel-23 cells treated with 10 μM of Compound C+, Compound D+ and Compound C- for 72 h were not statistically different from those obtained in the primary screening assay ($p > 0.05$). Contrarily, significantly lower melanin contents were obtained, after incubation with melanogenesis inducers Compound A+ and Compound B+ ($p \leq 0.05$). Lower than expected was also the inhibition of melanogenesis by Compound A-, Compound B- and Compound D-; however, only melanins contents of cells treated with Compound A- were considered statistically different. Despite some variations in the extent of melanogenesis modulation, for all compounds, the type of modulatory effect in SK-Mel-23 cells was according to the expected.

III.2.2. DOSE-RESPONSE ASSAY OF SELECTED HIT COMPOUNDS

As a second step in the confirmatory screening, the dose-response of selected hit compounds was tested,

and their performance compared with well-established agents of melanogenesis modulation. This analysis was performed in SK-Mel-23 and in SK-Mel-1 (a less pigmented melanoma cell line). A cell viability assay was included in this study to avoid envisaged outcomes due to possible cytotoxicity effects.

III.2.2.1. DEFINING THE NON-CYTOTOXIC RANGE OF DRUG CONCENTRATIONS

The extent of cytotoxicity from every condition tested was quantified as a percentage of cell viability, compared to vehicle control (DMSO 1%) – Figures III.2 to III.5. Treatment conditions where the mean viability was higher or not statistically lower than 80% (t test; $p > 0.05$) were considered as non-cytotoxic. Bellow that, the cytotoxicity was ranked as follow: within 80-60% as weak, 60-40% as moderate and below 40% as strong, respectively (ranges according to ISO 10993-5).

The treatments with 1 to 10 μM of hit inducers (Compound A+, Compound B+, Compound C+ and Compound D+) were mostly well tolerated by SK-Mel-23 cells – Figure III.2 (also Supplementary Material, Table III.S2). The exceptions were those using 10 μM of Compound C+ for 48 and 72 hours which caused weak cytotoxicity. For the two highest concentrations tested (50 and 100 μM), the cytotoxicity was evident for all inducers, though Compound A+ and especially Compound B+ were less toxic than Compound C+ and Compound D+.

Under the same range of concentrations, SK-Mel-1 cells were in general more sensitive to the tested inducers – Figure III.3 (also Supplementary Material, Table III.S2). Regarding Compound C+, besides the decrease in cell viability verified for the incubation with 10 μM for 48 h and 72 h, moderate cytotoxicity was already observed at 72 hours in cells treated with 5 μM of this compound (64.1 ± 1.5%). The treatment with 10 μM of Compound A+ for 72 h also caused a slight decrease in cell viability (60.8 ± 1.5%; weak to moderate cytotoxicity). Besides, Compound D+ was particularly toxic to SK-Mel-1 (5 μM, 48 h: 73.0 ± 2.3%; 5 μM, 72 h: 44.1 ± 4.2%; 10 μM, 48 h: 44.8 ± 2.3%; 10 μM, 72 h: 18.0 ± 1.9%). Among the four inducers tested, only Compound B+ was better tolerated by SK-Mel-1 cells, with mild cytotoxicity just being noticed at 72 h for the treatment with 50 (69.9 ± 3.3%) and 100 μM (73.5 ± 4.5%).

The inherent differences of adherent and suspension melanoma cells can justify the higher overall sensitivity of SK-Mel-1 to low concentrations of hit inducers (Supplementary Material, Table III.S2). Apart from this deferent sensitivity, the toxicity of the drugs was very consistent between the two cell models; the inducers less toxic were Compound B+ and Compound A+, followed by Compound C+ and Compound D+.

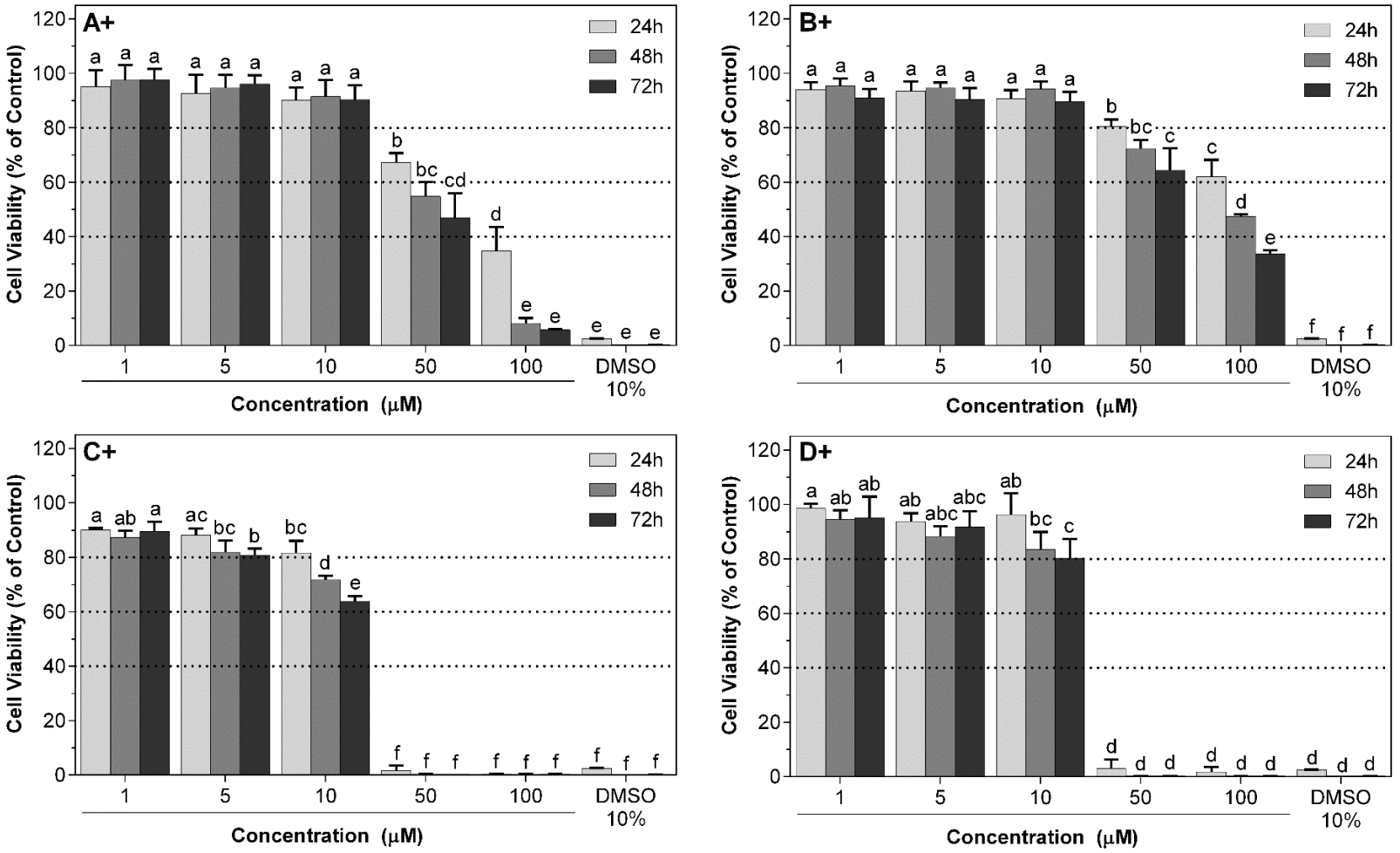


Figure III.2. SK-Mel-23 cell viabilities (MTT assay) determined at 24, 48 and 72 h of exposure to different concentration of hit inducers. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define cytotoxicity ranges: non-cytotoxicity > 80%; weak 80-60%; moderate 60-40%; strong < 40%. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

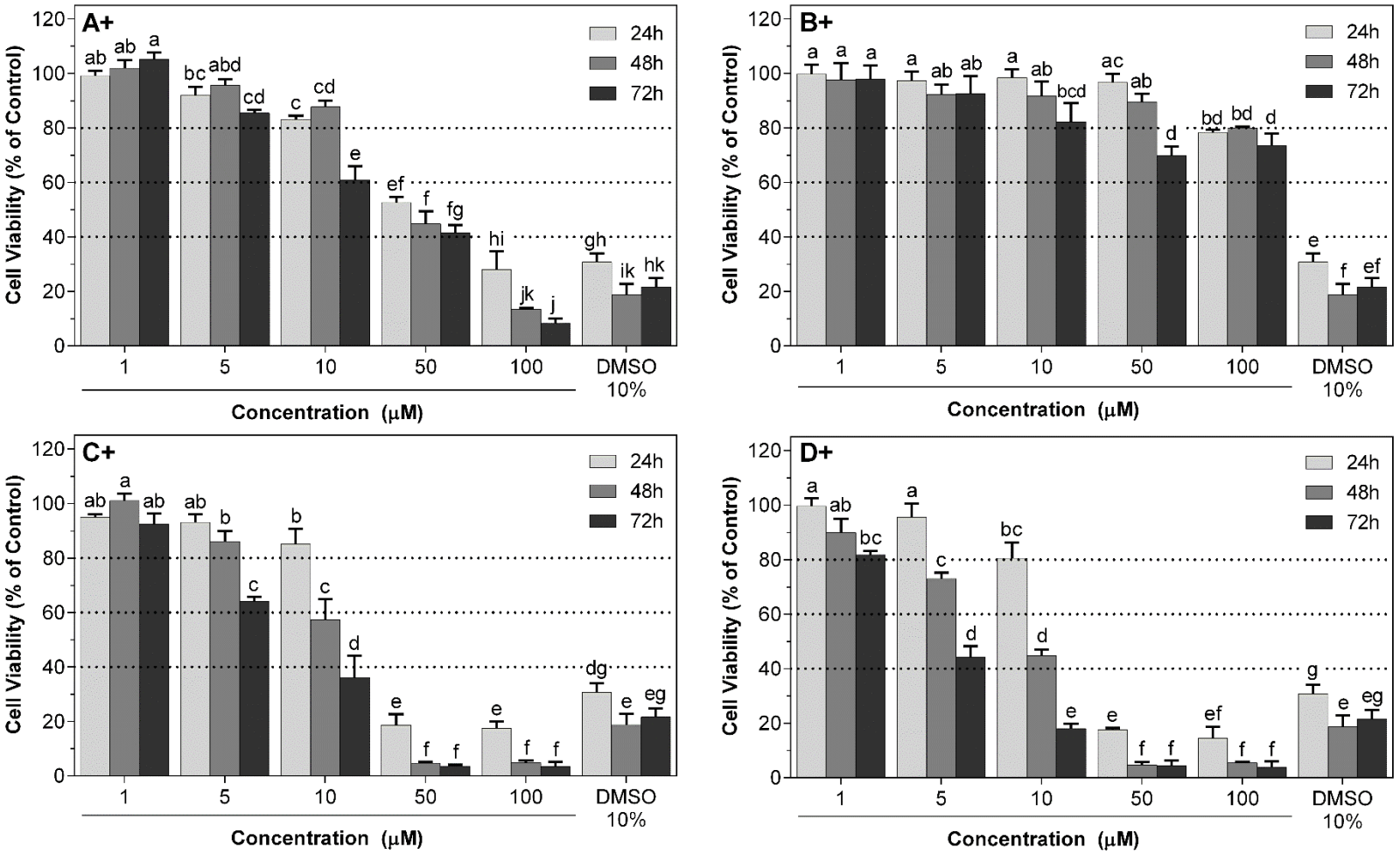


Figure III.3. SK-Mel-1 cell viabilities (MTT assay) determined at 24, 48 and 72 h of exposure to different concentration of hit inducers. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define cytotoxicity ranges: non-cytotoxicity > 80%; weak 80-60%; moderate 60-40%; strong < 40%. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

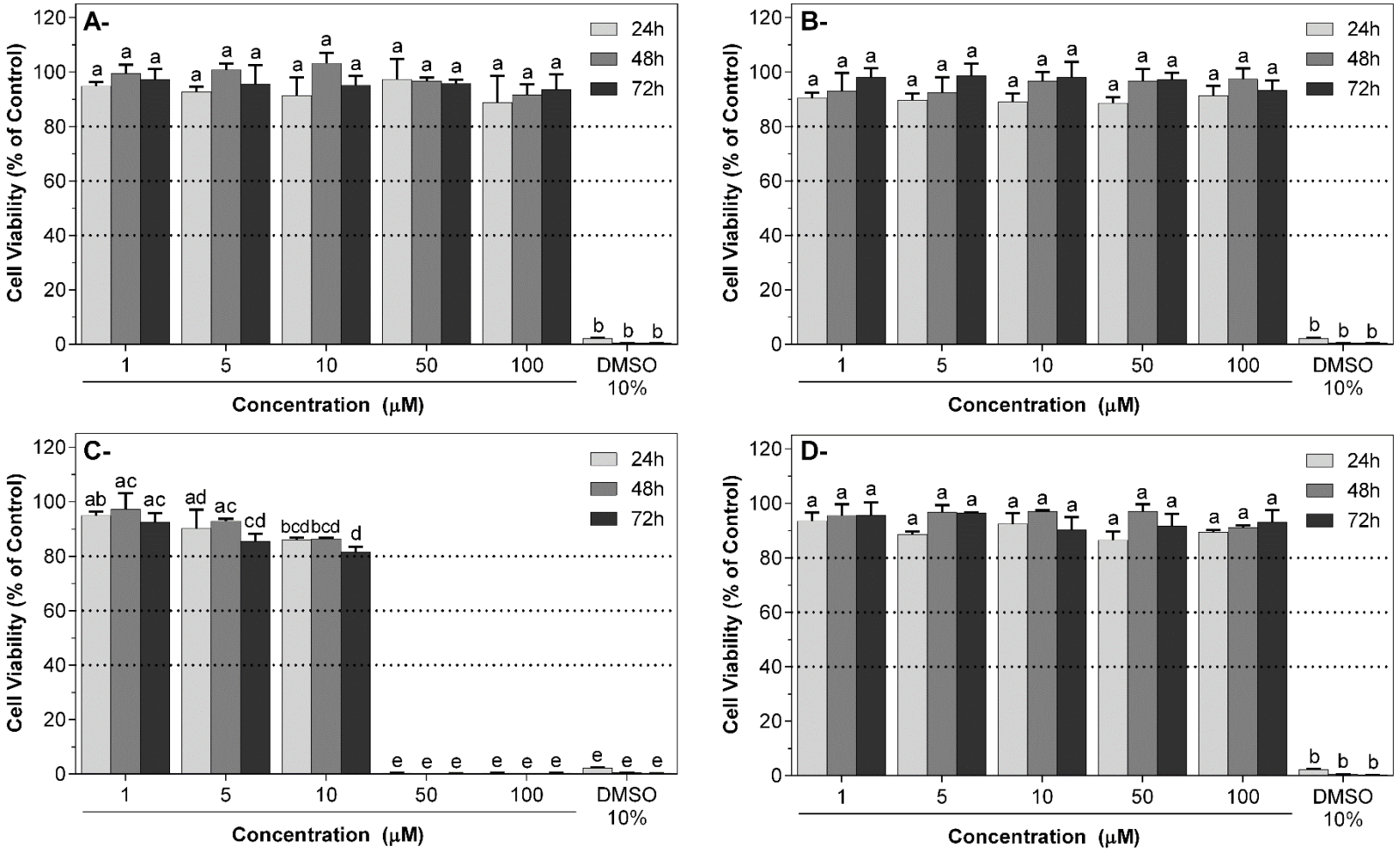


Figure III.4. SK-Mel-23 cell viabilities (MTT assay) determined at 24, 48 and 72 h of exposure to different concentration of hit inhibitors. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define cytotoxicity ranges: non-cytotoxicity > 80%; weak 80-60%; moderate 60-40%; strong < 40%. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

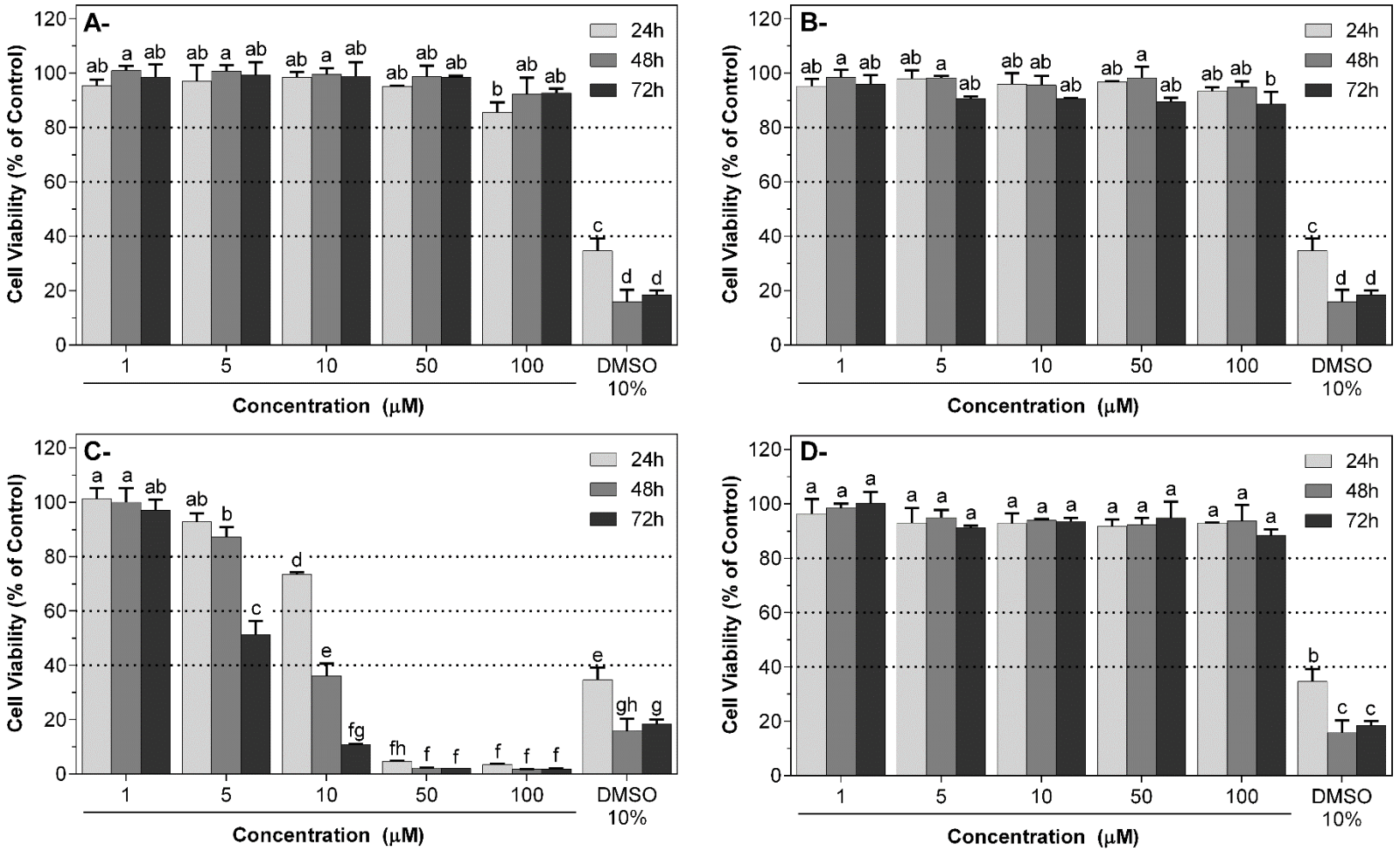


Figure III.5. SK-Mel-1 cell viabilities (MTT assay) determined at 24, 48 and 72 h of exposure to different concentration of hit inhibitors. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define cytotoxicity ranges: non-cytotoxicity > 80%; weak 80-60%; moderate 60-40%; strong < 40%. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

The hit inhibitors Compound A-, Compound B- and Compound D- were not cytotoxic to SK-Mel-23 or SK-Mel-1 in the whole range of concentrations tested (1-100 μM) – Figure III.4 and Figure III.5 (also Supplementary Material, Table III.S2). In contrast, the treatment with 50 or 100 μM of Compound C- induced an almost complete loss of viability in both cell lines. SK-Mel-1 seems to be particularly sensitive to this compound (Supplementary Material, Table III.S2), and cytotoxicity was verified even at lower concentrations (5 μM, 72h: 51.4 ± 4.9%, moderate cytotoxicity; 10 μM, 24h: 73.5 ± 0.7%, weak cytotoxicity; 10 μM, 48h: 36.1 ± 4.6%, strong cytotoxicity; 10 μM, 72h: 10.9 ± 0.2%, strong cytotoxicity).

III.2.2.2. MODULATION OF THE PRODUCTION OF MELANIN

The melanin production in SK-Mel-23 and SK-Mel-1 cells was evaluated as function of the hit drugs concentration. At every condition tested, intracellular melanin was determined as a percentage of the vehicle control (1% DMSO); this treatment do not interfere with the synthesis of melanin, as no significance differences were found between the melanin contents of cells cultured with vehicle control or without any supplement. A statistically significant effect of drug concentration on melanin content was verified (two-way ANOVA, $p \leq 0.05$); data obtained with concentrations that cause a cytotoxic effect across all times of incubations (24, 48 and 72 h) were excluded from the statistical analysis. Thus, the modulation of melanogenesis by all hit compounds can be considered dose-dependent.

In the whole range of non-cytotoxic concentrations, Compound A+ (1-10 μM), Compound B+ (1-50 μM), Compound C+ (1-10 μM) and Compound D+ (1-10 μM) promoted a mostly dose-dependent stimulation of melanogenesis in SK-Mel-23 – Figure III.6. The treatment with different concentrations of Compound A+, Compound B+, Compound C+ or Compound D+ was accountable for 81.42%, 93.64%, 90.38% and 73.93% of all variance in the melanin content of cells, respectively (Supplementary Material: Table III.S3, concentration as a source of variation). The time of incubation also contributed significantly to the modulation of melanogenesis, particularly when it involved the use of Compound A+ or Compound D+ (Supplementary Material: Table III.S3, time as a source of variation). As shown in Figure III.6, higher melanin contents were obtained when cells were incubated with 5 or 10 μM of Compound A+ for 72 hours ($p \leq 0.05$), compared to the incubations for 24 or 48 hours. In contrast to Compound A+, it is possible to observe an initial burst in pigment production at 24 h followed by a decrease over time in the melanin content of cells treated with 5 μM ($p \leq 0.05$, compared to treatment for 72 h) or 10 μM ($p \leq 0.05$, compared to treatments for 48 or 72 h) of Compound D+. This decrease could be related to a complete metabolization of the compound during the first 24 hours and/or extrusion phenomena

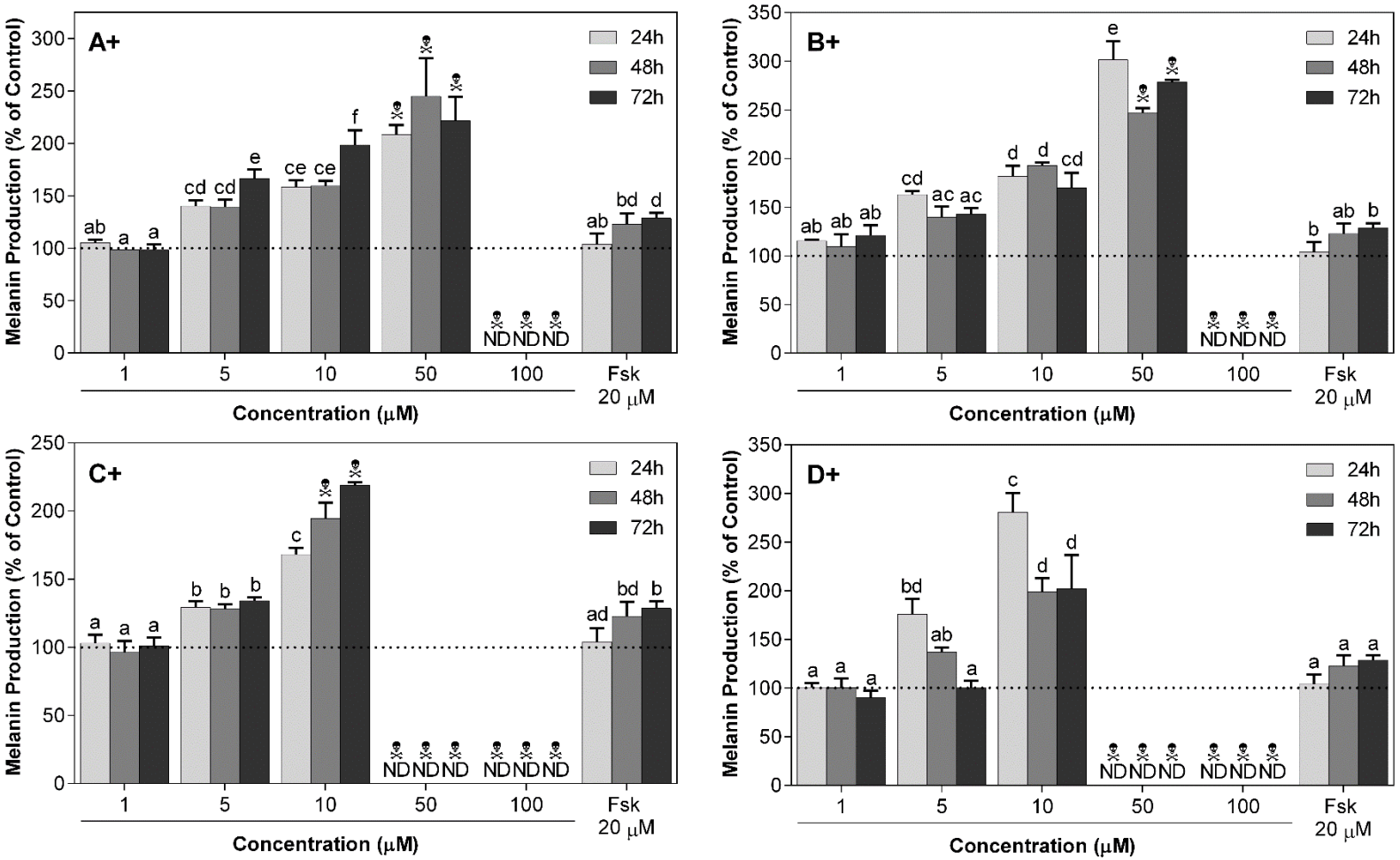


Figure III.6. Effect of various concentrations of hit inducers on melanin production of SK-Mel-23, over time. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

leading to decreased values of intracellular melanin per total protein.

In conclusion, the conditions for maximal *in vitro* stimulation of melanogenesis in SK-Mel-23 with the hit inducers were set as follow: Compound A+, 10 μM, 72 h (198.5 ± 13.9%); Compound B+, 50 μM, 24 h (301.5 ± 19.2%); Compound C+, 10 μM, 24 h (168.1 ± 5.0%); Compound D+, 10 μM, 24 h (280.4 ± 20.1%). Importantly, those melanin contents are higher (two-way ANOVA, $p \leq 0.001$) when compared to the melanin content of SK-Mel-23 cells incubated with Fsk (20 μM, 72 h: 128.6 ± 4.9%), a standard treatment for the stimulation of melanin production – Figure III.6. Fsk is an activator of adenylyl cyclase (AC), an enzyme of the cAMP/PKA signalling pathway involved in the positive regulation of melanogenesis [12,512].

The melanin contents of SK-Mel-1 cells treated with hit inducers were also significantly higher (two-way ANOVA, $p \leq 0.01$) when compared to melanin content of cells treated with Fsk (20 μM, 72 h: 140.5 ± 1.9%) – Figure III.7. However, some differences in the dose-response over time were found between the two melanoma cell lines.

Regarding Compound A+ (1-10 μM), the stimulation of melanogenesis was also mostly dose-dependent (80.60% of all variance in melanin content is attributable to concentration) but the time of incubation did not seem to have a direct effect on it – Figure III.7 (also Supplementary Material, Table III.S3). The production of melanin was maximal after an incubation of SK-Mel-1 cells with 10 μM of Compound A+ (48 h: 203.6 ± 14.0%).

For Compound B+, 87.56% of all variance in melanin content was attributable to the treatment of SK-Mel-1 with different concentrations of the compound (Supplementary Material, Table III.S3). A dose-dependent stimulation of melanogenesis was verified for concentrations ranging from 1 to 50 μM, but the incubation of cells with 100 μM of Compound B+ caused an almost complete loss of the cell pigment, with melanin being below the limits of detection or quantification – Figure III.7. This bleaching effect was also verified in SK-Mel-23 (Figure III.6), but the cytotoxicity caused by this concentration across all incubation times does not allow to infer about a possible dose-dependent dual effect of Compound B+ on melanogenesis; in SK-Mel-1, the depigmenting effects verified at 24 and 48 h are not related to cellular toxicity. Taking this into account, as an *in vitro* inducer of melanogenesis in SK-Mel-1, Compound B+ must be used at a concentration of 50 μM, preferably for 24 h (304.5 ± 47.0%); longer periods of incubation caused a decrease in melanin content across all stimulatory concentrations which it is probably the cause for the medium effect presented by time of incubation (5.137% of all variance in melanin in content;

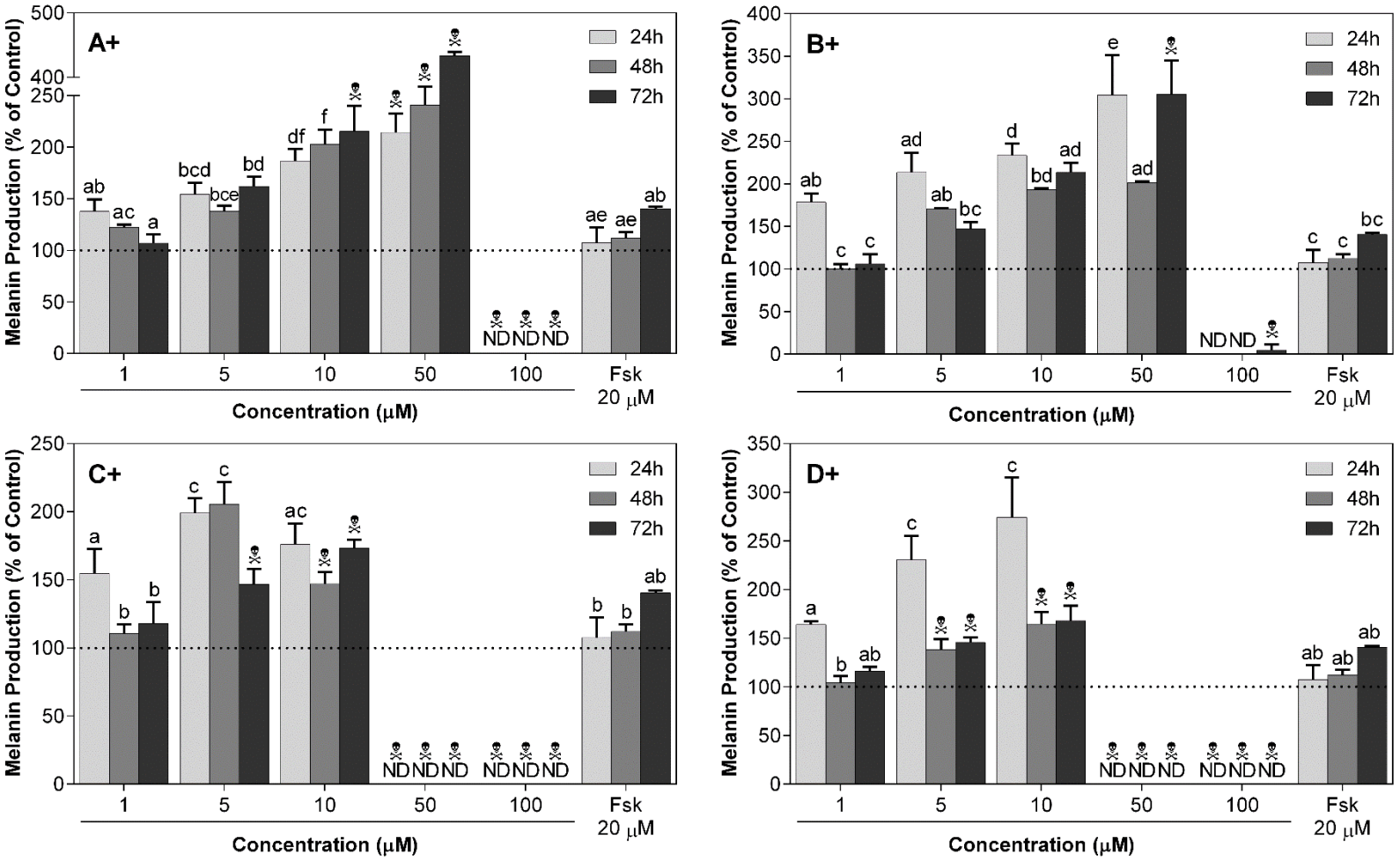


Figure III.7. Effect of various concentrations of hit inducers on melanin production of SK-Mel-1, over time. Fsk: Forskolin. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

Supplementary Material, Table III.S3).

Concerning the treatment of SK-Mel-1 cells with Compound C+ (1-10 μM) or Compound D+ (1-10 μM), the percentages of variation in melanin content attributed to each of the main effects (and their interaction) are also listed in Supplementary Material, Table III.S3. However, it is important to point out that the two-way ANOVA analysis took into consideration some melanin contents determined under conditions of moderate to strong cytotoxicity. These are melanin contents obtained for times of incubation with concentrations that do not cause a time-independent cytotoxicity; as an example, the melanin content of cytotoxic treatments with 10 μM of Compound C+ for 48 and 72 hours were included in the analysis in order to consider the non-cytotoxic treatment of cells with the same concentration for 24 hours. Consequently, the percentages of variation are expected to be misestimate at some level and a point to point analysis of the non-cytotoxic conditions must be considered instead for the assessment of the effect of Compound C+ and Compound D+ in SK-Mel-1 cells. Thus, attending to Figure III.7, cells treated with 1 μM of Compound C+ for 24 h presented higher melanin contents than cells treated with the same concentration for longer periods ($p \leq 0.05$); the same behaviour was shown by cells treated with Compound D+. Regarding the effect of concentration, the incubation of SK-Mel-1 cells with 5 μM of Compound C+ promoted an increase in melanin contents higher than those observed after the incubation with 1 μM for the same periods, 24 h ($p \leq 0.05$) and 48 h ($p \leq 0.0001$). The melanin content of cells treated with 1 μM of Compound D+ was also lower than those presented by cells treated with 5 μM ($p \leq 0.05$) or 10 μM ($p \leq 0.0001$); this comparison can only be performed for cells treated for 24 hours due to the extended cytotoxicity verified for other times of incubation. Summing up, the best non-cytotoxic treatments of SK-Mel-1 involve the incubation of cells with 5 μM of Compound C+ for 48 h (205.6 ± 16.2%) and incubation with 5 μM of Compound D+ for 24 h (230.3 ± 24.9%). Regarding Compound D+, 10 μM for 24 h was the treatment that provided the higher mean melanin content. However, although the cell viability is not statistically different from 80%, a trend towards a cytotoxic effect can be noticed. In this sense, it is preferable to use a safer concentration (5 μM) with a comparable ($p > 0.05$) melanogenic effect.

In SK-Mel-23, the profiles obtained with hit inhibitors Compound A-, Compound B- and Compound D- resemble a U-shaped dose-response relationship (hormetic response) – Figure III.8 [513]. In the range of concentrations from 1 to 10 μM, the melanin contents gradually decreased. Then, for higher concentrations, the inhibition of melanin production became less evident or even imperceptible. It is possible that compensatory pathways were activated in response to a disruption in homeostasis which

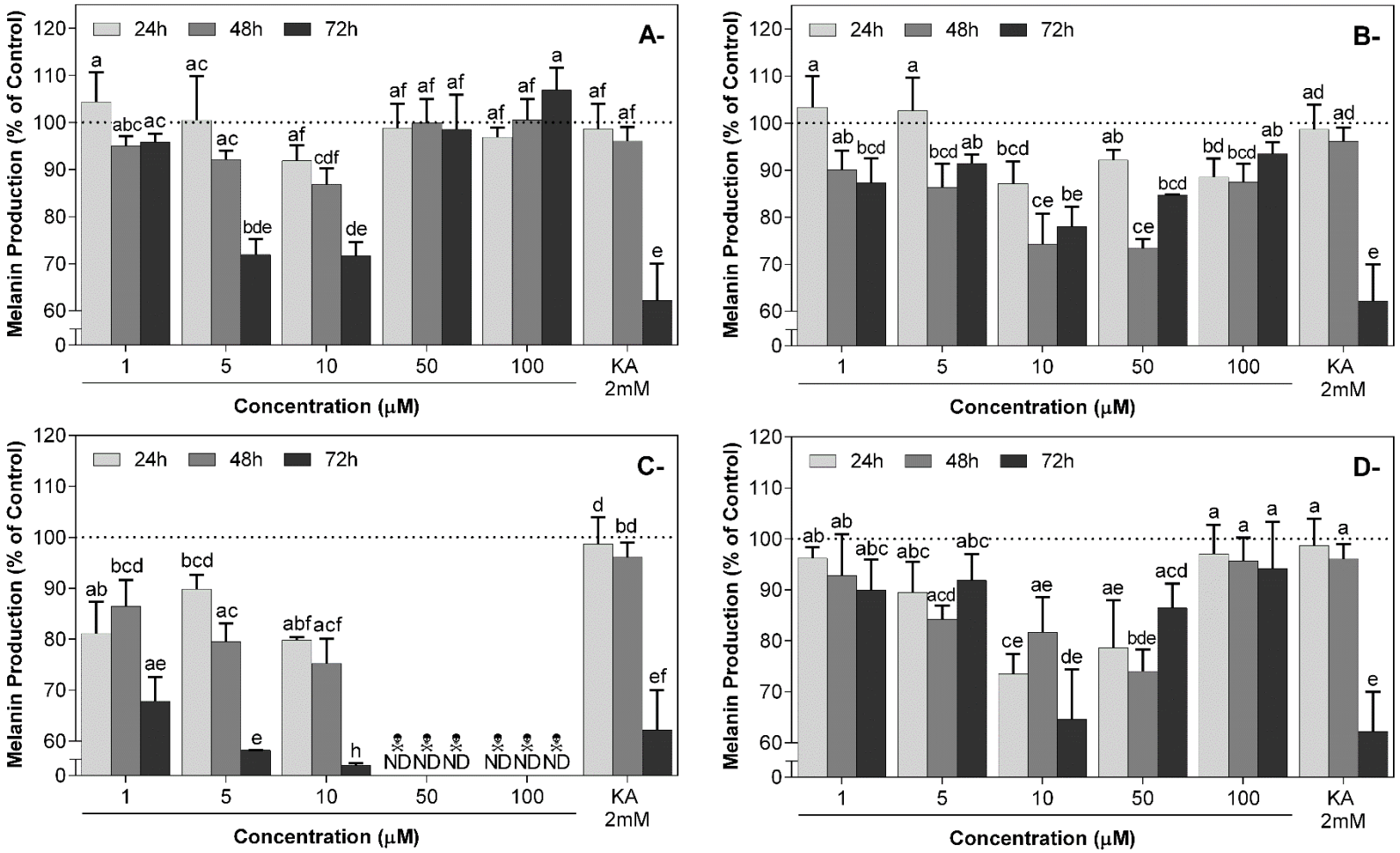


Figure III.8. Effect of various concentrations of hit inhibitors on melanin production of SK-Mel-23, over time. KA: Kojic acid. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

counteract the anti-melanogenic effects of these compounds. Alternatively, it is also possible that, depending on the dose, those compounds act on different (conflicting) pathways of melanogenesis regulation; although this kind of cellular response towards *in vitro* melanin production is very uncommon, it has been reported before [514]. Consequently, 41.45%, 33.64% and 61.81% of all variance in melanin content was attributable to the treatment of cells with different concentrations of Compound A-, Compound B- and Compound D-, respectively (Supplementary Material, Table III.S4). Nonetheless, the modulation of melanogenesis by Compound A- and Compound B- was not affected only by the concentration of compounds used. In the case of Compound A-, the interaction between concentration and time of incubation was accountable for 31.54% of all variance in melanin content (Supplementary Material: Table III.S4, interaction as a source of variation). As seen in Figure III.8, for concentrations that cause a depigmenting effect (5 and 10 μM), melanin production over time was lower, the higher the concentration used. In the case of Compound B-, the time of incubation was accountable for 32.15% of all variance in melanin content (Supplementary Material, Table III.S4). For most concentrations, and especially those causing the more pronounced depigmenting effect (10 and 50 μM), a decrease in melanin production occurred from 24 to 48 hours followed by a recover of pigment at 72 hours of incubation with Compound B-.

Compound C- was the only hit inducer that did not show signs of a dose-dependent dual effect on the production of melanin – Figure III.8. It inhibited melanogenesis across the whole range of non-cytotoxic concentrations used (1-10 μM), but mostly over the time of incubation (66.27% of all variance on melanin content; Supplementary Material, Table III.S4).

Considering the data presented, the *in vitro* conditions to obtain the lower melanin contents in SK-Mel-23 are as follow: Compound A-, 10 μM, 72 h (71.7 ± 2.9%); Compound B-, 10 μM, 48 h (74.3 ± 6.5%); Compound C-, 10 μM, 72 h (31.1 ± 6.9%); Compound D-, 10 μM, 72h: 64.6 ± 9.8%). Notably, those compounds had a depigmenting effect in SK-Mel-23 that it is better or at least comparable to KA; the treatments were considered comparable when no statistical significant differences (two-way ANOVA, $p > 0.05$) were found between melanin contents of cell treated with the hit inhibitors and KA. Kojic acid is a competitive inhibitor of tyrosinase activity during L-DOPA oxidation, and widely used as an inhibitor of *in vitro* and *in vivo* (skin) melanin production [515,516].

Compared to SK-Mel-23, the melanogenic effects of hit inhibitors in SK-Mel-1 were quite different, and a U-shaped dose-response relationship was not verified for any of the compounds. – Figure III.9. Despite the use of different concentrations of Compound A- or Compound D- had a medium effect (melanin

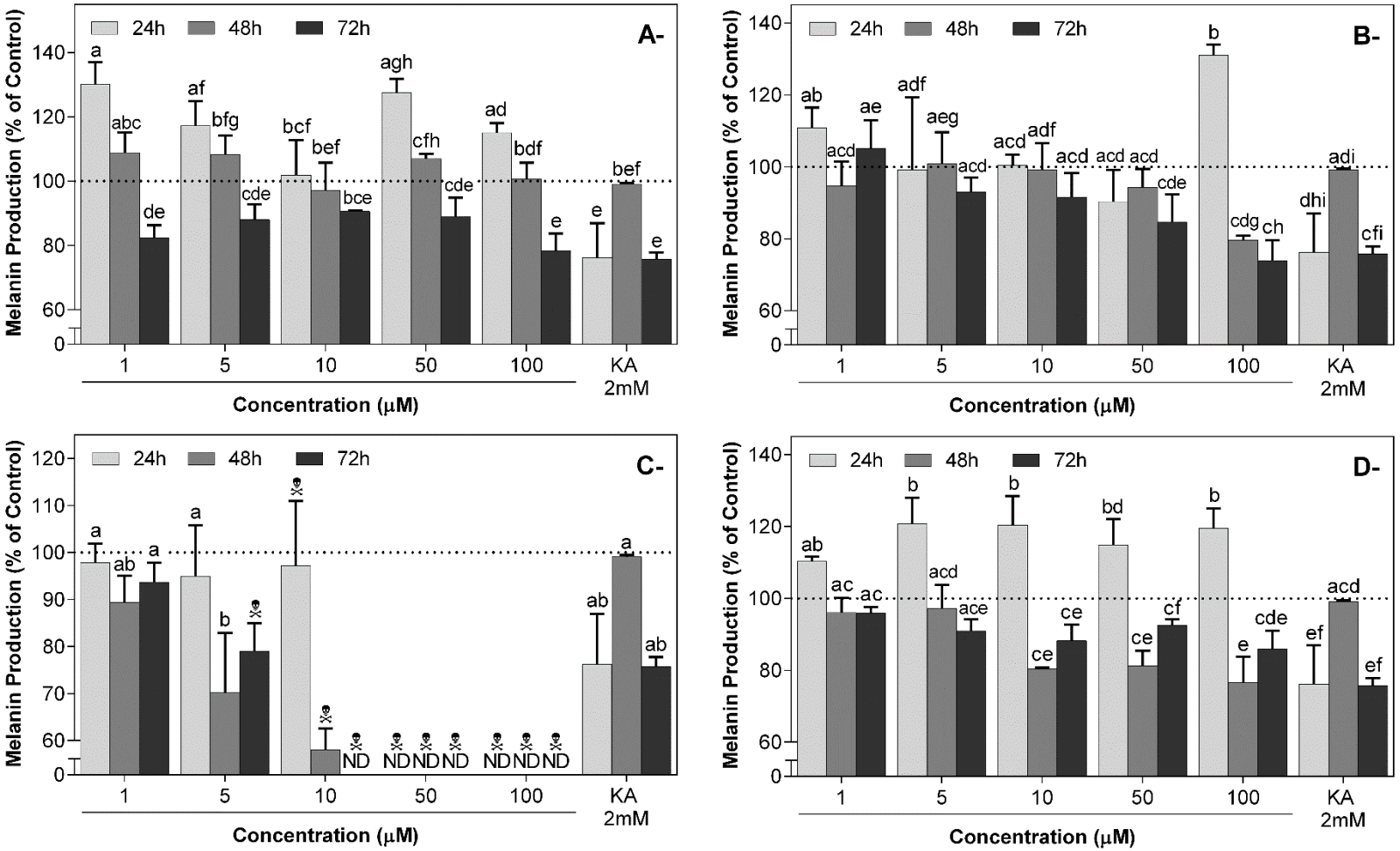


Figure III.9. Effect of various concentrations of hit inhibitors on melanin production of SK-Mel-1, over time. KA: Kojic acid. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

contents obtained with 100 μM were slight lower, compared to the other concentrations), their modulation of melanogenesis was mostly time-dependent: 71.76% and 77.57% of all variance is explained by the use of different times of incubation, respectively – Supplementary Material, Table III.S4. For Compound A-, after an increase at 24 h of incubation, melanin contents decreased over time with a depigmenting effect being noticed at 72 h (78.3 ± 5.4%). For Compound D-, a stimulation of melanogenesis also occurred within the first 24 h, but the depigmenting effect was verified earlier, at 48 h (76.5 ± 7.3%); at 72 h, the effect did not disappear, but becomes less evident with cells increasing melanin production. For both compounds, this behaviour over time was shown for every concentration tested.

Regarding Compound B- (1-100 μM), concentration and time also had a significant effect, but contrarily to Compound A- and Compound D-, their interaction explains most of all variance in melanin content (44.22%) – Supplementary Material, Table III.S4. This is well representative of the fact that noteworthy modulation of melanogenesis only occurs with high concentrations (especially 100 μM), with the kind of modulatory activity being dependent on the time of incubation: stimulation of melanogenesis at 24 h and inhibition of melanin production at longer periods of incubation (minimal melanin content at 72 h: 74.0 ± 5.7%).

Finally, in the case of Compound C- (1-10 μM), the melanin content of cells treated with 5 μM for 48 hours was 70.1 ± 12.8%. This was the only case of effective melanogenesis inhibition; the other treatments failed to promote a relevant decrease in melanin content (1 μM for 24, 48 and 72 h; 5 μM for 24 h) or induced moderate to strong cytotoxicity. Consequently, the data obtained with the two-way ANOVA is presented in Supplementary Material (Table III.S4), but it was not used to infer about the impact of Compound C- in the production of melanin in SK-Mel-1. Irrespective of the differences in melanogenesis inhibition amongst the two cell lines, in SK-Mel-1, the depigmenting effect of all compounds was still comparable (two-way ANOVA, $p > 0.05$) to the effect of KA (2 mM, 72 h: 75.8 ± 2.1%).

III.2.2.3. EFFECT ON THE ACTIVITY OF TYROSINASE

Tyrosinase is the rate-limiting enzyme in the canonical pathway of melanin biosynthesis, catalysing the two initial non-spontaneous steps: hydroxylation of L-tyrosine (L-Tyr) to L-DOPA, and the oxidation of the latter to dopaquinone [2,3,129,131]. The catalytic activity of tyrosinase can be directly regulated by allosteric modulators. Additionally, intracellular activity of tyrosinase can be regulated indirectly by interfering with its transcription, translation, processing, maturation, or degradation rate. Being closely

related to melanin production, the study of tyrosinase activity is a good starting point to disclose the cellular targets of melanogenic modulators.

In the first step of this study, the activity of mushroom tyrosinase was measured following incubation with hit compounds – Supplementary Material, Figure III.S1; mushroom tyrosinase is often used as a substitute model for the human homologue. None of the inducers or inhibitors under test influenced the rate of L-DOPA oxidation, suggesting that their melanogenic effect is not related to a direct interaction with the model enzyme. However, it is important to point out that, although mushroom tyrosinase has been used in many studies attempting to identify new melanogenesis modulators or their mechanism of action, there is emerging evidence that the modulation of its activity may require molecular motifs distinct from those of the human counterpart [517]. Thus, a direct interaction with human tyrosinase cannot be entirely ruled out as the mechanism leading to stimulation or inhibition of melanin production in SK-Mel-23 and SK-Mel-1 by the hit compounds.

In a second experiment, the intracellular tyrosinase activity was examined following the treatment of SK-Mel-23 and SK-Mel-1 cells with the hit compounds. As expected, under conditions of maximal *in vitro* stimulation of melanogenesis, the activity of tyrosinase from cells treated with Compound A+, Compound C+ and Compound D+ was found to be increased in a significant way – Figure III.10 (also Supplementary Material, Figure III.S2 and Figure III.S3). In contrast, mechanism other than the modulation of intracellular tyrosinase activity seems to govern the melanogenic effect of Compound B+. Despite some pro-melanogenic conditions are associated with increased activity (Supplementary Material, Figure III.S2 and Figure III.S3), maximal stimulation of melanogenesis by Compound B+ is attained without changes in the activity of enzyme or even under conditions that promotes its inhibition – Figure III.10. Although it is not common, this phenomenon had been reported before. Nakajima *et al.* showed that the treatment of *in vitro* cultured normal human melanocytes with arbutin cause an increase in melanin production along with the inhibition of intracellular tyrosinase activity; however, the possible mechanisms responsible for the stimulation of melanin production by arbutin were not further explored [518]. Since the mechanism of action involved in the therapeutic usage of Compound B+ is also unknown, without further studies, it is impossible at this moment to predict how Compound B+ may act on melanocytes to stimulate melanogenesis.

Other mechanism than the modulation of intracellular tyrosinase activity also seems to rule the inhibition of melanin production in SK-Mel-23 by Compound A-, Compound B- and Compound D-. For those compounds, none of the conditions that cause maximal inhibition of melanin production were

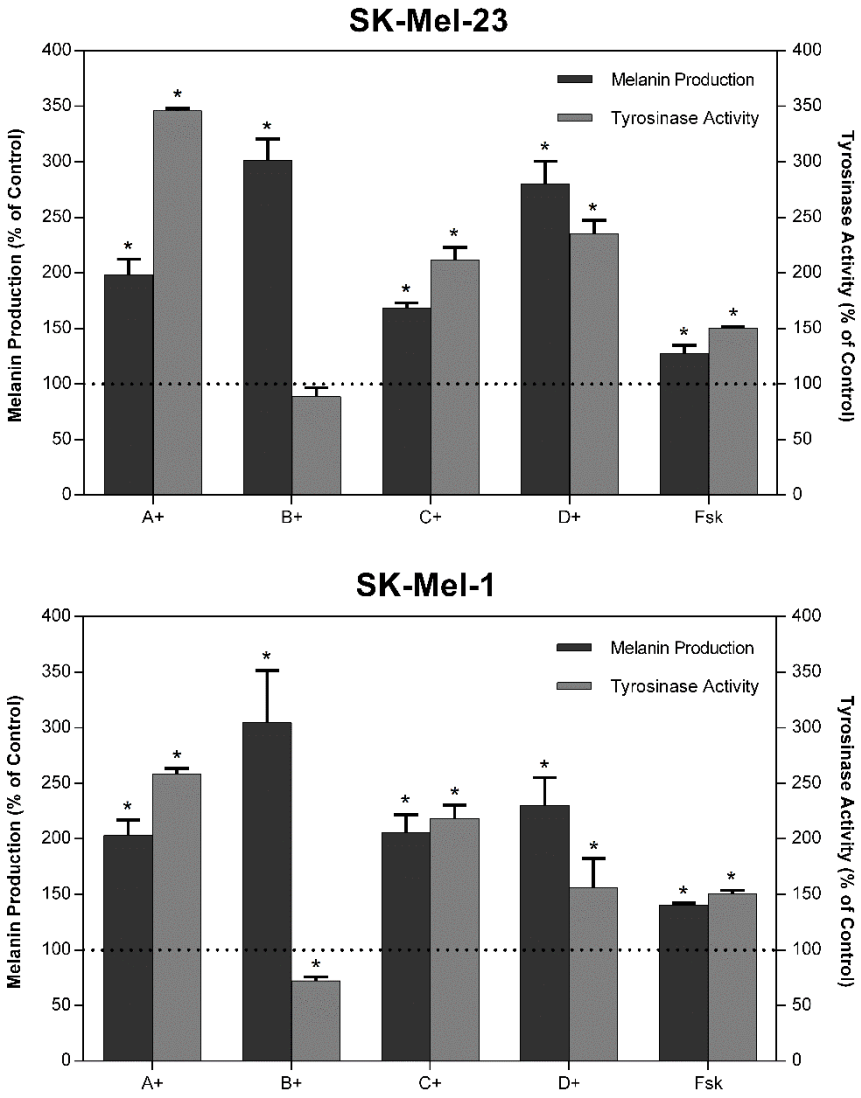


Figure III.10. Effect of hit inducers on melanin production and tyrosinase activity of SK-Mel-23 and SK-Mel-1, cultured under *in vitro* conditions for maximal melanogenesis stimulation. SK-Mel-23: 10 μM Compound A+, 72 h; 50 μM Compound B+, 24 h; 10 μM Compound C+, 24 h; 10 μM Compound D+, 24 h; 20 μM Fsk, 48 h. SK-Mel-1: 10 μM Compound A+, 48 h; 50 μM Compound B+, 24 h; 5 μM Compound C+, 48 h; 5 μM Compound D+, 24 h; 20 μM Fsk, 72 h. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin or tyrosinase activity (100% of vehicle control). Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to the 1% DMSO control (100%). Fsk: forskolin.

accompanied by significant changes in the intracellular tyrosinase activity – Figure III.11 (also Supplementary Material, Figure III.S4). In SK-Mel-1, the inhibition of melanogenesis by Compound D- was also largely independent of the intracellular tyrosinase activity, and a statistically significant increase was

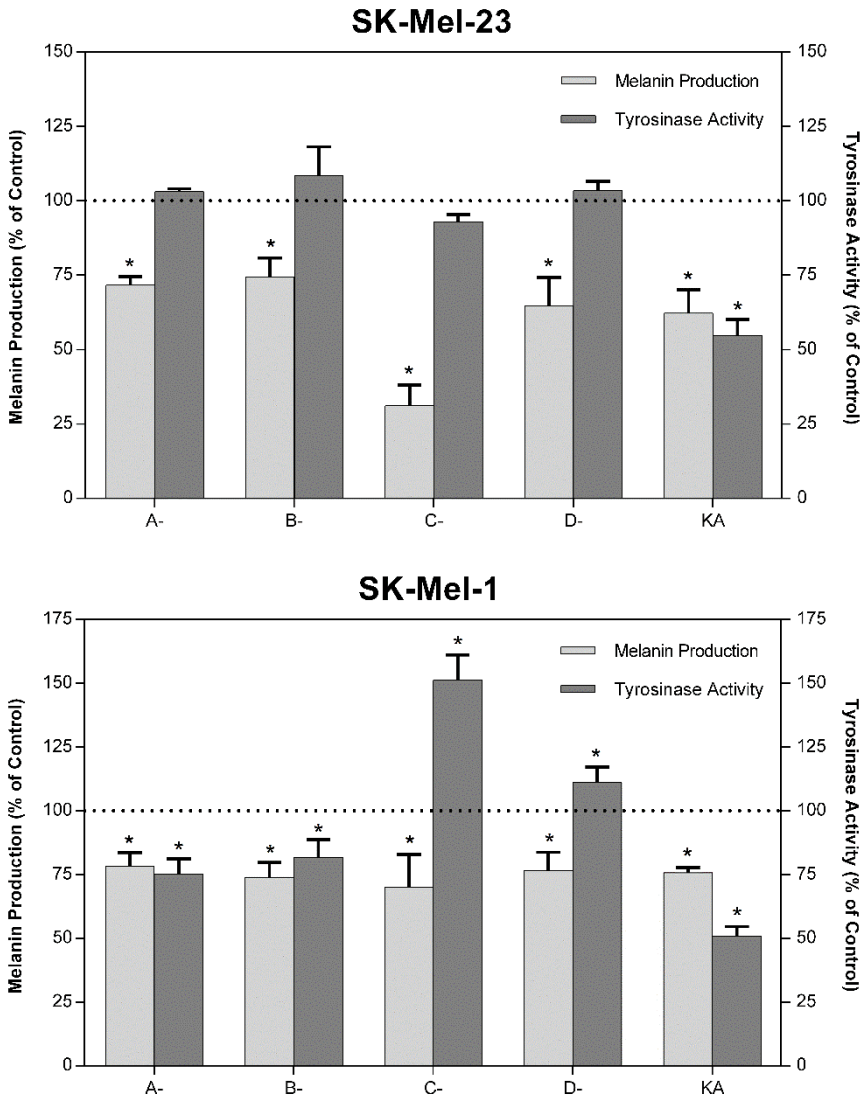


Figure III.11. Effect of hit inhibitors on melanin production and tyrosinase activity of SK-Mel-23 and SK-Mel-1, cultured under *in vitro* conditions for maximal melanogenesis inhibition. SK-Mel-23:10 μM Compound A-, 72 h; 10 μM Compound B-, 48 h; 10 μM Compound C-, 72 h; 10 μM Compound D-, 72 h; 2 mM KA, 72 h. SK-Mel-1: 100 μM Compound A-, 72 h; 100 μM Compound B-, 72 h;5 μM Compound C-, 48 h; 100 μM Compound D-, 48 h; 2 mM KA, 72 h. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal production of melanin or tyrosinase activity (100% of vehicle control). Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to 1% DMSO control (100%). KA: kojic acid.

even observed after the treatment of cells with 100 μM of the compound for 48 h – Figure III.11 (also Supplementary Material, Figure III.S5). Contrarily, the conditions for maximal inhibition of melanogenesis in SK-Mel-1 by Compound A- and Compound B- induced a significant decrease in the activity of the

enzyme activity, as expected – Figure III.11. Amongst the hit inhibitors, the use of Compound C- presented the most curious variations in tyrosinase activity. Attending to Figure III.11, melanin content obtained following incubation of SK-Mel-23 with 10 μM of Compound C- for 72 h (maximal inhibition of melanin production) do not seem to be related to a decrease in tyrosinase activity. Nonetheless, the incubation with low concentrations (1 or 5 μM) for the same period cause decreases in melanin contents that are escorted by inhibition of tyrosinase activity (Supplementary Material, Figure III.S4). Moreover, inhibition of melanogenesis is also attained under conditions that cause levels of intracellular tyrosinase activity (10 μM, 24 and 48 h) comparable to those presented by hit inducers (Supplementary Material, Figure III.S4). A considerable increase in tyrosinase activity was also found for the only case of significant melanogenesis inhibition by Compound C- in SK-Mel-1 cells – Figure III.11.

In the literature, there are several cases of inconsistencies between melanin content and intracellular tyrosinase activity that could help to explain the results here obtained with the hit inhibitors.

Inhibition of melanogenesis without relatable changes in the measurements of intracellular tyrosinase activity was attained with some tricyclic antidepressants [519] and quinolines [520]. The depigment effects verified were found to be associated with altered trafficking of tyrosinase and other related proteins. In melan-a cells treated with these compounds, although tyrosinase was normally expressed and maturated (assuring functionality), at some point, its normal trafficking was disrupted, and the enzyme ended trapped in organelles other than melanosomes. Homochlorcyclizine has likewise been reported to inhibit melanogenesis in B16 melanoma 4A5 cells without directly or indirectly inhibit tyrosinase activity [521]. Despite the particular drug mechanism had not been disclosed, the authors proposed that it could be related to: the blockage of L-phenylalanine transporters on the membrane of melanocytes, inhibition of phenylalanine hydroxylase (converts L-phenylalanine into L-Tyr) or inhibition of L-tyrosine hydroxylase isoform I (along with tyrosinase, also catalyses the conversion of L-Tyr to L-DOPA). The inhibition of solute carrier family 7 member 5 (SLC7A5) by JPH203 (now in clinical trials as an anti-cancer drug) or 2-amino-2-norbornanecarboxylic acid (BCH) had also been shown to cause a decrease in melanin content of B16F10 cells without affecting the intracellular tyrosinase activity [139]. SLC7A5 is a member of the L-type amino-acid transporter family, specialized in the transport of several aminoacids as L-Tyr; consequently, the levels of this tyrosinase substrate inside melanocytes might be reduced by inhibiting SLC7A5, causing a decrease in the production of melanin. Finally, the simplest reason for the discrepancies obtained in our study can be due to the measurement procedure itself. If tyrosinase is misplaced in the intact treated cells but not inactivated by the hit inhibitor, or if the enzyme is reversibly

inactivated, depending on the inhibitor concentration, when cell disruption occurs and the exogenous substrate is supplied to the cell lysate, the enzyme activity measured will not be representative of the anti-melanogenic effect of the compounds. Taxifolin and luteolin, which inhibit the *in vitro* production of melanin in B16F10 cells, were found to increase the protein levels of tyrosinase. When the lysates of cells, previously treated with those compounds, were used in the enzymatic assay, the rate of L-DOPA oxidation was found to be increased. On the other hand, when lysates of non-treated cells were mixed with the tested compounds, the formation of dopachrome was inhibited, suggesting that taxifolin and luteolin inhibit the production of melanin by directly inhibiting the enzyme, despite activating simultaneously some cellular pathways that cause an increase in the total enzyme content [522].

III.2.2.4. SELECTION OF COMPOUNDS FOR THE *IN VIVO* STUDY

The data available in the literature gathered with the data obtained in the dose-response assay were considered for the selection of drugs to be further tested in an *in vivo* study. Amongst the top hit inducers, Compound C+ and Compound D+ were the most cytotoxic compounds and especially harmful to SK-Mel-1 cells. Moreover, as a carcinogenic substance (category 1B), Compound C+ has a very limited applicability in cosmetic products, as stated in the Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. To avoid possible safety issues, none of them were selected for *in vivo* testing. Despite having been well tolerated and the most effective activator of melanogenesis, Compound B+ also promoted an almost complete loss of intracellular pigment content at the highest non-cytotoxic concentration tested. This phenomenon was quite unexpected, and it needs to be extensively explored before a cosmetic use in humans can be attempted. Consequently, Compound A+ was selected as the only agent of hair darkening for the *in vivo* study; this compound provided a sustained stimulation of *in vitro* melanogenesis across the whole range of non-cytotoxic concentrations.

In a clinical context, Compound A+ exerts its therapeutic effects by inhibiting PDEs, the enzymes responsible for normal degradation of second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). In melanocytes, cAMP and cGMP are known to increase the synthesis of melanin through a Protein Kinase A (PKA) [12,56,58,256,523,524] and Protein Kinase G (PKG) [525,526] dependent pathway, respectively; cGMP has been primarily associated with the UVB-induced pigmentation of skin. A cAMP- and/or cGMP-dependent stimulation of melanogenesis correlates well with the increased intracellular tyrosinase activity verified upon the treatment of SK-Mel-23 and SK-

Mel-1 cells with Compound A+, supporting the inhibition of PDEs as the trigger event responsible for its melanogenic effect. Several PDEs inhibitors have already shown a pro-melanogenic effect: cilostazol [527], sildenafil [526], vardenafil [526], theophylline and 3-Isobutyl-1-methylxanthine (IBMX) [528]. Of note, Compound A+ has been shown to act as a free radical scavenger and antioxidant. In the context of stimulation of melanin production in hair, these properties are of the highest importance since the inability of melanocytes to deal with the large amounts of oxidative species generated during the synthesis of melanin have been associated with the age-related depigmentation of hair [427,432,433].

Regarding the hit inhibitors, Compound A- and Compound D- were not included in the *in vivo* study due to safety concerns. Although none of the compounds have shown *in vitro* cytotoxicity in the melanotic cell lines used, Compound A- is a substance prohibited in cosmetic products, Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, and Compound D- have the potential to cause acute dermal toxicity. Thus, only Compound B- and Compound C- were selected as agents of hair lightening.

The pharmacological activity of Compound B- is mainly associated with the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), the enzymes responsible for the hydrolysis of neurotransmitter acetylcholine (ACh). The inhibition of cholinesterases can also explain the anti-melanogenic effect of Compound B-, at least in SK-Mel-1 cells where a decrease in intracellular tyrosinase activity was verified. By acting on the muscarinic receptors (M2R and M4R) of melanocytes, ACh is thought to inhibit AC and impair the synthesis of cAMP, causing a subsequent decrease in the expression of tyrosinase and synthesis of melanin. The negative modulation of pigment production by increased levels of ACh is reinforced by the observations of low activity of AChE in individuals with vitiligo, a disease characterized by the occurrence of depigmented spots (no melanin) in the skin [332,334,529–531].

Other known mechanisms of action of Compound B- can also account for the inhibition of melanin production verified in SK-Mel-1. Compound B- is a pharmacological activator of the heat shock factor 1 (HSF1), a facilitator of transcription, production, and accumulation of heat shock proteins (HSP) as HSP70. HSP70 provides negative feedback on melanogenesis as demonstrated by the impossibility to stimulate melanin production *in vitro* (mice melanocyte cell cultures) and *in vivo* (transgenic mice) models of HSP70 overexpression [395]. The upregulation of HSP70 has already been proposed as the action mechanism of some anti-melanogenic compounds, causing a decrease in tyrosinase protein levels [532,533].

In studies regarding its anti-inflammatory properties, Compound B- also inhibited the gene/protein

expression and activity of cyclooxygenases 1 and 2 (COX1 and COX2) and increased the levels of GSH (reduced form of glutathione). In melanocytes, COX2 knock-down markedly decreased the expression of several melanogenic factors and prevented the stimulation of melanin by α-MSH [534]. GSH causes an interruption of melanogenesis by inhibiting the translocation of tyrosinase; glutathione-treated cells exhibited complete formation of melanosomes but tyrosinase activity is predominantly located in the Golgi-associated endoplasmic reticulum of lysosomes, instead of melanosomes [535].

Recently, the ability of Compound B- to modulate the serotonergic system has also been studied. In turn, 5-HTR have been detected in pigmented cells, indicating a role in pigmentation [536]. However, a decrease in melanin production is reported to occur upon activation of 5-HT_{2B} receptor, and the current knowledge indicates that Compound B- is an agonist of 5-HT_{1A} receptor [537]. Although the actions of Compound B- towards 5-HT_{1A} receptor do not explain the inhibitory effect on melanogenesis, they can deliver some enlightenment regarding the hormetic response verified in SK-Mel-23. Activation of 5-HT_{1A} receptor is implicated in a cascade of events leading to an up-regulation of the pigmentary response and thus, it can be a competitive mechanism that obscure the anti-melanogenic effect in SK-Mel-23 cells treated with high concentrations of Compound B- – Figure III.8 [342,343].

Being a selective serotonin (5-HT) reuptake inhibitor (SSRI), Compound C- is used as an antidepressant medication. The modulation of melanogenesis by drugs with antidepressant properties is well documented in the literature, with both stimulatory [538–544] and inhibitory [519,545] effects being reported. The wide-spread effect of antidepressants on pigmentation can be related to the fact that melanocytes derived from the embryonic neural crest and share many of the same receptors and morphogenic features as neural cells [11,546]. Although the inhibition of 5-HT reuptake has not been directly implicated in modulation of melanin production, the ability of such antidepressants to exert agonist or antagonist actions towards 5-HT receptors have been proposed as their mechanisms of action. The agonistic activity of SSRI fluoxetine in the 5-HT_{1A} and 5-HT_{2A} receptors have been linked to an *in vitro* and *in vivo* increasing of pigmentation, through the upregulation of the expression of tyrosinase and other related proteins [538–540]. Since Compound C- is an inhibitor of melanogenesis, this is unlikely its mechanism of action, but it may be explanatory of the increased intracellular tyrosinase activity verified in SK-Mel-1 under *in vitro* conditions of maximal melanogenesis inhibition. Thus, to decrease the production of melanin, Compound C- may concomitantly depress the intracellular levels of L-DOPA, as showed for 6-nitroguipazine, another 5-HT uptake antagonist. The treatment with this compound inhibited the melanization of MM96E cells without affecting tyrosinase activity or tyrosine levels; consequently, the

authors proposed that 6-nitroguipazine directly decreases the intracellular levels of DOPA, either through facilitating its efflux from cells or through some other effects on its metabolism [547]. Making cells deficient in L-DOPA may not only explain the melanogenic effect of Compound C-, but also the effect of Compound A-, Compound B- and Compound D- in SK-Mel-23.

Compound C- was also able to regulate the mitogen-activated protein kinase (MAKP) pathways by elevating the levels of phosphorylated extracellular signal-regulated kinases (ERK) or decreasing the levels of phosphorylated p38-MAPK. In melanocytes, activated ERK can phosphorylate microphthalmia-associated transcription factor (MITF, Ser73), targeting it for ubiquitination and degradation [304,305,309]. Subsequently, there is a decrease in the transcription of tyrosinase (as well as other melanogenesis-related genes) and in the synthesis of melanin [56,523]. Activated p38-MAPK provides positive regulation of melanogenesis by phosphorylating CREB (cAMP responsive-element-binding protein) which in turn up-regulates the expression of MITF. Inhibiting the phosphorylation of p38-MAPK, melanin synthesis can be relieved [548–550]. Additionally, Compound C- showed the ability to decrease the intracellular concentrations of nitric oxide (NO), probably by inhibiting the nitric oxide synthases (NOS). It has been proposed that increased NO production through activation of different NOS isoforms (eNOS and inducible NOS) is responsible for excessive production of melanin in skin; NO activates guanylyl cyclase, promoting the synthesis of cGMP that up-regulates MITF [551,552]. Thus, the inhibition of NOS is expected to have an opposite effect and could be another mechanism by which Compound C- decrease the production of melanin. Finally, as for Compound B-, the inhibition of melanogenesis by Compound C- can be related to increased levels of GSH and decreased activities and gene/protein expression of COX1 and COX2 [534,535]. All those signalling pathways can explain the inhibition of melanogenesis in SK-Mel-23, for conditions where intracellular tyrosinase activity was decreased.

III.2.3. COSMETIC STUDY

A clinical study with intervention of cosmetics was conducted to disclosure the relevance and utility of the selected hit melanogenesis modulators in future hair cosmetics applications. Fourteen volunteers were assigned to three arms of the study: Formulation A (Compound A+), Formulation B (Compound B-) and Formulation C (Compound C-). Two distinct areas were shaved on the back of the volunteer's scalps, and one of the three formulations was applied to one area. The same formulation without any bioactive ingredient (placebo) was applied to the other area. The vehicle formulation was developed for maximal hair follicle permeation and accumulation of compounds, in compliance with the Regulation (EC)

No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Having a long history of use in humans, the therapeutic doses and safety profile of these drugs are well known. Consequently, this information was used to define their dosage regimen in this study. Formulations were applied three times a week, for five weeks. The doses used were sub-therapeutic and considerably lower (at least 1000 times) than the maximal dose described for each one of them. All volunteers completed the study.

The effect of the topical scalp application of Compound A+, Compound B- and Compound C- was evaluated based on qualitative (primary outcome) and quantitative (secondary outcome) data comparison. As the hair regrew, visually perceptible changes in hair colour were registered and documented by photographs. After the last application, a few hairs were plucked and other were cut from the treated areas and the melanin content determined. An overall analysis of the data collected shows that 57% of the volunteers (8 of 14) attained some stage of hair colour phenotype modulation by the end of the study – Figure III.12.

Regarding Compound A+, the specific effectiveness was set at 67%. A clear hair darkening was achieved in two volunteers presenting a natural medium brown shade while no appreciable effect was detected in the one displaying a dark brown shade. The mean hair melanin content of positive outcomes was 136.0 ± 30.97% of control – Figure III.12. Due to the small cohort size, no statistical significance was achieved comparing the overall effectiveness to the vehicle control (100%; one sample t-test: $t(1) = 1.644$; $p = 0.3479$). However, a trend towards significance was demonstrated; in individual analyses of each positive outcome, melanin contents of hair from areas of the scalp treated with the test formulation were statistically different, compared to those of the respective placebo-treated areas (two-way ANOVA, $p \leq 0.001$). In one case, the percentage was as higher as 157.9%, with the difference in melanin contents being indicative of a change in hair colour shade from medium to dark brown (11.97 and 18.86 μg of melanin/mg of hair for placebo and Compound A+, respectively) – Figure III.13.

Volunteers with medium brown, dark brown and black hairs were assigned to the treatment with Compound B- – Figure III.14. For three of them (50% specific effectiveness), the hair growing under the influence of this compound presented significantly lower melanin contents than hair from areas where no melanogenesis modulator was applied (two-way ANOVA, $p \leq 0.05$). The mean melanin content obtained (80.67 ± 6.070%) was also significantly lower than the control (one sample t-test: $t(2) = 5.515$; $p = 0.0313$) – Figure III.12. In agreement with the numerical data, the dark brown hair from the scalp area of Volunteer #2 treated with Compound B- was visibly lighter than the hair from placebo-treated area

– Figure III.12. Noteworthy, despite the most pronounced melanogenesis inhibitor effect of Compound B- had been achieved in one volunteer with black hair (26.2% decrease in melanin content, Volunteer #4), no visually perceptible change of colour was noticed.

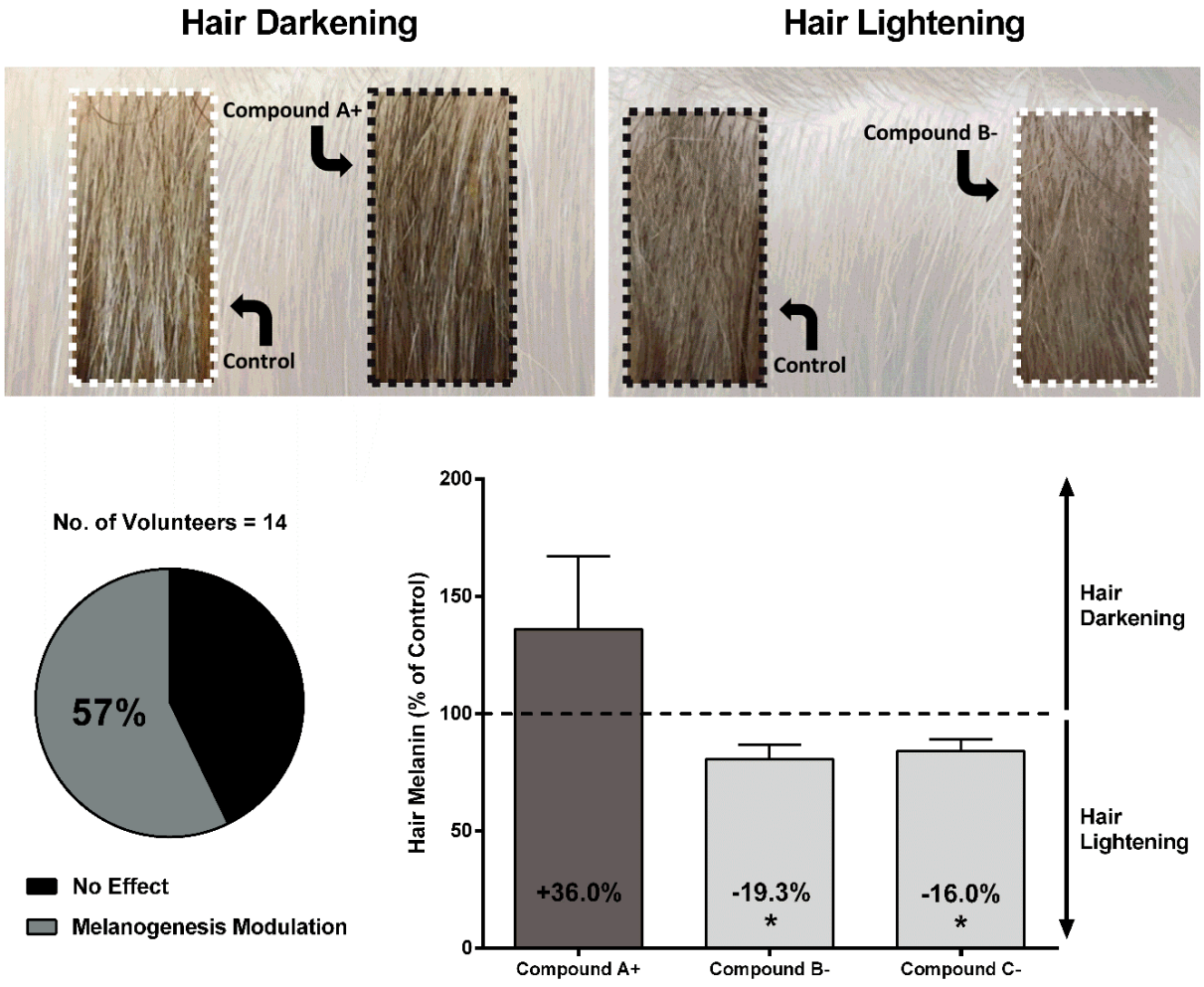


Figure III.12. Main outcomes of the clinical study with intervention of cosmetics, conducted to assess the feasibility of a drug-based approach for hair colour modulation. Top: Representative photographs of the hair darkening and lightening promoted by the treatment of scalps with Compound A+ and Compound B-. Bottom Left: Combined effectiveness of the different drugs used for hair colour phenotype modulation. Bottom Right: Mean hair melanin contents of positive outcomes, regarding the treatment with Compound A+, Compound B- or Compound C-. Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to the hypothetical value 100%.

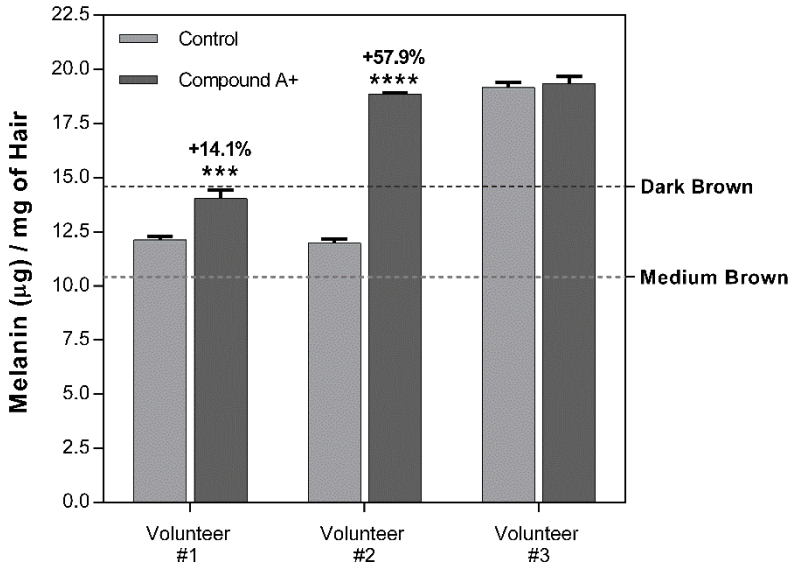


Figure III.13. Quantitative analysis of melanin contents in hairs collected from scalp of volunteers treated with Compound A+. Data were analysed by two-way ANOVA, followed by *post-hoc* Sidak's test. ***$p \leq 0.001$ or ****$p \leq 0.0001$, when melanin contents were compared with the corresponding control (hairs from areas of the scalp treated with the placebo formulation).

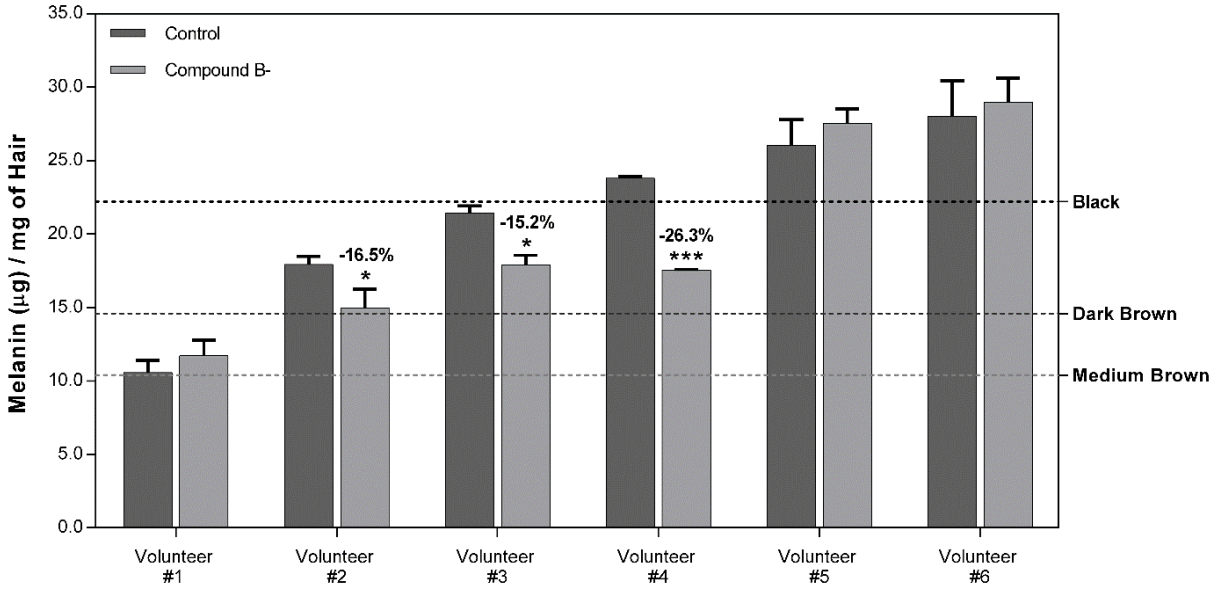


Figure III.14. Quantitative analysis of melanin contents in hairs collected from volunteers treated with Compound B-. Data were analysed by two-way ANOVA, followed by *post-hoc* Sidak's test. *$p \leq 0.05$ or ***$p \leq 0.001$, when melanin contents were compared with the corresponding control (hairs from scalp areas treated with control formulation).

Compound C- did not promote any visual lightening of hair colour. Nonetheless, melanin contents for the three volunteers (#3, #4 and #5) were statistically lower than the respective controls (two-way ANOVA, $p \leq 0.05$) – Figure III.15. As for Compound B-, the highest decrease in melanin content was determined in black hair (19.9%), and the overall mean of melanin content of positive outcomes (84.02 ± 4.953%) was statistically lower than the control (one sample t-test: $t(2) = 5.589$; $p = 0.0306$) – Figure III.12. The specific effectiveness of melanin inhibition by Compound C- was 60%.

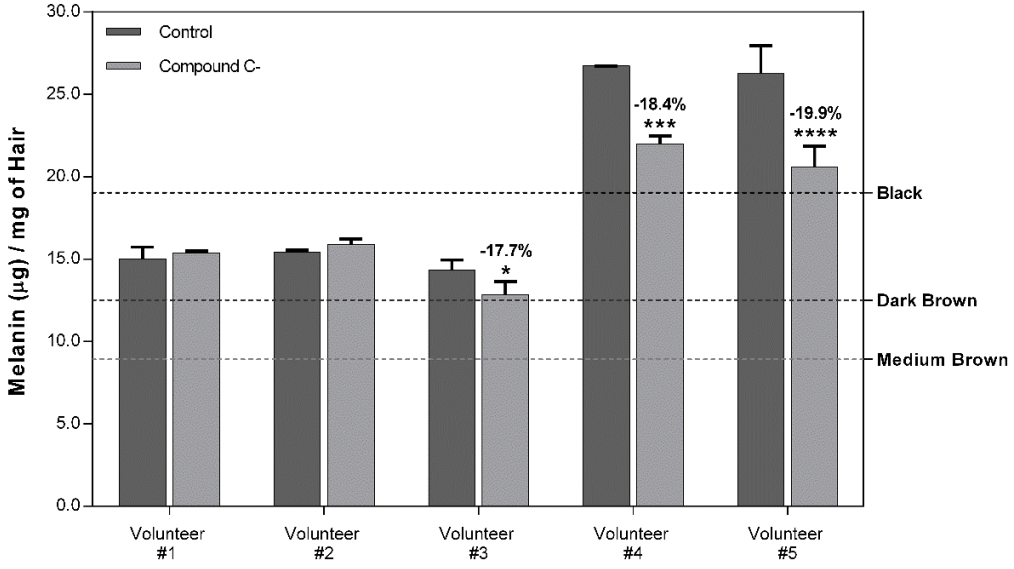


Figure III.15. Quantitative analysis of melanin contents in hairs collected from volunteers treated with Compound C-. Data were analysed by two-way ANOVA, followed by *post-hoc* Sidak's test. *$p \leq 0.05$; ***$p \leq 0.001$ or ****$p \leq 0.0001$, when melanin contents were compared with the corresponding control (hairs from scalp areas treated with control formulation).

No significant differences were detected between the length of hair in scalp areas treated with placebo and test formulations. More important, no systemic or serious adverse side effects were reported by the volunteers during the entire duration of the study. These results unarguably prove that Compound A+, Compound B- and Compound C- can safely and effectively modulate the melanin production in hair follicles and eventually change hair colour which was the main goal of this pilot study. Nevertheless, other clinical trials must be conducted to address the major limitations of the present one: small number of participants and short treatment duration. A trial with many volunteers will portray the real effectiveness of those agents, and positive results that were not statistically significant will certainly be validated. Larger and more representative populations will also provide information regarding responsiveness to the

treatment according to age, gender, and basal hair pigmentation; different hair phenotypes seemed to respond differently to the two melanogenesis inhibitors under test, but there is no robustness in the data that can confirm those observations. Some cases of proven melanogenesis modulation could have been heightened or even translated into visual perceptible hair colour changes with a more prolonged study. The *in vitro* effect of Compound B- and Compound C- was time-dependent which can be indicative that a pronounced *in vivo* effect will only be achieved with long periods of exposure to these drugs. Additionally, a different dose regimen can also accomplish even more satisfactory results. The doses used in our study were far below the usual therapeutic range for those drugs and largely determined by the solubility in the formulation used. The frequency of application intended to improve the recruitment process and adherence of volunteers to the entire study. In a real-life situation, both could easily be increased for an improved effect of compounds; for instance, Compound A+ showed *in vitro* dose-dependent increase of melanin production so, the use of higher concentrations would certainly boost the darkening effect. Finally, the use of particulate vehicles (polymeric nanoparticles, liposomes, among others) can also increase the hair follicle targeting and improve the effect of these compounds. In testing those hypotheses, more exhaustive safety studies will have to be considered.

III.3. CONCLUSION

This work proves the feasibility of pharmacological modulation of hair colour. In a clinical study at the pilot scale, the hair of volunteers became darker or lighter following the treatment of the scalp with formulations containing different agents of hair colour modulation: Compound A+, Compound B- or Compound C-. These molecules are repurposed drugs, targeted as inducers or inhibitors of melanin biosynthesis in an *in vitro* screening of Prestwick Chemical Library®. This screening is a comprehensive survey of melanogenesis modulation by existing drugs and, besides the reported cosmetic implications, the data generated has the potential to reveal new therapeutic approaches to deal with diseases related with abnormal production of melanin. Based on tyrosinase activity assays and the known clinical function of these compounds, some hypothesis regarding the onset of their melanogenesis modulatory effect are presented, but wide-ranging follow up studies will need to be carried out to uncover their cellular targets in melanocytes and to confirm whether the clinical and cosmetic functions of these compounds share the same mechanisms. All treatments with hair colour modulation agents were well tolerated, and no adverse event was reported. Further larger trials will be performed in wider contexts, but data generated already supports the topical use of those drugs as a safe and effective approach to change the natural hair colour.

III.4. SUPPLEMENTARY MATERIAL

Table III.S1. Hit compounds and their effect on intracellular melanin content of SK-Mel-23

Inducers		Inhibitors	
Compound	**Melanin Content (% of control)**	**Compound**	**Melanin Content (% of control)**
Compound A+	327.16	Compound A-	22.22
Compound B+	243.48	Compound B-	33.55
Compound C+	232.21	Compound C-	35.98
Compound D+	191.91	Compound D-	39.75
Compound E+	178.19	Compound E-	49.27
Compound F+	171.58	Compound F-	53.59
Compound G+	170.45	Compound G-	54.79
Compound H+	169.90	Compound H-	55.83
Compound I+	167.09	Compound I-	56.79
Compound J+	163.50	Compound J-	57.16
-	-	Compound K-	57.57

Table III.S2. IC 20 and 50 of hit melanogenesis modulators for 24, 48 and 72 hours of incubations with SK-Mel-23 and SK-Mel-1 cells

		SK-Mel-23			SK-Mel-1		
		24h	**48h**	**72h**	**24h**	**48h**	**72h**
Compound A+	IC20	25.48	35.69	30.47	13.70	16.82	5.914
	IC50	71.33	52.57	47.43	48.23	40.21	22.21
	R^2	0.909	0.957	0.959	0.980	0.984	0.921
Compound B+	IC20	36.00	33.04	20.12	96.63	>100	28.10
	IC50	>100	96.28	66.76	>100	>100	>100
	R^2	0.860	0.966	0.907	0.840	-	0.724
Compound C+	IC20	9.767	7.188	6.029	11.17	5.993	2.809
	IC50	16.32	13.88	12.23	24.76	11.75	7.033
	R^2	18.96	16.82	15.04	31.24	14.31	9.198
Compound D+	IC20	15.30	9.969	9.785	10.33	3.618	1.194
	IC50	21.59	16.08	15.45	23.85	8.998	3.649
	R^2	0.989	0.982	0.983	0.982	0.989	0.986
Compound A-	IC20	>100	>100	>100	>100	>100	>100
	IC50	>100	>100	>100	>100	>100	>100
	R^2	-	-	-	-	-	-
Compound B-	IC20	>100	>100	>100	>100	>100	>100
	IC50	>100	>100	>100	>100	>100	>100
	R^2	-	-	-	-	-	-
Compound C-	IC20	10.74	11.14	9.610	8.493	5.782	3.192
	IC50	16.59	16.28	15.67	15.33	8.532	5.071
	R^2	0.983	0.994	0.978	0.997	0.995	0.994
Compound D-	IC20	>100	>100	>100	>100	>100	>100
	IC50	>100	>100	>100	>100	>100	>100
	R^2	-	-	-	-	-	-

Table III.S3. Output of the two-way ANOVA analyses performed, regarding the effect of using different hit inducers concentrations and different times of incubations in the production of melanin by SK-Mel-23 and SK-Mel-1 cells

SK-Mel-23				
Compound	**Source of Variation**	**F (DFn, DFd)**	**P value**	**% of variation**
Compound A+	Concentration	F (2,18) = 255.9	< 0.0001****	81.42
	Time	F (2,18) = 28.30	< 0.0001****	9.005
	Interaction	F (4,18) = 10.54	0.0001***	6.710
Compound B+	Concentration	F (3,19) = 335.6	< 0.0001****	93.64
	Time	F (2,19) = 8.019	0.0030**	1.492
	Interaction	F (6,19) = 5.564	0.0018**	3.105
Compound C+	Concentration	F (2,16) = 404.4	< 0.0001****	90.38
	Time	F (2,16) = 12.99	0.0004***	2.903
	Interaction	F (4,16) = 11.02	0.0002***	4.925
Compound D+	Concentration	F (2,12) = 121.6	< 0.0001****	73.93
	Time	F (2,12) = 24.97	< 0.0001****	15.18
	Interaction	F (4,12) = 5.966	0.0070**	7.252
SK-Mel-1				
Compound	**Source of Variation**	**F (DFn, DFd)**	**P value**	**% of variation**
Compound A+	Concentration	F (2,15) = 83.42	< 0.0001****	80.60
	Time	F (2,15) = 0.7571	0.4861ns	0.7313
	Interaction	F (4,15) = 5.915	0.0046**	11.43
Compound B+	Concentration	F (4,22) = 249.5	< 0.0001****	87.56
	Time	F (2,22) = 29.27	< 0.0001****	5.137
	Interaction	F (8,22) = 7.657	< 0.0001****	5.374
Compound C+	Concentration	F (2,15) = 34.48	< 0.0001****	50.90
	Time	F (2,15) = 11.20	0.0011**	16.54
	Interaction	F (4,15) = 7.277	0.0018**	21.49
Compound D+	Concentration	F (2,16) = 32.31	< 0.0001****	32.93
	Time	F (2,16) = 54.19	< 0.0001****	55.23
	Interaction	F (4,16) = 1.807	0.1768ns	3.684

Table III.S4. Output of the two-way ANOVA analyses performed, regarding the effect of using different hit inhibitors concentrations and different times of incubations in the production of melanin by SK-Mel-23 and SK-Mel-1 cells

SK-Mel-23				
Compound	**Source of Variation**	**F (DFn, DFd)**	**P value**	**% of variation**
Compound A-	Concentration	F (4,24) = 18.60	< 0.0001****	41.45
	Time	F (2,24) = 12.23	0.0002***	13.63
	Interaction	F (8,24) = 7.077	< 0.0001****	31.54
Compound B-	Concentration	F (4,28) = 14.06	< 0.0001****	33.64
	Time	F (2,30) = 26.88	< 0.0001****	32.15
	Interaction	F (8,28) = 3.652	0.0050**	17.46
Compound C-	Concentration	F (2,14) = 21.57	< 0.0001****	15.31
	Time	F (2,14) = 93.36	< 0.0001****	66.27
	Interaction	F (4,14) = 9.480	0.0006***	13.46
Compound D-	Concentration	F (4,28) = 18.16	< 0.0001****	61.81
	Time	F (2,28) = 0.2355	0.7918ns	0.4006
	Interaction	F (8,28) = 2.053	0.0763ns	13.97
SK-Mel-1				
Compound	**Source of Variation**	**F (DFn, DFd)**	**P value**	**% of variation**
Compound A-	Concentration	F (4,24) = 5.507	0.0027**	8.591
	Time	F (2,24) = 92.01	< 0.0001****	71.76
	Interaction	F (8,24) = 3.298	0.0110*	10.29
Compound B-	Concentration	F (4,30) = 4.269	0.0075**	11.03
	Time	F (2,30) = 19.62	< 0.0001****	25.36
	Interaction	F (8,30) = 8.556	< 0.0001****	44.22
Compound C-	Concentration	F (1,11) = 10.57	0.0077**	28.07
	Time	F (2,11) = 6.432	0.0141*	34.17
	Interaction	F (2,11) = 1.607	0.2442ns	8.538
Compound D-	Concentration	F (4,23) = 3.998	0.0132*	5.091
	Time	F (2,23) = 121.8	< 0.0001****	77.57
	Interaction	F (8,23) = 3.934	0.0046**	10.02

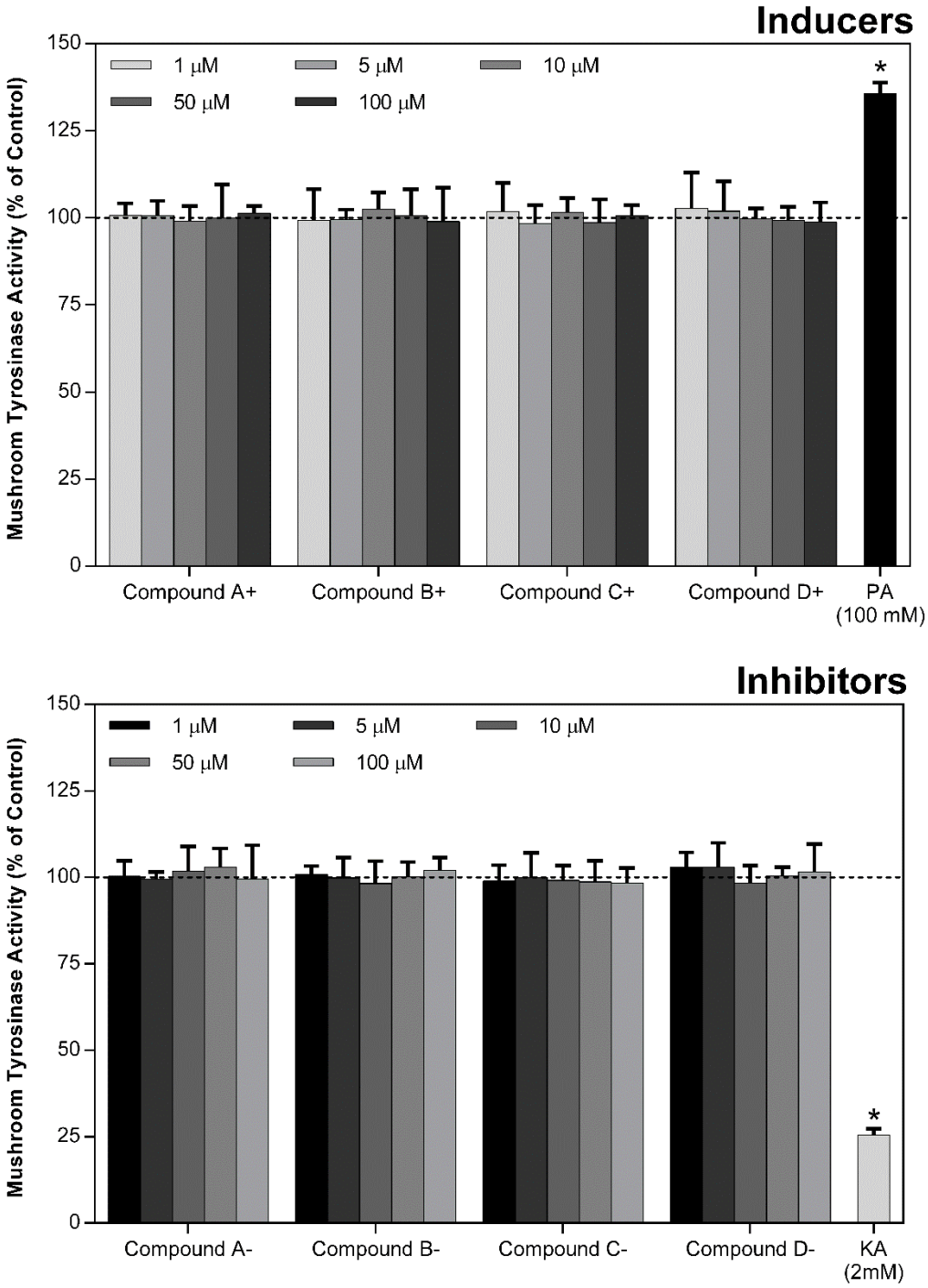


Figure III.S1. Effect of hit inducers and inhibitors on mushroom tyrosinase activity. Dotted lines define basal levels of enzyme activity (100% of control). Data were analysed by one-sample t test. *$p \leq 0.05$, when conditions were compared to the hypothetical value 100%. PA: palmitic acid. KA: kojic acid.

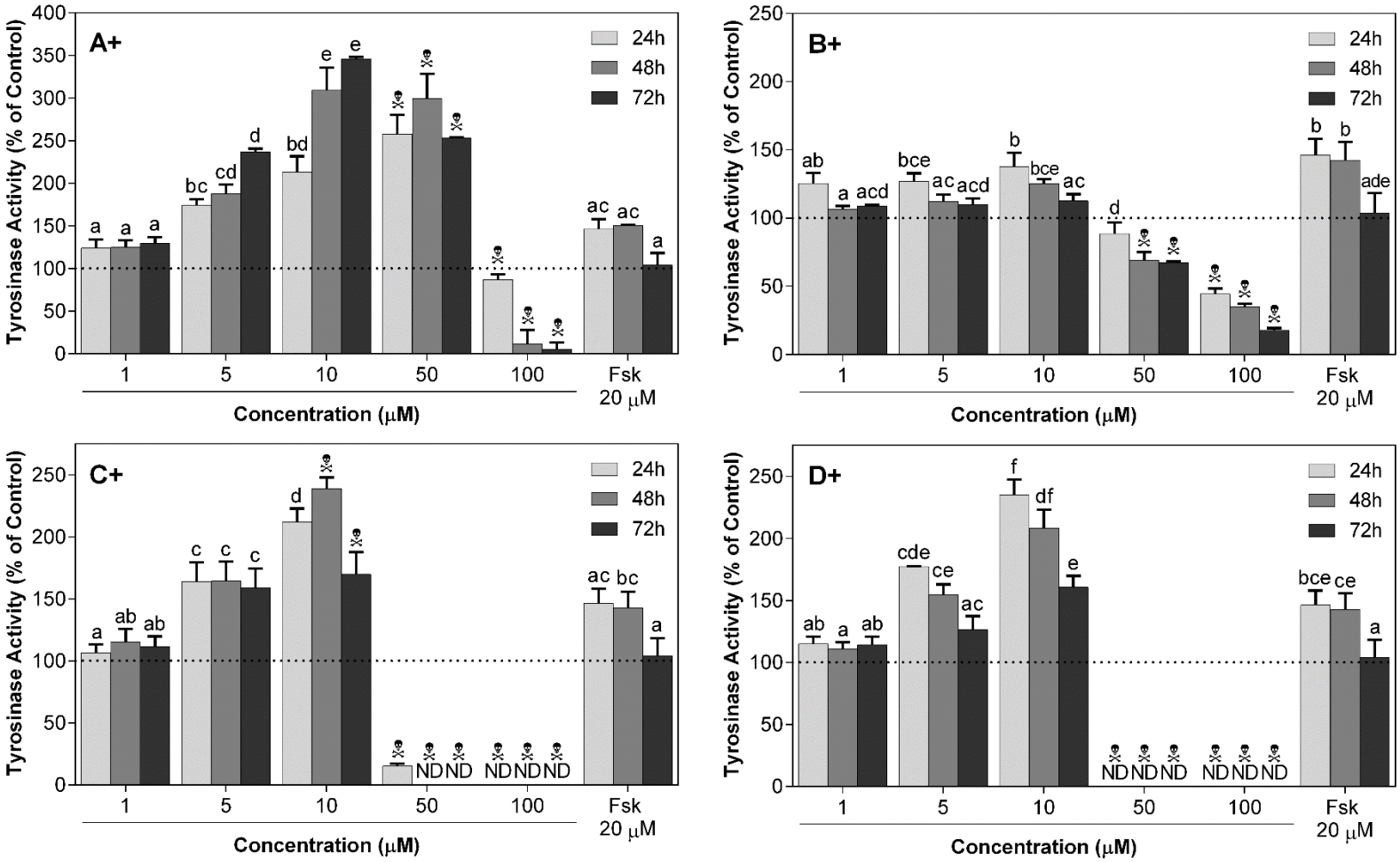


Figure III.S2. Effect of various concentrations of hit inducers on tyrosinase activity of SK-Mel-23, over time. Fsk: Forskolin. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal tyrosinase activity (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

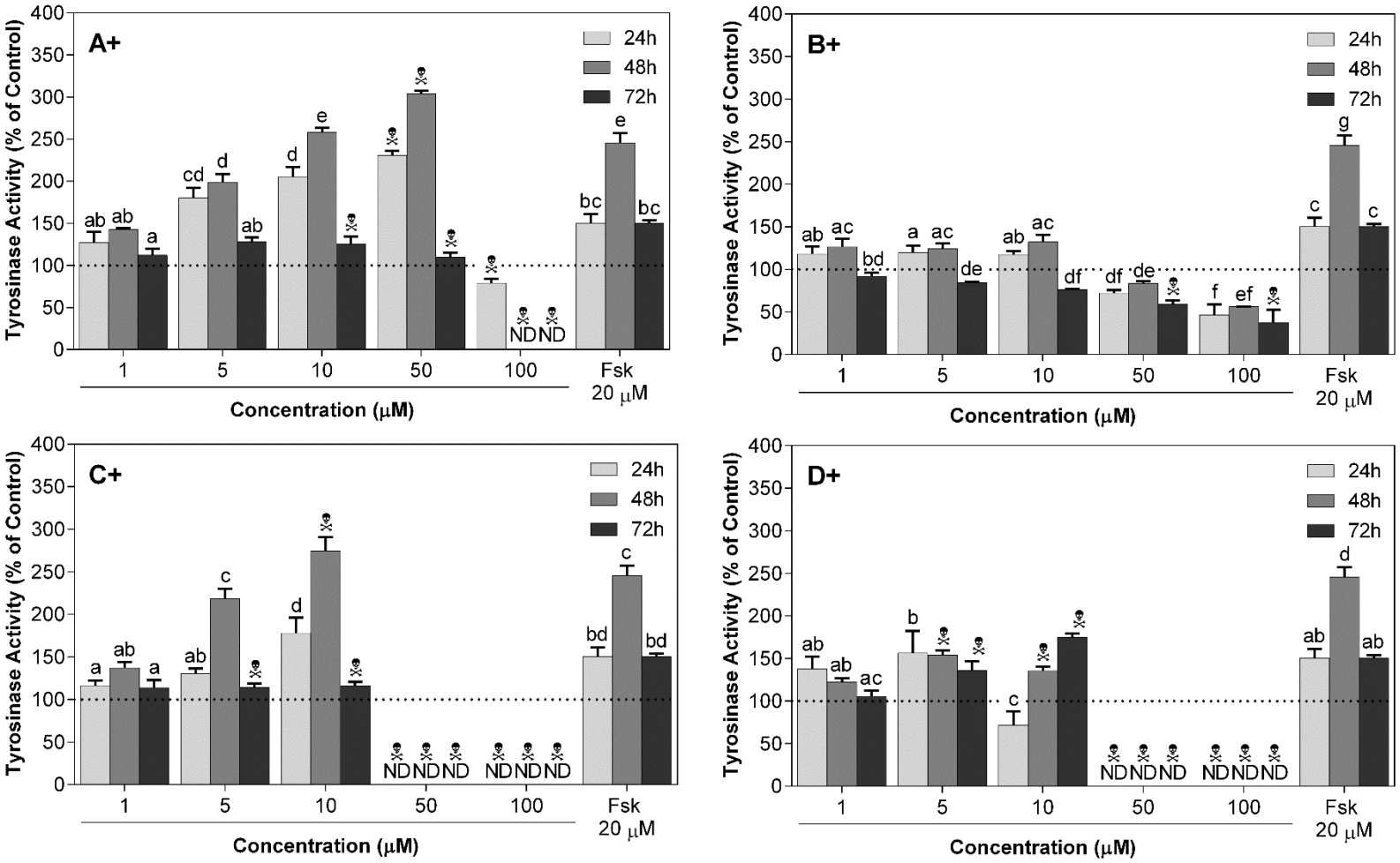


Figure III.S3. Effect of various concentrations of hit inducers on tyrosinase activity of SK-Mel-1, over time. Fsk: Forskolin. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal tyrosinase activity (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

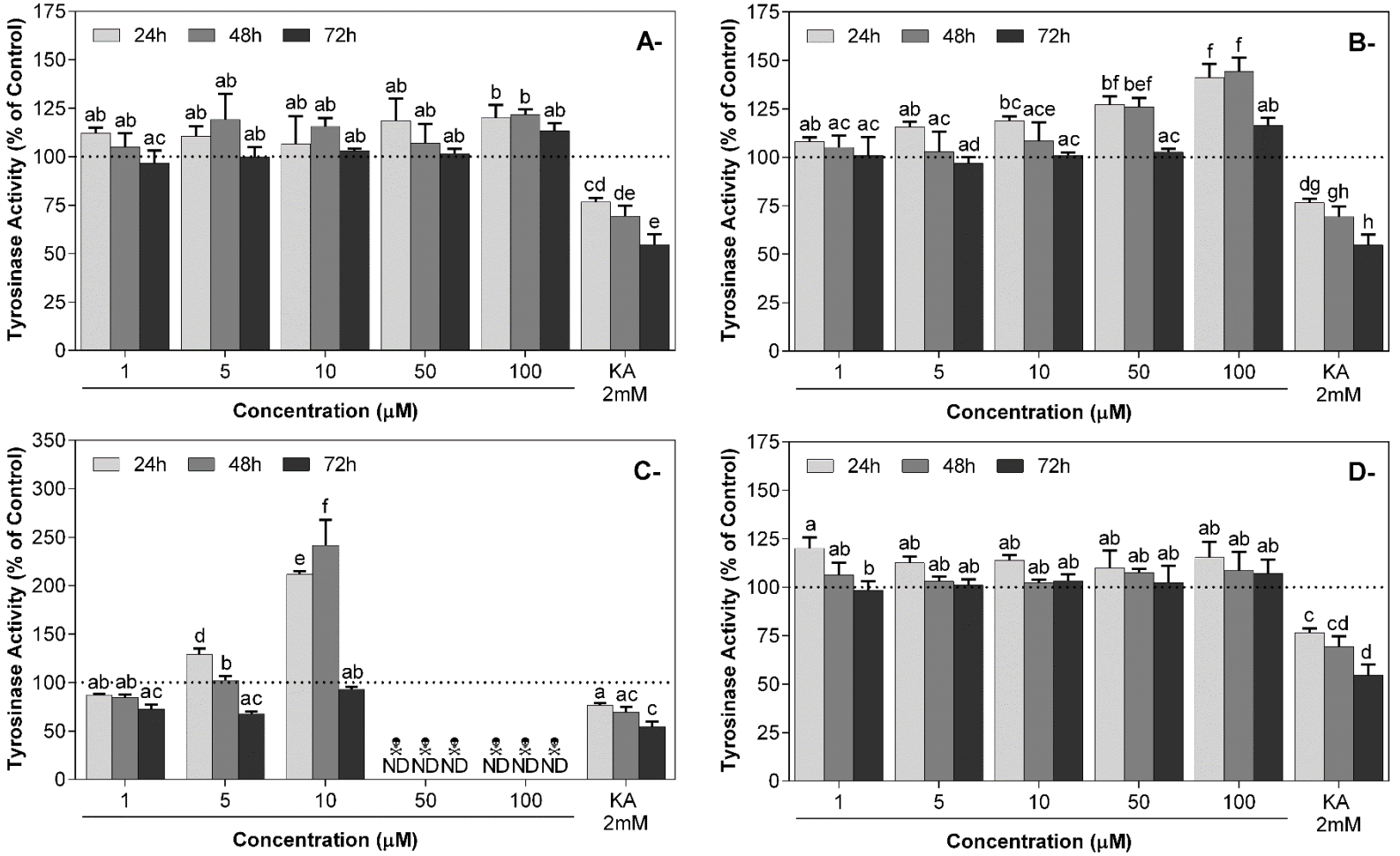


Figure III.S4. Effect of various concentrations of hit inhibitors on tyrosinase activity of SK-Mel-23, over time. KA: Kojic acid. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal tyrosinase activity (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

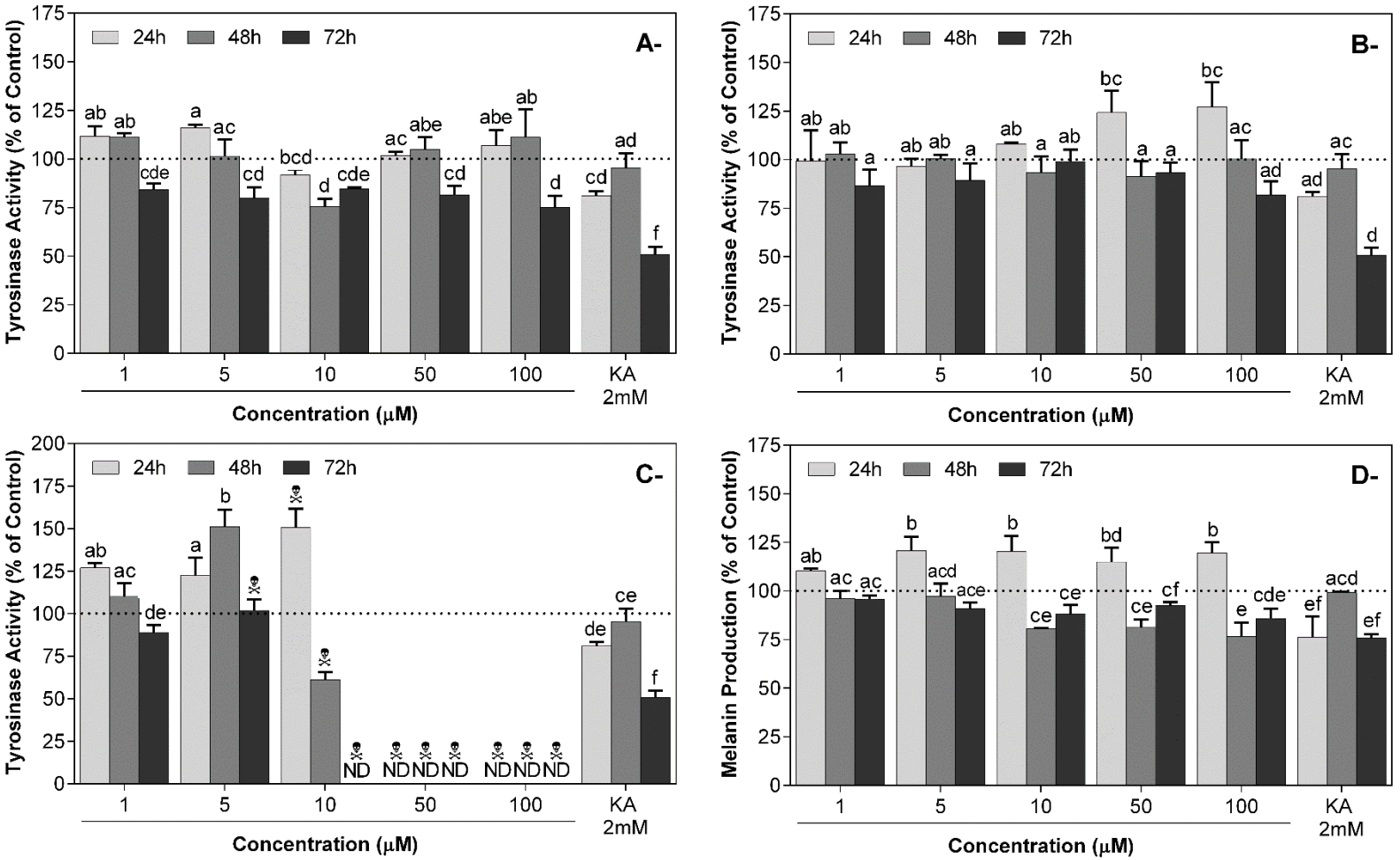


Figure III.S5. Effect of various concentrations of hit inhibitors on tyrosinase activity of SK-Mel-1, over time. KA: Kojic acid. Values are presented as a percentage of vehicle control (1% DMSO). Dotted lines define basal tyrosinase activity (100% of vehicle control). Cytotoxic conditions are noted as ☠. Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different. ND: not detectable.

CHAPTER IV

CYCLOSPORIN A-LOADED POLY (D,L-LACTIDE) NANOPARTICLES: A PROMISING TOOL FOR TREATING ALOPECIA

Chapter IV*

Cyclosporin A-loaded Poly (D, L-lactide) nanoparticles: a promising tool for treating alopecia

ABSTRACT

Alopecia treatments are scarce and lack efficacy. Cyclosporin A (CsA) has hair growth-inducing properties but its poor cutaneous absorption undermines its use in topical treatments. In this work, Poly (D, L-lactide) (PLA) nanoparticles were prepared to improve skin permeation and hair follicle targeting of CsA. A monodispersed population of CsA-loaded PLA nanoparticles with approximately 150 nm and loading efficiency of 2.3% was obtained by nanoprecipitation. Over 6 months of storage at 4 °C, no significant variations in these parameters were observed, indicating good physical and chemically stability. In *in vitro* simulated hair follicle-like lipophilic conditions, a sustained release of CsA occurred over 48 h. Penetration studies in *ex vivo* porcine skin confirmed that PLA nanoparticles increase CsA skin permeation/hair follicle accumulation, compared to a non-colloidal vehicle referenced for alopecia treatment. Since CsA-loaded PLA nanoparticles proved to be non-cytotoxic for a reference skin cell line (NCTC2455, human skin keratinocytes), this work fosters their further *in vivo* investigation as a promising new strategy to treat alopecia, a very traumatic, possibly autoimmune, disease.

*** This chapter is based on the following scientific paper:**

Bruno Fernandes, Teresa Matamá, Andreia C. Gomes and Artur Cavaco-Paulo. Cyclosporin A-loaded Poly (D, L-lactide) nanoparticles: a promising tool for treating alopecia. *Nanomedicine.* 2020, 15(15): 1459-1469.

IV.1 INTRODUCTION

Alopecia is a disease characterized by progressive hair loss affecting mainly the scalp. It is thought to have an underlying autoimmune cause and it is a common complaint in dermatological practice. Topical minoxidil and oral finasteride are the only approved treatments for alopecia; despite being widely used, none of them are robustly efficacious. Given the psychological burden associated to hair loss, the development of new or the improvement of already existing therapies for alopecia treatment is a very pertinent issue [553,554].

Cyclosporin A (CsA) is an immunosuppressive drug that is used in the prevention of organ transplant rejection and in the treatment of a variety of autoimmune skin disorders (e.g., psoriasis, atopic dermatitis). Interestingly, CsA shows hair growth-stimulating properties; reversible, dose-dependent hypertrichosis is one of the most common side effects of CsA systemic therapy [555,556]. The hypertrichotic effect of CsA is attributed to the induction and prolongation of the anagen phase (active hair growth) as well as to the suppression of the catagen phase onset (hair follicle regression). The mechanism by which CsA affects hair cycle appears to be multifactorial [557–562].

Over the last decades, several attempts have been made to treat hair growth disorders with CsA. When administered orally, this immunosuppressive agent yielded good and consistent results in the treatment of alopecias with different aetiologies (e.g. alopecia areata, male pattern alopecia) [563–567]. However, the numerous side effects of long-term systemic administration (e.g., nephrotoxicity) led to the exploration of its topical delivery. Unfortunately, skin application of CsA has produced inconsistent results. Given its highly lipophilic nature, cyclic molecular structure and large molecular weight, CsA cutaneous permeation is not easily achieved which can explain the failure to produce clinically acceptable results in the treatment of alopecia by topical administration [568–574].

Chemical penetration enhancers are frequently used to reduce the diffusional resistance of the *stratum corneum*, the main barrier to percutaneous absorption of drugs. Several enhancers have already shown significant influence on skin permeation of CsA; however, most of them have the potential to induce irritation, burning allergic dermatitis, redness and scalp dryness following repeated applications. Moreover, they frequently increase the transdermal diffusion of drugs into the bloodstream which can result in the known undesirable systemic effects of CsA [575–577].

Nanoparticles can efficiently mask the physicochemical properties of the encapsulated molecules, facilitating the transport of therapeutic drugs that do not easily permeate into/across the skin. More

importantly, when applied to the skin, nanoparticles tend to penetrate along the follicular ducts, becoming trapped inside these structures. The natural tendency of nanoparticles to accumulate inside the follicle duct has important implications in the treatment of several dermatological diseases, especially when the target site of the entrapped drugs is the hair follicle itself [578–581]. In recent years, several particulate systems for topical delivery of anti-hair loss drugs have been reported: liposomes [582], transfersomes [583,584], polymeric nanoparticles [585,586], phospholipid-polymer hybrid nanoparticles [587], among others [588].

The main idea of the work here reported is to present a new solution to suppress the major CsA limitations, which are low skin permeability and serious side effects when administered by systemic routes. In this sense, our approach was to use nanoparticles. We chose to encapsulate CsA in Poly (D, L-lactide) nanoparticles because they already proved to efficiently and selectively penetrate into hair follicles upon skin application [589]. We prepared CsA-loaded PLA nanoparticles by nanoprecipitation. Then, we determined the physical and chemical properties of the new CsA delivery system, as well as the *in vitro* release profile of CsA. We used a reference cell line of human skin keratinocytes to evaluate the potential cytotoxicity of CsA nanoparticles. The ability of these nanoparticles to improve CsA delivery into *ex vivo* porcine skin was evaluated by immunostaining and compared with a published non-particulate vehicle [568].

IV.2 MATERIALS AND METHODS

IV.2.1 CHEMICAL AND SOLVENTS

Poly (D,L-lactide) (Mw = 18-24 kDa), Pluronic® F68 (suitable for cell culture), Cyclosporin A (pharmaceutical secondary standard), thiazolyl blue tetrazolium bromide (MTT), isopropyl myristate (Kosher grade, ≥ 98%), acetonitrile (LC-MS CHROMASOLV® grade, ≥ 99.9%) and water (CHROMASOLV® Plus grade, ≥ 99.9%) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Acetone, ethanol, and dimethyl sulfoxide (DMSO) were of the highest commercially available grade and purchased from several suppliers.

IV.2.2 PREPARATION OF CSA-LOADED PLA NANOPARTICLES

Nanoparticles were prepared based on an improved nanoprecipitation method, previously reported [589]. The polymer was dissolved in a mixture of acetone:ethanol (50:50, v/v) at a concentration of 2% (w/w).

Different amounts of CsA were dissolved along with the polymer in the binary mixture (100, 200 and 400 μg/mL). The organic solvent phase was added drop wise into the aqueous phase containing 0.6% (w/w) of Pluronic® F68, under magnetic stirring – organic phase to aqueous phase ratio was 1:2. The organic phase was completely removed with a rotary evaporator and the suspensions filtered to remove polymeric aggregates above the nano sizes. To remove non-encapsulated drug, suspensions were passed through a gel filtration chromatography column (PD-10 Desalting Column, 5 kDa cut-off; GE Healthcare, UK) and then stored at 4 °C.

IV.2.3 PHYSICAL CHARACTERIZATION

The size and polydispersity index (PDI, from 0.0 to 1.0) of nanoparticles were assessed by Photon Correlation Spectroscopy (PCS). Laser Doppler anemometry (LDA) was employed for zeta (ζ)-potential. All measurements were performed at 25 ± 1 °C, using a ZetaSizer Nano ZS equipment (Malvern Instruments Ltd, Worcestershire, UK).

IV.2.4 YIELD OF NANOPARTICLES, CsA ENTRAPMENT AND LOADING EFFICIENCIES

A known volume of aqueous suspension of CsA-loaded nanoparticles was freeze-dried and the powder obtained dissolved in acetonitrile:water (90:10, v/v). The polymer and the drug were quantified by High Performance Liquid Chromatography (HPLC). The yield of nanoparticles (YN) was calculated as weight percentage regarding the initial amount of polymer used in the formulation (Equation IV.1). The CsA entrapment efficiency (EE) was calculated according to the Equation IV.2. The loading Efficiency (LE) was also determined – Equation IV.3.

$$\text{Yield of Nanoparticles (\%)} = \frac{\text{mass of PLA in nanoparticles}}{\text{initial mass of PLA}} \times 100$$

Equation IV.1. Yield of nanoparticles obtained in the production of cyclosporin A-loaded poly (D,L-lactide) nanoparticles. PLA: poly (D,L-lactide).

$$\text{Entrapment Efficiency (\%)} = \frac{\text{mass of CsA in nanoparticles}}{\text{initial mass of CsA}} \times 100$$

Equation IV.2. Entrapment efficiency of cyclosporin A in poly (D,L-lactide) nanoparticles. CsA: Cyclosporin A.

$$\text{Loading Efficiency } (\%) = \frac{\text{mass of CsA in nanoparticles}}{\text{mass of nanoparticles}} \times 100$$

Equation IV.3. Loading efficiency of cyclosporin A in the poly (D,L-lactide) nanoparticles. CsA: Cyclosporin A.

IV.2.5 STABILITY STUDIES

The physical and chemical stability of CsA-loaded nanoparticles were evaluated monthly over a period of 6 months. Before each evaluation, suspensions of nanoparticles were treated again to remove polymeric aggregates and free drug. Size, PDI and LE were determined.

IV.2.6 *IN VITRO* CsA RELEASE PROFILING

In vitro release profile of CsA from PLA nanoparticles was carried out using isopropyl myristate (IPM) as the lipophilic receptor phase. IPM is a simple model that mimics the hydrophobic character of sebum and *stratum corneum*. Aqueous suspensions of CsA-loaded nanoparticles were added to vials containing the same volume of IPM and the biphasic systems were constantly stirred at 37 °C. At fixed time points, a vial was recovered, and the hydrophilic phase collected. Nanoparticles were freeze-dried and dissolved in acetonitrile:water (90:10, v/v). The amount of CsA still inside nanoparticles was determined by HPLC and used to indirectly calculate the percentage of released drug with respect to the initial amount present in the formulation.

IV.2.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The HPLC system used to quantify PLA and CsA consisted of a WellChrom K-1001 pump (KNAUER, Berlin, Germany), a MIDAS autosampler (Spark Holland, Emmen, Netherlands) and a WellChrom K-2501 spectrophotometer (KNAUER, Berlin, Germany). Separation was achieved using a Nucleogel® GFC 300-8 column (300 x 7.7 mm), maintained at 35 °C. The flow rate of the mobile phase, a 90:10 (v/v) mixture of acetonitrile and water, was set at 0.8 mL/min. The mobile phase was filtered and degassed prior to use. The sample injection volume was 10 µL with PLA and CsA being detected at 210 nm; the retention times for polymer and drug were 7.0 and 15.4 min, respectively. The HPLC method was validated with respect to linearity, reproducibility, limits of quantification and detection.

IV.2.8 *EX VIVO* SKIN PENETRATION ASSAY

Abdominal porcine skin was excised from freshly slaughtered pigs at Seara S.A. (Vila Nova de Famalicão, Portugal), following approved protocol by DGV-Direção Geral de Veterinária. The subcutaneous fat was carefully removed, and the hair shafts trimmed. Skin pieces were washed with phosphate-buffered saline (PBS, pH 7.4) and placed in Franz Diffusion Cells (PermeGear Inc., Bethlehem, PA, USA) with the *stratum corneum* facing the donor compartment and the dermis facing the receptor compartment. After the acclimatization of the tissue to the receptor phase (PBS, pH 7.4, thermostatically maintained at 37 °C), 300 µL of the test formulations were applied to the exposed skin surface in the donor compartment: aqueous suspension of CsA-loaded PLA nanoparticles or CsA-containing non-particulate solution. Both formulations contained 60 µg/mL of CsA. Empty PLA nanoparticles and non-colloidal solution without drug (propylene glycol:ethanol:water [80:15:10]) were used as controls. All skin samples were incubated for 24 hours.

IV.2.9 IMMUNOHISTOCHEMISTRY

Vertical histological cuts (20 µm thickness) of frozen skin blocks were made using a Leica CM1900 cryostat (Leica Microsystems, Numsloch, Germany) and mounted onto slides. Skin sections were dried at room temperature for 2 h and then, fixed with cooled methanol (10 min). A 10% (v/v) solution of goat serum in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.6 containing 70mM NaCl and 0.3% Triton X-100) was used for blocking (4 h). A monoclonal mouse IgG1 anti-CsA (1:100; Thermo Fischer Scientific, MA, USA) was used as primary antibody and a monoclonal mouse IgG1 anti-Keratin 10 Ab-2 (1:200; Thermo Fischer Scientific, MA, USA) assisted as a positive control; incubations were performed overnight, at 4 °C, in a humidified chamber. A 3% (v/v) H_2O_2 solution in methanol (15 min) was used to block endogenous peroxidases. Anti-mouse IgG (whole molecule)-peroxidase produced in rabbit (1:300) was used as secondary antibody (Sigma-Aldrich®, St. Louis, MO, USA)– 1 h incubation, at room temperature. After staining with HIGHDEF® Red IHC Chromogen (HRP; Enzo Biochem Inc., New York, USA), the distribution of CsA inside the skin was observed by bright field microscopy (Olympus BX51 Microscope; Olympus Corporation, Tokyo, Japan).

IV.2.10 *IN VITRO* CYTOTOXICITY

NCTC2544 cells (spontaneously immortalized human skin keratinocytes, purchased from Instituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna, Brescia, Italy) were cultured in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% (v/v) fetal bovine serum (FBS) and 1% (v/v) of penicillin/streptomycin solution. At every 2-3 days, cell culture medium was refreshed. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Keratinocytes ($4.5x10^4$ cells/well) were seeded on 24-well tissue culture plates (TPP, Switzerland). Cells attached for one day before they were treated with a 5, 10 or 20% volume fraction of sterilized aqueous suspension of PLA nanoparticles (0.45 µm filter; SARSTEDT, Nümbrecht, Germany) in fresh medium. The death control (10% DMSO, v/v), the 100% viability control (just fresh medium) and the medium dilution controls (5, 10 and 20% (v/v) of water instead of nanoparticles' suspensions) were all performed. Cell viability, calculated as a percentage of the 100% cell viability control, was determined after 24, 48 and 72 hours of exposure to nanoparticles by MTT assay. A Spectramax 340PC microplate reader (Molecular Devices, CA, USA) was used to measure the absorbance values (570 and 690 nm).

IV.2.11 STATISTICAL ANALYSIS

All the results are expressed as mean value ± standard deviation (SD) of at least three experiments. Statistical analysis was performed with GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA), using analysis of variance (ANOVA). The differences were considered statistically significant at $p \leq 0.05$.

IV.3 RESULTS

IV.3.1 CHARACTERIZATION OF CSA-LOADED PLA NANOPARTICLES

The physical properties of CsA-loaded PLA nanoparticles are presented in Table IV.1. Regardless of the drug concentration, monodispersed populations of nanoparticles with negative surface charge were obtained. No statistically significant differences were found in size (one-way ANOVA: $F(3,8) = 0.3739$; $p = 0.7743$; $\eta^2 = 0.1230$), size distribution (one-way ANOVA: $F(3,8) = 0.0604$; $p = 0.9792$; $\eta^2 = 0.0222$) or ζ-potential (one-way ANOVA: $F(3,8) = 1.131$; $p = 0.3928$; $\eta^2 = 0.2979$).

The HPLC assay used to determine the chemical composition of nanoparticles showed good linearity ($R2 = 0.999$) for PLA and CsA. The limits of detection and quantification for the polymer were 60 µg/mL and 190 µg/mL, respectively. The limits of detection and quantification for the drug were 1.0 µg/mL and 3.0 µg/mL, respectively. No interference by other components of the formulation was observed. The inter-day and intra-day variations accounted for less than 10% (good to excellent reproducibility).

Table IV.1. Effect of cyclosporin A concentration on size, size distribution and ζ-potential of poly (D,L-lactide) nanoparticles. CsA: Cyclosporin A. PDI: Polydispersity index

CsA (µg/mL)	Z-Average (nm) ± SD	PDI ± SD	Zeta-Potential (mV) ± SD
0	145.8 ± 2.90	0.067 ± 0.006	-16.3 ± 0.8
100	152.2 ± 5.80	0.068 ± 0.006	-16.0 ± 0.2
200	151.9 ± 8.81	0.069 ± 0.005	-15.9 ± 0.1
400	149.7 ± 9.79	0.069 ± 0.005	-16.5 ± 0.6

These particles were prepared by nanoprecipitation, with different amounts of CsA being dissolved in the organic phase along with the polymer. Data were analysed by one-way ANOVA. No statistically significant differences were found when nanoparticles prepared with or without CsA were compared among each other. CsA: Cyclosporin A; PDI: Polydispersity index; SD: Standard deviation.

Table IV.2. Effect of cyclosporin A concentration on the yield of nanoparticles, entrapment and loading efficiencies. CsA: Cyclosporin A. PDI: Polydispersity index

CsA (µg/mL)	Yield of Nanoparticles (%) ± SD	Entrapment Efficiency (%) ± SD	Loading Efficiency (%) ± SD
100	70.4 ± 3.9^{a}	65.1 ± 4.4^{a}	0.60 ± 0.04^{a}
200	64.0 ± 3.5^{a}	56.1 ± 1.2^{b}	1.08 ± 0.02^{b}
400	67.8 ± 4.5^{a}	54.3 ± 1.9^{b}	2.30 ± 0.17^{c}

These particles were prepared by nanoprecipitation, with different amounts of CsA being dissolved in the organic phase along with the polymer. Data were analysed by one-way ANOVA, followed by *post-hoc* Tukey's test. Means that do not share a letter are significantly different. CsA: Cyclosporin A; SD: Standard deviation.

Table IV.2. summarizes the obtained yield of nanoparticles, entrapment and loading efficiencies. Formulations prepared with different concentrations of CsA had similar yields of nanoparticles (one-way ANOVA: $F(2,6) = 1.964$; $p = 0.2207$; $\eta^2 = 0.3957$). In contrast, the entrapment efficiency was statistically different between groups (one-way ANOVA: $F(2,6) = 12.37$; $p = 0.0074$; $\eta^2 = 0.8048$). Nanoparticles prepared with 100 µg/mL of CsA showed a significantly higher entrapment efficiency when compared to those prepared with 200 µg/mL (Tukey's *post hoc* test: $p \leq 0.05$) and 400 µg/mL (Tukey's *post hoc* test: $p \leq 0.01$). Loading efficiencies were also statistically different between groups (one-way ANOVA:

$F(2,6) = 232.3$; $p < 0.0001$; $\eta^2 = 0.9872$). Nanoparticles prepared with 400 μg/mL of CsA presented the highest drug content (Tukey's *post hoc* test; $p \leq 0.0001$).

IV.3.2 CsA RELEASE PROFILE AND FORMULATION STABILITY

In vitro drug release kinetics of CsA was evaluated using IPM – Figure IV.1. The profile obtained exhibit a typical biphasic release phenomenon: initial burst release (63.5 ± 2.0% in 2 h) followed by a constant slow release over the remaining time (99.7 ± 0.5% in 48 h).

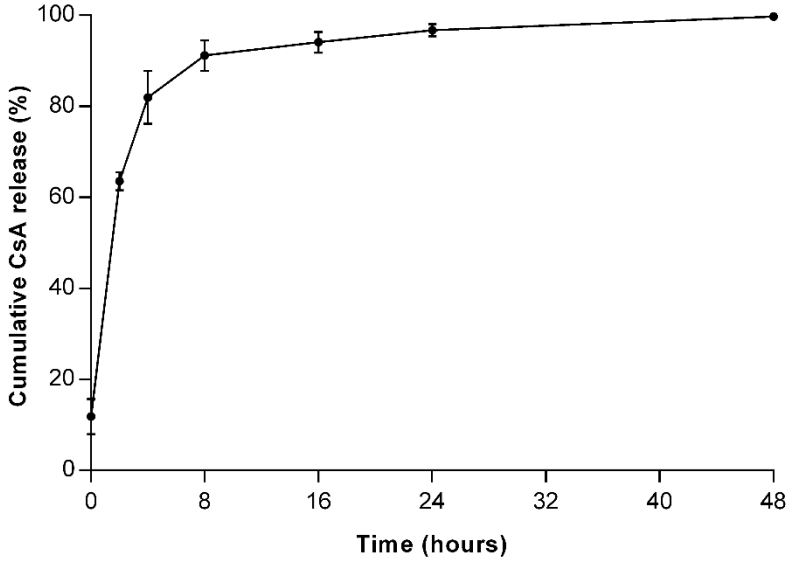


Figure IV.1. *In vitro* release profile of cyclosporin A from poly (D,L-lactide) nanoparticles. These particles were prepared by nanoprecipitation, dissolving CsA in the organic phase at a concentration of 400 μg/mL. The release profile was obtained using the biphasic system IPM/aqueous suspension of CsA-loaded PLA nanoparticles. At each time point, aliquots from aqueous phase were collected. HPLC was used to quantify the remaining CsA inside nanoparticles; subsequently, the percentage of drug released was determined. CsA: Cyclosporin A.

The formulation stability, as the maintenance of nanoparticles properties, was evaluated over a storage period of 6 months at 4 °C – Figure IV.2. The properties of CsA-loaded PLA nanoparticles were assessed monthly. No significant variations in size (one-way ANOVA: $F(6,14) = 1.163$; $p = 0.3787$; $\eta^2 = 0.3327$), PDI (one-way ANOVA: $F(6,14) = 1.577$; $p = 0.2257$; $\eta^2 = 0.4033$) and loading efficiency (one-way ANOVA: $F(6,14) = 0.1632$; $p = 0.9826$; $\eta^2 = 0.0654$) were found, compared to the time point 0 months.

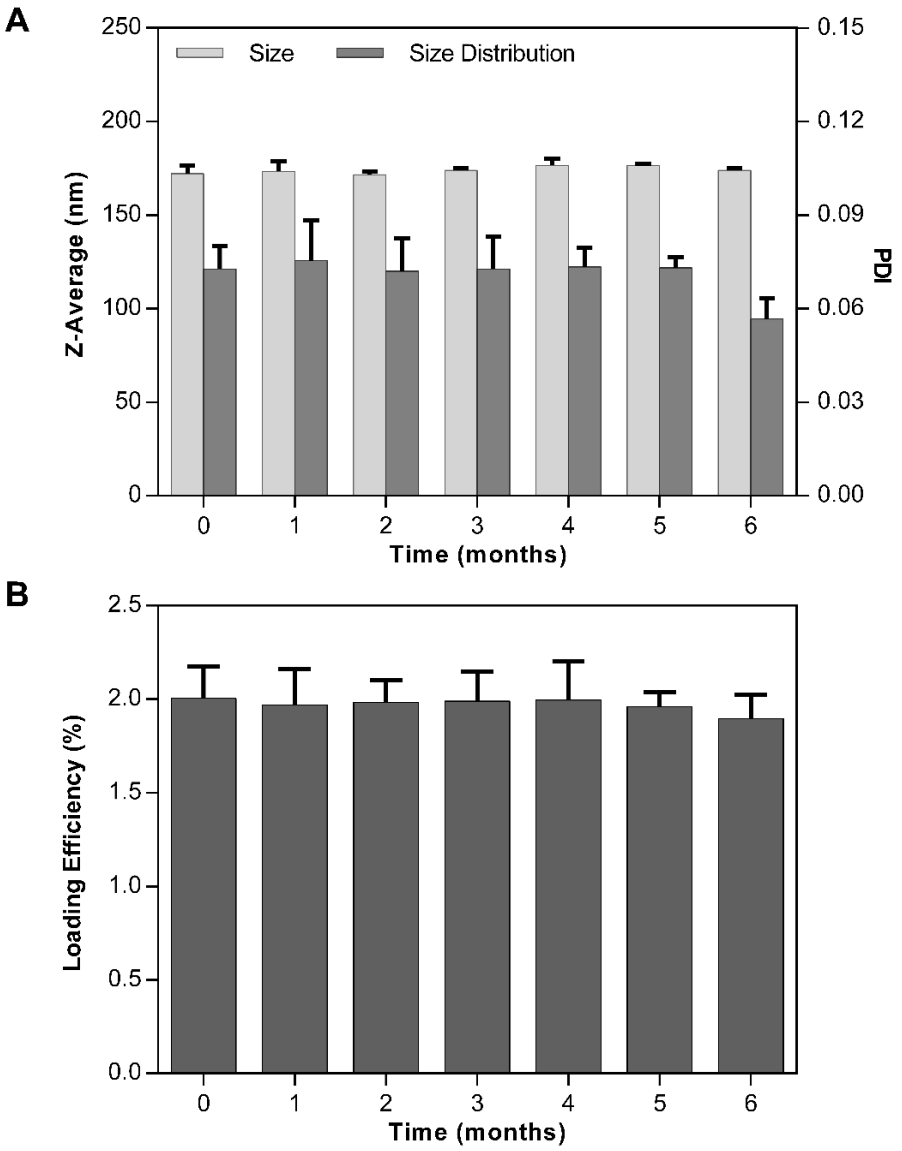


Figure IV.2. Physical and chemical stability of cyclosporin A-loaded poly (D,L-lactide) nanoparticles. Size and size distribution (A) and loading efficiency (B) of CsA-loaded PLA nanoparticles over 6 months storage at 4 °C. These particles were prepared by nanoprecipitation, dissolving CsA in the organic phase at a concentration of 400 µg/mL. Data were analysed by one-way ANOVA. No statistically significant differences were found, compared to the time point 0 months. PDI: Polydispersity index.

IV.3.3 DERMAL PERMEATION OF CSA UPON TOPICAL APPLICATION

An immunohistochemical staining protocol was established for the specific detection of CsA in porcine skin cryosections, in order to study the efficacy of CsA topical delivery of PLA system comparing it to a non-particulate CsA vehicle. This protocol was validated through the presence of cytokeratin 10 staining (positive control; Supplementary Material, Figure IV.S1A) and absence of CsA staining (negative control)

in sections of skin treated with empty PLA nanoparticles (Figure IV.3A) or non-particulate vehicle solution containing no drug (negative control; Supplementary Material, Figure IV.S1B).

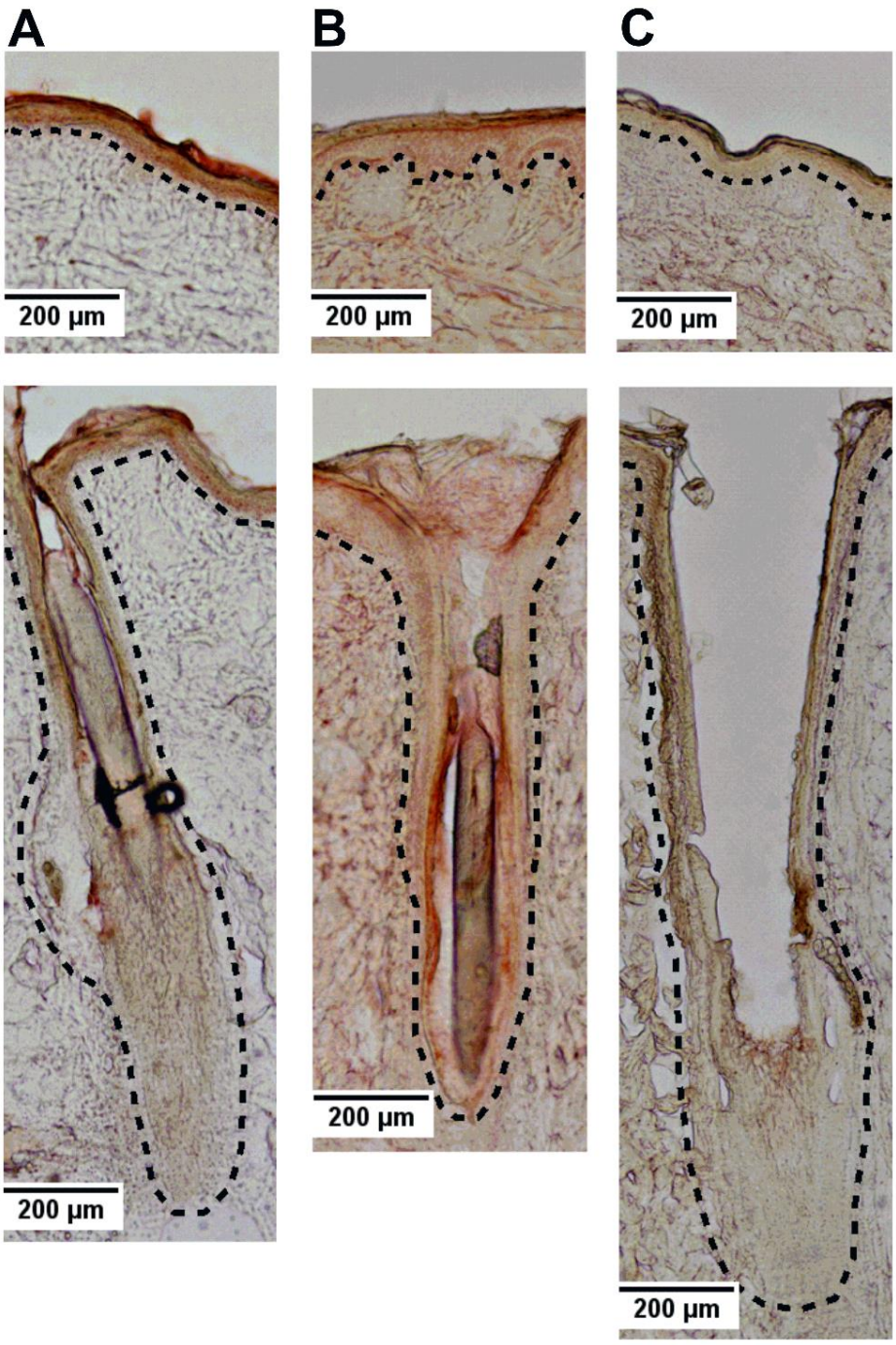


Figure IV.3. Cyclosporin A (CsA)-immunostained (red) cryosections of porcine skin after 24 hours of incubation with poly (D,L-lactide) nanoparticles (A), CsA-loaded PLA nanoparticles (B) or CsA non-colloidal solution (C). The concentration of CsA in the suspension of PLA nanoparticles and in the non-particulate solution was 60 µg/mL. Top: *Stratum corneum*. Bottom: Hair follicles.

Figure IV.3B depicts the result of CsA staining in sections of skin treated with CsA-loaded PLA nanoparticles. It clearly demonstrates permeation of CsA into the skin; the drug was accumulated inside the hair follicles along the entire follicular duct. CsA also accumulated slightly in the *stratum corneum*. On the other hand, no staining was detected in sections of skin treated with non-particulate solution – Figure IV.3C.

IV.3.4 CYTOTOXICITY OF CSA-LOADED PLA NANOPARTICLES

NCTC2544 cells were incubated with CsA-loaded PLA nanoparticles for 24, 48 and 72 hours, after which the viability was assessed – Figure IV.4. For cells exposed to 194 and 388 μg/mL of nanoparticles (5 and 10% of formulation in culture medium), no significant variations in cell viability occurred over time. Exposure of cells to 775 μg/mL of nanoparticles (20% of formulation in culture medium) promoted a minor decrease in cell viability at 48 hours ($p \leq 0.05$, compared to 24 h). Nevertheless, none of the conditions tested differed significantly from their respective medium dilution controls.

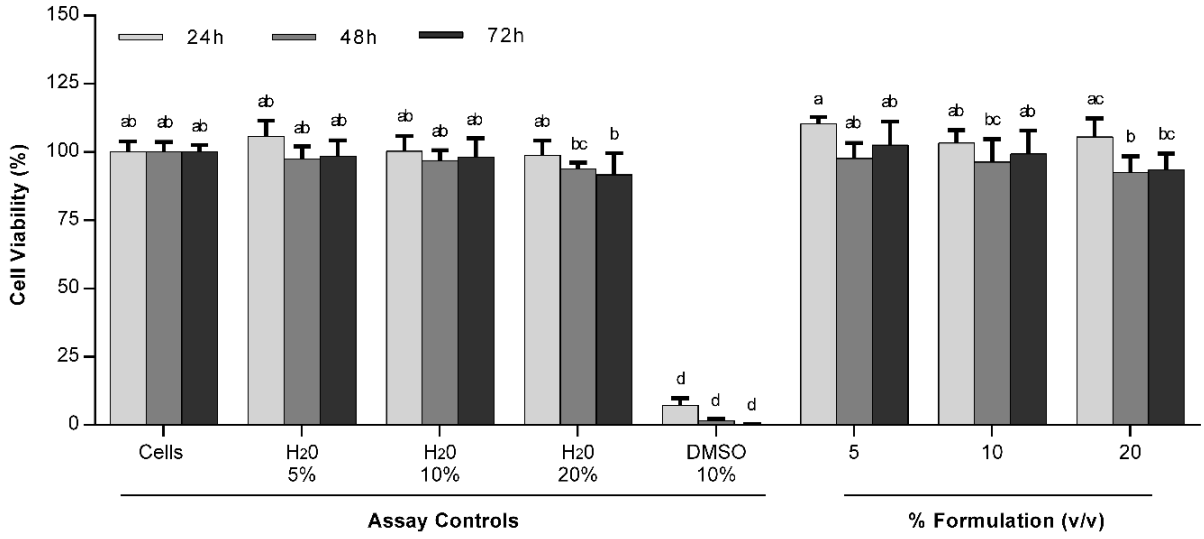


Figure IV.4. NCTC2544 cell viability (MTT assay) determined at 24, 48 and 72 hours of exposure to different volume fractions of a suspension of cyclosporin A-loaded poly (D,L-lactide) nanoparticles. These particles were prepared by nanoprecipitation, dissolving CsA in the organic phase at a concentration of 400 μg/mL. Values for tested samples are presented in relation to the Cells control (cells cultured with medium scored for 100% of viability). Data were analysed by two-way ANOVA, followed by *post hoc* Tukey's test. Means that do not share a letter are significantly different.

IV.4 DISCUSSION

This study focused on preparing nanoparticles to improve cutaneous absorption of Cyclosporin A (CsA). The different amounts of CsA, used in the preparation of PLA nanoparticles by a nanoprecipitation method, previously optimized [589], did not affect their physical properties (Table IV.1). Nonetheless, the loading efficiencies obtained were quite different: particles prepared with 400 μg/mL of CsA presented the highest loading efficiency value (Table IV.2). These particles are the most interesting for a future *in vivo* application because they are able to deliver the highest amount of drug using the lowest quantity of carrier [590]. They were thus selected for subsequent experiments.

In vitro CsA release from PLA nanoparticles was evaluated using IPM. The lipophilicity of this solvent resembles that of sebum and *stratum corneum*. IPM is a very simple model of the *stratum corneum* lipophilic character, traditionally used in *In Vitro* Release Tests (IVRTs) for the development of topical formulations [591–594]. More recently, IPM was used in the development of high throughput screening methodologies that could work on the initial assessment of the rate and extent of absorption of compounds through skin; IPM showed a good correlation with porcine skin diffusion cell data [595]. A fast release of CsA occurred within the first hours – Figure IV.1. This behaviour is according to the expected [40]. When in contact with the hydrophobic phase, PLA nanoparticles become destabilized by the IPM molecules that start to infiltrate into their cores. Having high affinity to IPM, encapsulated lipophilic compounds, such as CsA, are easily partitioned between the core of nanoparticles and IPM, rapidly leaking out of the particles. The leakage process destabilizes the particles even further, accelerating the compound escape [596]. Based on the *in vitro* release profile of CsA, we can foresee that *in vivo* drug release inside the hair follicle canal will occur in a shorter time period than the one needed for the excretion of nanoparticles due to sebum production (8 days) [597]. Despite their behaviour upon contact with IPM, aqueous suspension of CsA-loaded PLA nanoparticles presented great physical and chemical stability over a long period of time, under storage conditions – Figure IV.2.

The ability of PLA nanoparticles to permeate the skin and deliver loaded dyes along the entire hair follicles was already verified in previous *ex vivo* permeation assays, using fluorescence and confocal microscopy [589,591]. Here, we choose an immunohistochemical staining to prove that PLA nanoparticles can promote the follicular delivery of a compound with such low permeability as CsA. This detection of the drug was performed for bright field microscopy, which allows to distinguish skin morphology details simultaneously. CsA permeation studies were conducted on porcine skin as it resembles human skin regarding the permeability properties [598]. The immunohistochemical staining of skin sections proved

that PLA nanoparticles promote the follicular delivery of substantial amounts of CsA, a drug whose cutaneous permeation is hardly achieved – Figure IV.3B. In contrast, there was a somehow unexpected absence of staining in sections of skin treated with a non-particulate solution containing CsA – Figure IV.3C. This vehicle solution was used for CsA delivery in a case of successful topical treatment of alopecia universalis in a 23-years-old male [568]. However, considering that hair regrowth was only achieved by chronical application of 2.38 mg/mL of CsA (forty times higher than the concentration used in this study), it is possible that the permeation of detectable amounts of drug inside porcine skin samples would require a longer incubation time or higher drug concentrations. In summary, the solution failed to promote the accumulation of CsA inside the hair follicles in the work here reported. Since this accumulation is essential for the desired application, our data emphasizes even more the advantage of using PLA nanoparticles for alopecia treatment by topical CsA administration.

A reference skin cell line (NCTC2544 keratinocytes) was used to test the *in vitro* cytocompatibility of CsA-loaded PLA nanoparticles. This study is a preliminary indication of the overall possible skin toxicity of the CsA formulation for future dermatological applications. As cell viabilities obtained were all above 90% (Figure IV.4), the range 194-775 μg/mL of PLA nanoparticles (corresponding to 4-16 μg/mL of CsA) can be considered nontoxic to keratinocytes. The human melanoma cell lines SK-Mel-23 (a kind gift of Doctor Francisco X. Real) and A375 (purchased from American Type Culture Collection, Virginia, USA) were also used to complement the *in vitro* toxicity profile and to predict the compatibility of these nanoparticles with a potential collateral target of their follicular delivery: the melanocytes. Regarding SK-Mel-23 cells, viability was decreased (<80%) after 48 hours of incubation with 388 and 775 μg/mL PLA nanoparticles. For the same concentrations, a significant decrease in A375 cell viability was registered only after 72 hours of incubation (data not shown). The lower melanoma cell viabilities obtained are according to the expected, given their higher susceptibilities to free CsA (A375 IC50 of 8.5 μg/mL at 72 h and SK-Mel-23 IC50 of 6 μg/mL at 72 h, data not shown), when compared to NCTC2544 cells (IC50 of 9 μg/mL at 72 h, data not shown). Noteworthy is the fact that nontoxic concentrations of CsA do not significantly influence the production of melanin by SK-Mel-23 cells (data not shown). Abnormal production of melanin, the pigment responsible for skin and hair colours, could influence the acceptance of a future product for alopecia treatment based on CsA-loaded PLA nanoparticles.

IV.5 CONCLUSION

PLA nanoparticles were chosen as a follicular delivery system of CsA, in view of a promising treatment

for alopecia. CsA-loaded PLA nanoparticles, produced by nanoprecipitation method, showed low particle size, narrow polydispersity, and efficient drug entrapment. Over 6 months, no significant changes were observed in their main physical and chemical properties, indicating good storage stability. In *ex vivo* skin permeation assays, these particles proved to be suitable for the delivery of CsA into hair follicles, performing even better than an already established non-colloidal vehicle. *In vitro* studies suggested that nanoparticles will not present toxicity to skin and that they will maintain a sustained release of the drug for over 48 hours, prolonging its therapeutic effect.

IV.6 FUTURE PERSPECTIVES

Taken together, the results of this study suggest that CsA-loaded PLA nanoparticles can be an effective strategy for *in vivo* hair follicle delivery of CsA and, consequently, they can relaunch this drug as a promising treatment of alopecia. The quantitative analysis of CsA deposition in the skin layers/hair follicles and transdermal permeation (preferentially using human skin) will be the subject of further pharmacokinetics studies. More important, pharmacodynamics studies in animal models and human volunteers will precisely assess the efficacy of this proposed delivery system.

IV.7 SUPPLEMENTARY MATERIAL

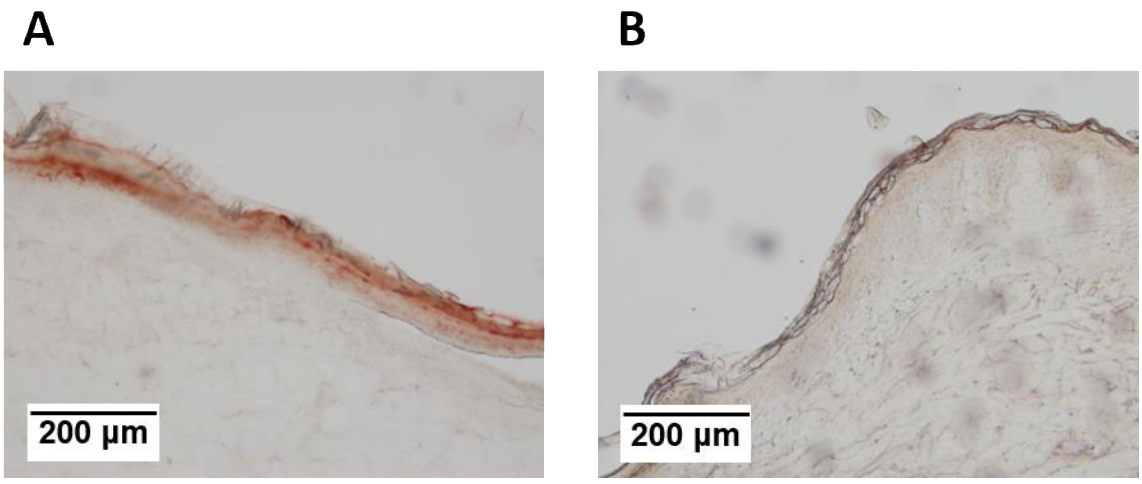


Figure IV.S1. A) Immunohistochemical staining (red) of cytokeratin 10 (positive control); B) Immunohistochemical staining of CsA in sections of porcine skin treated with the non-particulate vehicle solution without drug (negative control).

CHAPTER V

PHARMACOLOGICAL MODULATION OF MELANOGENESIS: WHERE DO WE STAND NOW AND WHICH DRUGS TO REPURPOSE NEXT?

Chapter V*

Pharmacological modulation of melanogenesis: where do we stand now and which drugs to repurpose next?

ABSTRACT

Controlling the production of melanins, the pigments responsible for hair and skin colour, is widely recognized as an important approach to address several dermatological conditions and skin cosmetic purposes. Under the scope of this PhD thesis, the ability of melanogenesis modulators, applied topically, to change the human hair colour was demonstrated, providing a new field of opportunities for the development of ground-breaking hair colour-modifying cosmetics. Although some modulators of melanin synthesis have already been formulated into commercially available products, they present very limited *in vivo* efficacy. Consequently, new agents for topical control of pigmentation are still needed. In this context, and as a way of concluding the thesis, the findings of this work were paired with the existing literature to generate the most up-to-date overview on pharmaceutical drugs with melanogenic effects. By focusing the scope on melanogenesis modulators consisting of repurposed drugs, this review provides a state-of-art on molecules with well-defined safety profiles and high levels of readiness for exploitation into dermatological and/or cosmetic applications. Additionally, this overview is given from a new perspective that emphasises the most represented classes of drugs with melanogenic effects on a clear attempt to expedite future discoveries.

*** This chapter is based on the following scientific paper:**

Bruno Fernandes, Teresa Matamá and Artur Cavaco-Paulo. Pharmacological modulation of melanogenesis: where do we stand now and which drugs to repurpose next? To be submitted.

V.1 INTRODUCTION

Melanins are pigments synthesized by melanocytes, through a series of oxidative reactions catalysed by tyrosinase (TYR) along with tyrosinase related protein 1 (TYRP1) and tyrosinase related protein 2 (TYRP2, also known as dopachrome tautomerase or DCT). The type and amount of melanins are the main determinants of skin and hair colours. The modulation of their synthesis may be used to address a variety of dermatological conditions (melasma, post-inflammatory melanoderma, solar lentigines, among others) as well as cosmetic objectives (sunless tanning, modification of hair colour, etc.) [302,528,599–606].

In recent years, many modulators of the biosynthetic pathway of melanins (termed melanogenesis) have been discovered [302,528,599–606]. However, due to the lack of clinical trials to demonstrate the safety for human usage, only a few of them have been approved as pharmaceutical and/or cosmetic ingredients, being used for skin darkening (afamelanotide) and lightening (4-n-butylresorcinol, α-arbutin, arbutin, deoxyarbutin, ascorbic acid, azelaic acid, hydroquinone, kojic acid) [607–611]. The scarcity of such trials is related to the considerable amount of time and resources needed to perform them; besides the enormous investment required, the chance of a new molecule to reach the market is often minimal. In this sense, one sustainable option for product development in the field of pigmentation modulators is the repurpose of drugs.

Drug repurposing (also known as drug repositioning, drug reprofiling, indication expansion and indication shift) is the establishment of approved, discontinued, shelved or experimental drugs for uses that are outside the scope of its original medical intention. The main advantage of repurposed drugs is that they have already proved to be safe at some level (at least, early stage-trials in humans or pre-clinical models), being unlikely to fail from a safety point of view in new trials. By repositioning marketed drugs, this risk of failing is even lower as they already passed all phases of clinical trials, regulatory scrutiny and undergone post-market surveillance. Since repurposing can bypass much of the safety assessment, once a drug is found to have an off-target effect or a newly recognized on-target effect, only the *in vivo* efficacy for the new indication has to be confirmed. This represents a major shortening in the time and investment required for turning it into a profitable application.

Over the last decade, this strategy has been of considerable importance for the pharmaceutical industry, with about one-third of new approved therapies corresponding to repurposed drugs which generate already 25% of the annual revenue. In the academic field, this approach is also of increasing interest, as shown by an exponential growth in related publications [612–614]. Regarding the cosmetic industry, this tactic will certainly assume an even higher importance given the worldwide effort to phasing out animal

testing, which until recently was viewed as the most reliable way to assure that newly developed cosmetic products/ingredients presented low risk in subsequent human trials or even for direct market entry.

In this review, therapeutic agents with melanogenic effects are examined. As most of them are generic drugs, they have a long history of human usage and can easily be rendered into new dermatological and cosmetic ingredients without the necessity of extended/expensive trials on safety. Although most of these modulators of melanogenesis are still in *in vitro* phase of discovery, a substantial number have already undergone *ex vivo* and/or *in vivo* testing with good results (Table V.1 and Table V.2).

V.2 MODULATORS OF MELANOGENESIS

Drugs with the ability to modulate melanogenesis are grouped according to their current clinical usage. For all of them, efforts were made to provide a description regarding the basis of their melanogenic activity.

V.2.1 ANALGESICS, ANTIPYRETICS, AND ANTI-INFLAMMATORY DRUGS

Paracetamol (Table V.3) is an anilide drug commonly used in the management of pain and fever. It demonstrated an inhibitory effect on the melanization process of normal human melanocytes (NHM) by decreasing the intracellular tyrosinase activity [615]. Contrarily, Compound B+ (Table V.3) increased melanin content of SK-Mel-23 and SK-Mel-1 human melanoma cell lines without causing a statistically significant change in the activity of the enzyme (Chapter III, Section III.2.2, Figure III.10); this drug has an analgesic effect in the urinary tract.

The class of non-steroidal anti-inflammatory drugs (NSAIDs) are used in the management of inflammation, pain, and fever. They are broadly reported to modulate melanogenesis, in particular causing its inhibition – Table V.3. Compound D- and Compound G- were flagged as hit melanogenesis inhibitors in a screening of generic drugs performed in SK-Mel-23 cells (Chapter III, Section III.2.1). Additionally, the former showed antimelanogenic effects on SK-Mel-1, with the mechanism of action in both cell lines seeming to be independent of the modulation of tyrosinase activity (Chapter III, Section III.2.2, Figure III.11). Diclofenac [616], mefenamic acid [616], nimesulide [616] and indomethacin [617] prevented the stimulation of melanogenesis by α-MSH (alpha melanocyte stimulating hormone) in mouse melanoma cells (B16F1). All these drugs inhibited melanogenesis by decreasing the mRNA and/or protein levels of tyrosinase and related proteins, with indomethacin also inhibiting microphthalmia associated transcription

Table V.1. Drugs with an *ex vivo* or *in vivo* effect on skin pigmentation. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. NHM: Normal human melanocytes

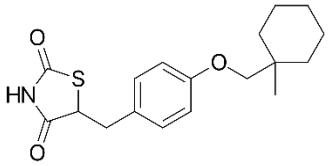
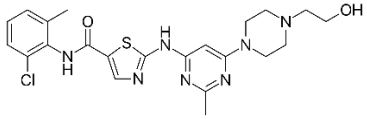
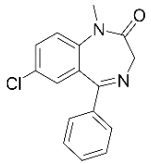
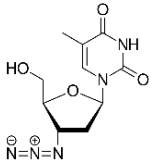
	ATC code	Type of Modulator	*In vitro* effect			*Ex vivo* and/or i*n vivo* effects	Ref.
			Cell line	Melanin content	Tyrosinase activity		
Ciglitazone 	*None*	Inducer	NHM	143% (50 μM)	144% (50 μM)	***Ex vivo*** Increased pigmentation of human skin explants	[264]
Dasatinib 	L01XE06	Inducer	NHM	175-200% (200 nM)	150-175% (200 nM)	***Ex vivo*** Increased pigmentation of human skin explants	[618]
Diazepam 	N05BA01	Inducer	NHM	125-150% (40 μM)	150-175% (40 μM)	***Ex vivo*** Increased pigmentation of human skin explants	[619]
Zidovudine 	J05AF01	Inducer	–	–	–	***In vivo*** Hyperpigmentation of tail and footpads of mice	[620]

Table V.1. (Continued)

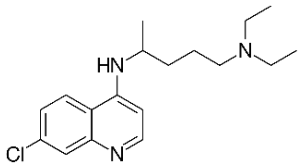
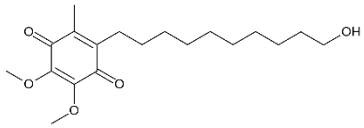
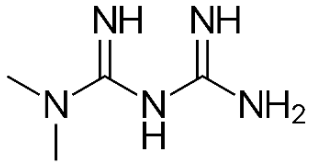
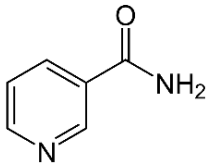
	ATC code	Type of Modulator	*In vitro* effect			*Ex vivo* and/or *in vivo* effects	Ref.
			Cell line	Melanin content	Tyrosinase activity		
Chloroquine 	P01BA01	Inhibitor	Melan-a	*Decreased*	*No change*	***Ex vivo*** Decreased pigmentation of human skin equivalent	[520]
Idebenone 	N06BX13	Inhibitor	SK-Mel-28	40%	–	***In vivo*** Attenuated skin hyperpigmentation in humans	[621]
Metformin 	A10BA02	Inhibitor	B16F10	*Decreased* (10 mM)	–	***Ex vivo*** Decreased pigmentation of human skin equivalent and explants ***In vivo*** Whitening of mice tail	[622]
Nicotinamide 	A11HA01	Inhibitor	–	–	–	***Ex vivo*** Decreased pigmentation of human skin equivalent ***In vivo*** Decreased axillary skin hyperpigmentation	[638, 639]

Table V.1. (Continued)

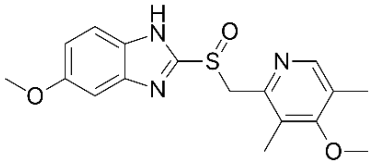
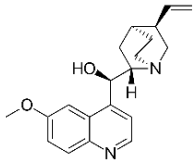
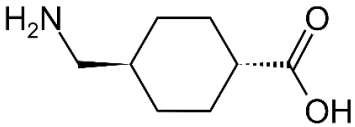
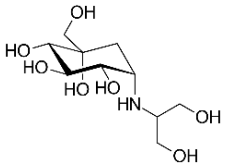
	ATC code	Type of Modulator	*In vitro* effect			*Ex vivo* and/or *in vivo* effects	Ref.
			Cell line	Melanin content	Tyrosinase activity		
Omeprazole 	A02BC01	Inhibitor	NHM	50-60% (300 μM)	–	***Ex vivo*** Decreased pigmentation of human skin equivalent ***In vivo*** Decreased UV-induced skin pigmentation of human subjects	[640, 641]
Quinine 	P01BC01	Inhibitor	Melan-a	*Decreased*	*No change*	***Ex vivo*** Decreased pigmentation of human skin equivalent	[520]
Tranexamic acid 	B02AA02	Inhibitor	NHM	70-80% (≈ 318 μM)	–	***In vivo*** Decreased hyperpigmentation associated with melasma in human subjects	[627–630]
Voglibose 	A10BF03	Inhibitor	MNT-1	*Decreased* (4 mM)	*Decreased*	***Ex vivo*** Decreased pigmentation of human skin equivalent	[631]

Table V.2. Drugs with an *ex vivo* or *in vivo* effect on hair pigmentation. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. NHM: Normal human melanocytes

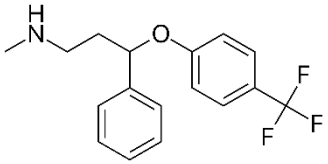
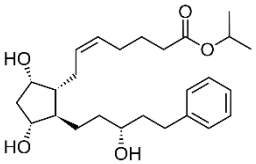
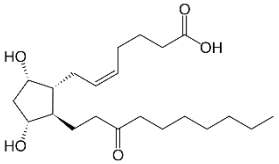
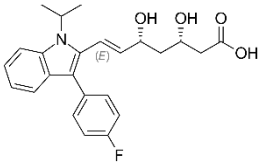
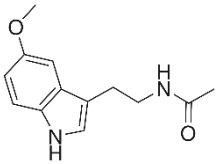
	ATC code	Type of Modulator	*In vitro* effect			*Ex vivo* and/or *in vivo* effects	Ref.
			Cell line	Melanin content	Tyrosinase activity		
Compound A+	–	Inducer	SK-Mel-23	199% (10 μM)	346% (10 μM)	***In vivo*** Darkening of human hair	*Current thesis*
Fluoxetine 	N06AB03	Inducer	NHM	625-650% (10 μM)	225-250% (10 μM)	***Ex vivo*** Stimulation of human follicular melanogenesis ***In vivo*** Increased pigmentation of hairs in mice	[548, 549, 647]
Latanoprost 	S01EE01	Inducer	–	–	–	***In vivo*** Stimulation of mice follicular melanogenesis	[633]
Unoprostone 	S01EE02	Inducer	–	–	–	***In vivo*** Stimulation of mice follicular melanogenesis	[633]

Table V.2. (Continued)

	ATC code	Type of Modulator	*In vitro* effect			*Ex vivo* and/or *in vivo* effects	Ref.
			Cell line	Melanin content	Tyrosinase activity		
Compound B-	–	Inhibitor	SK-Mel-23	74% (10 μM)	109% (10 μM)	***In vivo*** Lightening of human hair	*Current thesis*
Compound C-	–	Inhibitor	SK-Mel-23	31% (10 μM)	93% (10 μM)	***In vivo*** Inhibition of human follicular melanogenesis	*Current thesis*
Fluvastatin 	C10AA04	Inhibitor	–	–	–	***In vivo*** Depigmentation of hairs in mice	[634]
Melatonin 	N05CH01	Inhibitor	MNT-1	77% (10 mM)	–	***Ex vivo*** Inhibited melanin synthesis in hair follicles of Siberian hamster	[650, 651]

factor (MITF). Acetylsalicylic acid also inhibited the melanin synthesis in B16F1 (α-MSH-stimulated) and B16F10 (unstimulated and α-MSH-stimulated) by lowering the protein levels of tyrosinase. Such reduction correlated with low levels of MITF, a result of impaired transcription and activation of extracellular signal-regulated kinases (ERK) that triggered MITF proteasomal degradation via enhanced ubiquitination [637,638] Only ethenzamide, a salicylic acid derivative, was found to significantly enhance the synthesis of melanin in B16F1, via phosphorylation of CREB (cAMP response element-binding protein) and subsequent up-regulation of MITF, TYR, TYRP1 and TYRP2 expression [639].

Table V.3. Analgesics, antipyretics, and anti-inflammatory drugs with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. α-MSH: alpha melanocyte stimulating hormone. NHM: Normal human melanocytes

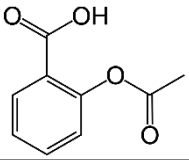
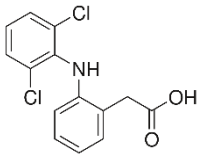
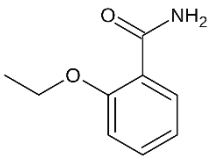
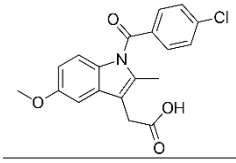
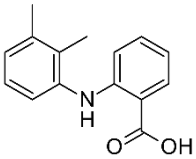
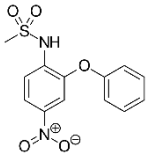
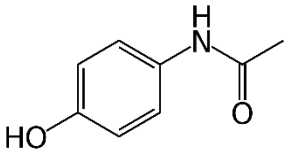
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Acetylsalicylic acid 	N02BA01	B16F1 (+α-MSH)	27% (3 mM)	*Decreased* (3 mM)	[638]
Compound B+	–	SK-Mel-23	302% (50 μM)	89% (50 μM)	*Current thesis*
Compound D-	–	SK-Mel-23	65% (10 μM)	103% (10 μM)	*Current thesis*
Compound G-	–	SK-Mel-23	55% (10 μM)	–	*Current thesis*
Diclofenac 	M01AB05	B16F1 (+α-MSH)	22% (100 μM)	*Decreased* (100 μM)	[616]
Ethenzamide 	N02BA07	B16F1	*Increased* (500 μM)	*Increased* (500 μM)	[639]

Table V.3. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Indomethacin 	M01AB01	B16F1 (+α-MSH)	20-30% (200 μM)	*Decreased* (200 μM)	[617]
Mefenamic acid 	M01AG01	B16F1 (+α-MSH)	22% (100 μM)	*Decreased* (100 μM)	[616]
Nimesulide 	M01AX17	B16F1 (+α-MSH)	37% (100 μM)	*Decreased* (100 μM)	[616]
Paracetamol 	N02BE01	NHM	93% (2 mM)	83% (2 mM)	[615]

V.2.2 ANTI-ACNE MEDICATION

Retinol, tretinoin (all-trans retinoic acid) and isotretinoin (13-cis-retinoic acid) are retinoids for the topical treatment of acne – Table V.4 [640,641]. Retinol and tretinoin prevented α-MSH or 3-isobutyl-1-methyl-xanthine (IBMX) stimulation of melanogenesis in cultured B16F1 cells [640]. Isotretinoin and tretinoin avoided the ultraviolet (UV)B-stimulation of melanogenesis in Cloudman S91-M3 mouse melanoma cells and NHM [641]. Those drugs revealed inhibitory activity towards the production of melanin by down-regulating the protein levels of tyrosinase and tyrosinase-related proteins. Curiously, in the absence of a UVB stimulus, tretinoin and isotretinoin increased intracellular tyrosinase activity and melanin contents in S91-M3 cells. Nevertheless, in unstimulated NHM, the melanin content was still decreased [641].

V.2.3 ANTIEPILEPTICS

Cannabidiol and Compound F+ are antiepileptic drugs with proved melanogenic effects – Table V.5

Table V.4. Anti-acne medication with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. IBMX: 3-isobutyl-1-methyl-xanthine. NHM: Normal human melanocytes

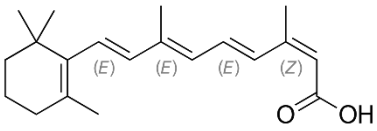
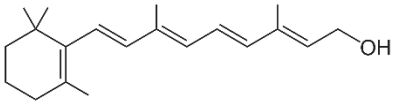
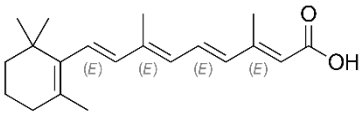
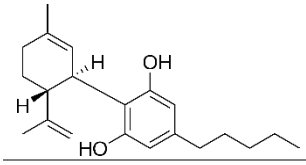
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Isotretinoin 	D10AD04	NHM	*Decreased* (1 μM)	63% (1 μM)	[641]
Retinol 	D10AD02	B16F1 (+IBMX)	33% (10 μM)	*Decreased* (10 μM)	[640]
Tretinoin 	D10AD01	NHM	*Decreased* (1 μM)	61% (1 μM)	[641]

Table V.5. Antiepileptics with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. NHM: Normal human melanocytes

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Cannabidiol 	N03AX24	NHM	140-150% (6 μM)	130-140% (6 μM)	[642]
Compound F+	–	SK-Mel-23	172% (10 μM)	–	*Current thesis*

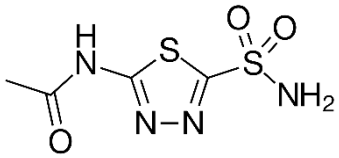
Compound F+, used in the management of epilepsy and migraines, was here identified as a hit inducer of melanogenesis in SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1). Cannabidiol was found to be a stimulator of the synthesis of melanin by up-regulating the gene and protein expression of MITF, TYR, TYRP1 and TYRP2. Up-regulation of MITF was related to increased phosphorylation of p38 mitogen-

activated protein kinases (MAPK) and ERK At the most upstream level, the melanogenic effect of cannabidiol was proved to be mediated by the cannabinoid CB1 receptor [642].

V.2.4 ANTI-GLAUCOMA MEDICATION

The application of prostaglandin analogues latanoprost and unoprostone to the dorsal skin of 7-week-old C57BL/6 mice showed stimulatory effects on follicular melanogenesis – Table V.2 [633]. On the other hand, the carbonic anhydrase inhibitor acetazolamide prevented the 3,4-dihydroxyphenylalanine (DOPA)-stimulated synthesis of melanin in cultured A375 human melanoma cells and decreased the pigmentation of zebrafish embryos – Table V.6. The melanogenic effect of acetazolamide was coupled with decreased protein levels of tyrosinase; additionally, this drug demonstrated inhibitory activity against mushroom tyrosinase [643].

Table V.6. Anti-glaucoma medication with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. DOPA: 3,4-dihydroxyphenylalanine

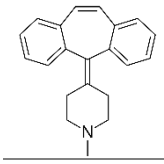
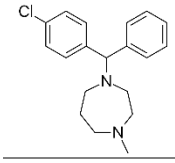
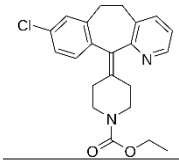
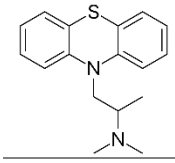
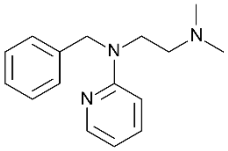
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Acetazolamide 	S01EC01	A375 (+DOPA)	20-30% (40 μM)	10-20% (40 μM)	[643]

V.2.5 ANTIHISTAMINES

Several antagonists of the histamine H1 receptor have displayed *in vitro* anti-melanogenic activities – Table V.7. Compound E- and Compound I- were identified by us as hit inhibitors of melanogenesis in SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1). Tripelennamine, promethazine and cyproheptadine decreased production of melanin in Melan-a mouse melanocyte cell line [519]. Loratadine inhibited melanogenesis in NHM and Mel-Ab mouse melanocytes by downregulating the mRNA/protein levels of MITF and TYR, via increased Akt and glycogen synthase kinase 3 beta (GSK3β) phosphorylation, and reduced Protein Kinase C (PKC)-βII activity [644]. Homochlorcyclizine (marketed in Japan) hindered the stimulation (α-MSH or IBMX) of melanogenesis in B16 melanoma 4A5 cells, without affecting the gene

or protein expression of tyrosinase or by acting as a direct inhibitor of the enzyme. The authors suggested that homochlorcyclizine may affect the levels of tyrosine by interfering with the phenylalanine transporters or with phenylalanine hydroxylase (PAH) [521].

Table V.7. Antihistamines with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. α-MSH: alpha melanocyte stimulating hormone. NHM: Normal human melanocytes

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound E-	–	SK-Mel-23	49% (10 μM)	–	*Current thesis*
Compound I-	–	SK-Mel-23	57% (10 μM)	–	*Current thesis*
Cyproheptadine 	R06AX02	Melan-a	46% (5 μM)	–	[519]
Homochlorcyclizine 	*None*	B16 melanoma 4A5 (+ α-MSH)	64% (4 μM)	≈ 100% (4 μM)	[521]
Loratadine 	R06AX13	NHM	30-40% (7.5 μM)	50-60% (7.5 μM)	[644]
Promethazine 	R06AD02	Melan-a	55% (5 μM)	–	[519]
Tripelennamine 	R06AC04	Melan-a	44% (5 μM)	–	[519]

V.2.6 ANTIHYPERGLYCEMIC AGENTS

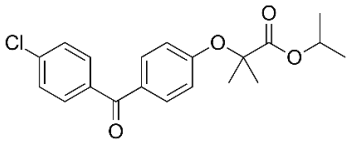
Metformin (Table V.1) is a biguanide derivative and the most widely used antidiabetic drug in the world. It inhibited the *in vitro* production of melanin by decreasing the expression of MITF, TYR, TYRP1 and TYRP2 (unstimulated and forskolin- or α-MSH-stimulated) in B16F10 cells. The events leading to decreased expression of melanogenic proteins were the inhibition of the accumulation of cyclic adenosine monophosphate (cAMP) and downstream reduction of PKA and CREB phosphorylations. The antimelanogenic effect of metformin was confirmed in *ex vivo* (SkinEthic™ Reconstituted Human Epidermis and human skin explants) and *in vivo* (topical application in 8-week-old C57BL/6J mice) assays [622]. Another anti-diabetic drug that showed potent *in vitro* (MNT-1 human melanoma cells) and *ex vivo* (Neoderm®-ME human reconstructed skin model) antimelanogenic effects is voglibose, a valiolamine derivative – Table V.1. Although it increased the gene expression of tyrosinase, being a α-glucosidase inhibitor, voglibose prevented the proper post-transcriptional N-glycan processing of the enzyme (altering its stability), subsequently decreasing the synthesis of melanin [631].

The antihyperglycemic agent ciglitazone increased the synthesis of melanin in NHM and the pigmentation of *ex vivo* cultured skin explants by increasing the protein levels of MITF and TYR – Table V.1. Although ciglitazone was never marketed, it served as the prototypical drug for anti-diabetic thiazolidinediones, compounds that activate PPARs (peroxisome proliferator-activated receptors). The melanogenic effect of ciglitazone was suggested to be dependent on the ability to serve as a PPARγ agonist [264].

V.2.7 ANTIHYPERLIPIDEMIC AGENTS

Compound J+ and fluvastatin are two 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase inhibitors, with proved melanogenic effects. In the screening of generic drugs performed during this thesis work, Compound J+ (Table V.8) increased the production of melanin in SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1). On the other hand, subcutaneous injection of fluvastatin in 4-week-old C57BL/6 mice caused the growing of depigmented hairs, suggesting an antimelanogenic effect – Table V.2; the same study found that fluvastatin decreases the protein levels of tyrosinase in B16F10 cells [634]. Fenofibrate (Table V.8) is a lipid modifying agent that exerts its therapeutic effect by activating the PPARα. Fenofibrate was found to inhibit melanogenesis via downregulation of melanogenic proteins [melanocortin 1 receptor (MC1R), MITF and TYR), up-regulation of p38-MAPK, and possibly by interfering with the nuclear oxysterol liver X receptor (LXR) signalling pathway [645].

Table V.8. Antihyperlipidemic agents with *in vitro* effects on melanogenesis. Melanin contents and tyrosinase activity are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound J+	–	SK-Mel-23	164% (500 nM)	–	*Current thesis*
Fenofibrate 	C10AB05	B16F10	≈ 60% (10 μM)	55% (6 μM)	[645]

V.2.8 ANTIMICROBIALS

The lincosamide antibacterial agent lincomycin caused a decrease in melanin content of B16 melanoma cells without affecting the gene expression of TYR, TYRP1 or TYRP2 [646]. The aminoglycosides antibiotics gentamicin [647] and netilmicin [648] reduced the synthesis of melanin in NHM by affecting the intracellular tyrosinase activity. Decreased intracellular tyrosinase activity was also associated with the antimelanogenic effect of ciprofloxacin, an antibacterial quinoline [649]. On the other hand, minocycline (a tetracycline) [650], the aminoglycoside tobramycin [651] and the quinoline flumequine [652] have shown to stimulate melanogenesis by increasing the expression of TYR, TYRP1, TYRP2 and/or MITF via activation of the p38-MAPK signalling pathway. Additionally, increased phosphorylation of c-Jun N-terminal kinase (JNK) was also observed for the treatment of cells with the flumequine, and this drug was even able to increase pigmentation of zebrafish larvae. These antibacterial agents are listed in Table V.9.

Compound D+, another quinoline that it is used in the treatment of malaria, have also demonstrated *in vitro* pro-melanogenic effects in SK-Mel-23 and SK-Mel-1 cells by increasing the intracellular activity of tyrosinase – Table V.9 (Chapter III, Section III.2.2, Figure III.10). Contrarily, the antimalarial quinolines chloroquine and quinine inhibited the *in vitro* synthesis of pigments in Melan-a cells without affecting the expression of tyrosinase and related proteins or the enzymatic activity of TYR; additionally, they presented a lightening effect in an *ex vivo* cultured human skin equivalent model (MelanoDerm™) – Table V.1. These drugs were found to disrupt the intracellular trafficking of tyrosinase and related proteins, leading to their accumulation in other organelles than melanosomes, thus preventing melanogenesis [520].

The imidazole antifungals miconazole and Compound H- decreased the melanin content of B16 [653] and SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1), respectively – Table V.9; the effect of the first was associated with a decrease in the protein expression of tyrosinase. The feeding of C57BL/6 with zidovudine (an antiretroviral medication) increased the number of melanosomes in epidermal melanocytes, leading to increased hyperpigmentation of the tail and footpads – Table V.1 [620]. Compound C+ (once used as an anthelmintic, having also completed phase II trials for advanced malignant lymphomas) induced the synthesis of melanin in SK-MEL-23 and SK-Mel-1 through increased intracellular tyrosinase activity (Chapter III, Section III.2.2, Figure III.10) – Table V.9.

Table V.9. Antimicrobials with *in vitro* effects on melanogenesis. Melanin contents and intracellular and tyrosinase activity are presented as a percentage of the control. NHM: Normal human melanocytes

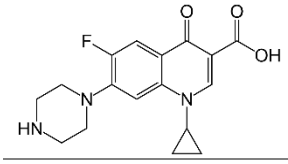
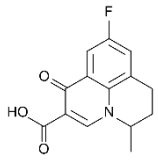
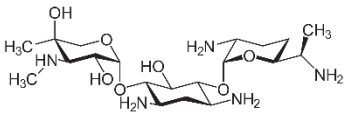
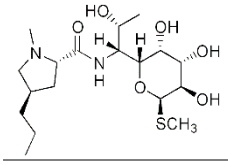
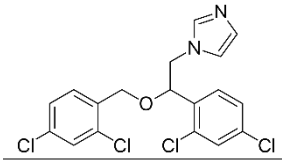
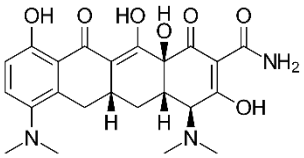
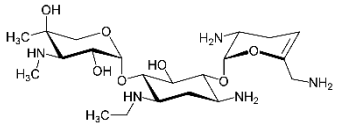
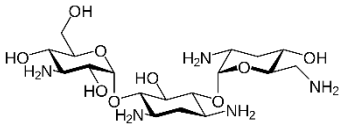
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Ciprofloxacin 	J01MA02	NHM	92% (50 μM)	79% (50 μM)	[649]
Compound C+	–	SK-Mel-23	168% (10 μM)	212% (10 μM)	*Current thesis*
Compound D+	–	SK-Mel-23	280% (10 μM)	235% (10 μM)	*Current thesis*
Compound H-	–	SK-Mel-23	56% (10 μM)	-	*Current thesis*
Flumequine 	J01MB07	B16F10	159% (50 μM)	–	[652]
Gentamicin 	J01GB03	NHM	83% (750 μM)	81% (750 μM)	[647]

Table V.9. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Lincomycin 	J01FF02	B16	≈ 75% (1 μM)	130% (1 μM)	[646]
Miconazole 	D01AC02	B16	77% (10 μM)	68% (10 μM)	[653]
Minocycline 	J01AA08	B16	175-200% (≈ 11 μM)	–	[650]
Netilmicin 	J01GB07	NHM	90% (7.5 μM)	91% (7.5 μM)	[648]
Tobramycin 	J01GB01	B16F10	350-375% (≈ 9 mM)	350-375% (≈ 9 mM)	[651]

V.2.9 ANTINEOPLASTIC AGENTS

Mercaptopurine and thioguanine, two purine analogues used in the treatment of leukaemia, were found to abrogate IBMX-stimulated melanogenesis by directly inhibiting the activity of tyrosinase – Table V.10 [654]. Some protein kinase inhibitors, also used in the treatment of leukaemia, were found to modulate the synthesis of melanin in other ways. By impairing the activation of receptor tyrosine-protein kinase KIT and downstream MAPK signalling, imatinib (Table V.10) supressed the expression of MITF and TYR, inhibiting melanogenesis [655]. On the other hand, dasatinib (Table V.1) increased pigmentation of *in vitro* cultured cells and *ex vivo* cultured human skin explants by up-regulating the gene and protein expression of MITF and TYR via ERK-dependent phosphorylation of CREB; phosphorylations of p38-MAPK

and JNK were additionally suggested to be involved in dasatinib-induced melanogenesis [618]. Nilotinib (Table V.10) reduced the phosphorylation of Akt and increased the PKA-dependent phosphorylation of CREB. Consequently, the levels of MITF, TYR and TRP1 were enhanced, and the melanin content of HM3KO human melanoma cells improved [656]. In B16F0 cells, nilotinib was also shown to effectively induce melanogenesis through phosphorylation of JNK [657].

Table V.10. Antineoplastic agents with *in vitro* effects on melanogenesis. Melanin contents and intracellular and tyrosinase activity are presented as a percentage of the control. IBMX: 3-isobutyl-1-methyl-xanthine. NHM: Normal human melanocytes

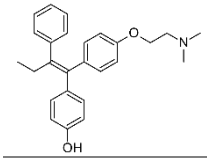
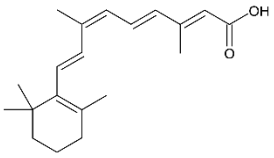
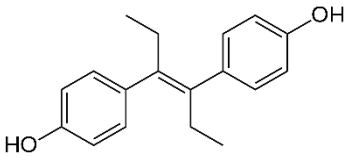
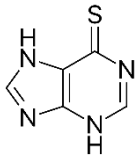
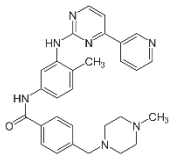
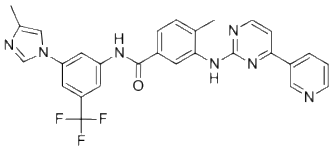
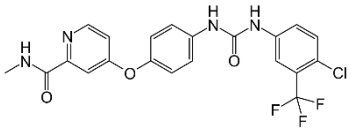
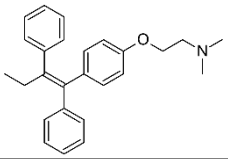
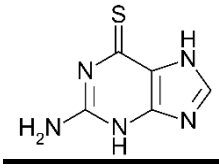
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Afimoxifene 	*None*	NHM	175-200% (1 μM)	–	[658]
Alitretinoin 	L01XX22	MNT-1	140-145% (256 nM)	–	[659]
Compound I+	-	SK-MEL-23	167% (10 μM)	–	*Current thesis*
Diethylstilbestrol 	L02AA01	B16	425-450% (10 nM)	200-225% (10 nM)	[361]
Mercaptopurine 	L01BB02	B16 (+IBMX)	80-90% (10 μM)	–	[654]
Imatinib 	L01XE01	NHM	80-90% (4 μM)	80-90% (4 μM)	[655]

Table V.10. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Nilotinib 	L01XE08	HM3KO	250-275% (1 μM)	250-275% (1 μM)	[671, 672]
Sorafenib 	L01XE05	HM3KO	425-450% (5 μM)	725-750% (5 μM)	[660]
Tamoxifen 	L02BA01	NHM	150-175% (1 μM)	–	[658]
Thioguanine 	L01BB03	B16 (+IBMX)	43% (50 μM)	–	[654]

Sorafenib (Table V.10) is another protein kinase inhibitor drug, indicated in the treatment of unresectable hepatocellular carcinoma, advanced renal cell carcinoma and locally recurrent or metastatic, progressive, differentiated thyroid carcinoma refractory to radioactive iodine treatment; its pro-melanogenic effect was associated with increased mRNA and protein levels of MITF, TYR and TRP1. Reduced phosphorylation of Akt and ERK, along with increased phosphorylation of tyrosine 216 of GSK3β and further activation of β-catenin signalling seems to rule the melanogenic mechanism of action for sorafenib [660].

Diethylstilbestrol (non-steroidal oestrogen medication) and tamoxifen (anti-oestrogen medication) are used in the treatment of breast cancer; nonetheless, the first is more commonly applied in the treatment of prostate cancer. Both drugs stimulate *in vitro* melanogenesis – Table V.10. Diethylstilbestrol fuelled the synthesis of melanin via cAMP/PKA-mediated up-regulation of MITF and TYR (as well as related proteins, TRP1 and TRP2) [361]. The transcript levels of genes encoding for premelanosome protein (PMEL) and protein melan-a (MLANA), both involved in the biogenesis of melanosomes, showed an increased tendency upon treatment of NHM with selective oestrogen receptor modulator tamoxifen as well as

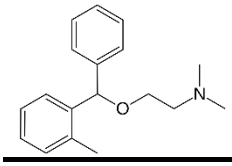
afimoxifene (under clinical trials for the treatment of breast cancer) [658]. Compound I+ (marketed in Japan) is another anti-oestrogen therapy for the treatment of breast cancer; as an aromatase inhibitor, it prevents the synthesis of oestrogens from androgens. Compound I+ was found to increase the production of melanin in SK-Mel-23 cells – Table V.10 (Chapter III, Section III.2.1, Table III.S1).

The retinoid alitretinoin (9-*cis* retinoic acid), used in the treatment of Kaposi's sarcoma, increased tyrosinase protein levels in MNT-1 cells, with consequent overproduction of melanin – Table V.10 [659].

V.2.10 ANTIPARKINSONIAN DRUGS

The dopaminergic agent Compound E+ (Table V.11) was selected as a hit inducer of melanogenesis in a screening of generic drugs performed in SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1). Orphenadrine (Table V.11) is an anticholinergic drug that decreased the production of melanin in Melan-a cells [519].

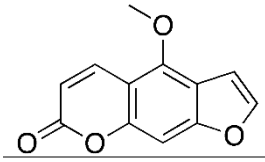
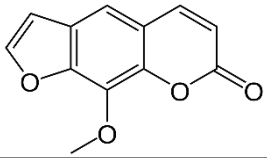
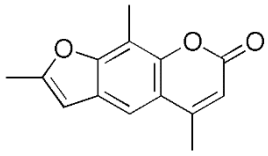
Table V.11. Antiparkinsonian drugs with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound E+	-	SK-Mel-23	179% (10 μM)	–	*Current thesis*
Orphenadrine 	N04AB02	Melan-a	55% (5 μM)	–	[519]

V.2.11 ANTIPSORIATICS

Three psoralens, used in the treatment of psoriasis, have demonstrated the ability to increase the production of melanin – Table V.12. Bergapten increased the expression of TYR and TYRP1, with PKA and PKC systems playing an important role in its melanogenic effect [661]. Methoxsalen increased the protein expression of MITF and TYR via activation of the PKA signalling pathway [662]. Trioxysalen acted on melanogenesis of B16 cells by increasing intracellular tyrosinase activity [663].

Table V.12. Antipsoriatics with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Bergapten 	D05BA03	S91-M3	600-650% (50 M)	*Increased* (50 μM)	[661]
Methoxsalen 	D05BA02	Mel-Ab	325-350% (100 μM)	600-700% (100 μM)	[662]
Trioxysalen 	D05BA01	B16	250-275% (100 μM)	150-175% (100 μM)	[663]

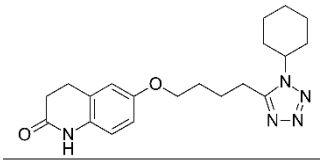
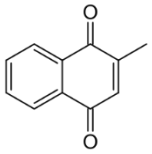
V.2.12 ANTITHROMBOTIC AND ANTIHEMORRHAGIC AGENTS

Cilostazol and Compound A+ are antithrombotic agents, affecting platelet aggregation by inhibiting phosphodiesterase type 3 (PDE_3), an enzyme that degrade cAMP; additionally, Compound A+ also inhibits PDE_5. Both drugs have shown to stimulate the production of melanin. Cilostazol (Table V.13) increased the melanin content of B16F10 cells by enhancing the phosphorylation of CREB (via PKA), leading to further upregulation of MITF and TYR expression [527]. Compound A+ was recognized as a stimulator of melanogenesis in a screening of generic drugs performed on SK-Mel-23 cells (Table V.2; Chapter III, Section III.2.1). Increased melanin synthesis was also observed in SK-Mel-1 human melanoma cell line and the melanogenic effect in both cell lines was related to increased intracellular tyrosinase activity, possibly caused by accumulation of cAMP and further up-regulation of tyrosinase expression (Chapter III, Section III.2.2, Figure III.10). Importantly, the treatment of human scalps with topical Compound A+ caused a darkening of hair in virtue of the increased follicular production of melanins and incorporation into the hair shafts (Chapter IIII, Section III.2.3, Figure III.12).

The antithrombotics tranexamic acid (Table V.1) and menadione (Table V.13) have shown antimelanogenic effects. Tranexamic acid is an antifibrinolytic with proved efficacy in the treatment of

melasma, an acquired, chronic, and recurrent form of hypermelanosis [627–629]. The melanogenic effect of tranexamic acid has been associated with decreased expression of TYR, TYRP1 and TYRP2 caused by enhanced degradation of MITF upon the activation of the ERK signalling pathway [630]. Additionally, the inhibition of melanogenesis by tranexamic acid have been associated with the activation of the autophagy system, involved in the degradation of melanosomes [664]. Menadione (Vitamin K3) also caused a reduction in the melanin content of Mel-Ab cells by increasing the phosphorylation (activation) of ERK, further reducing MITF and TYR expression [665].

Table V.13. Antithrombotic and antihemorrhagic agents with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Cilostazol 	B01AC23	B16F10	350% (10 μM)	325-350% (10 μM)	[527]
Menadione 	B02BA02	Mel-Ab	50-60% (5 μM)	60-70% (5 μM)	[665]

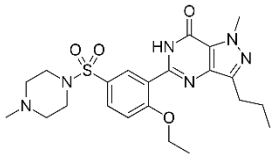
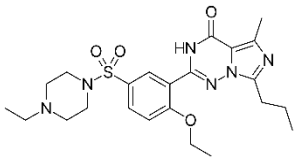
V.2.13 DRUGS USED IN ERECTILE DYSFUNCTION

Sildenafil and vardenafil exert their therapeutic effects by inhibiting phosphodiesterase type 5 (PDE_5), thus elevating the cyclic guanosine monophosphate (cGMP) levels. In B16 cells, these drugs stimulated the phosphorylation of CREB in a PKG-dependent manner, leading to increased tyrosinase expression and melanin synthesis – Table V.14. The activation of PKG was suggested to be mediated by the accumulation of intracellular concentrations of cGMP upon inhibition of PDE_5 [526].

V.2.14 IMMUNOSUPPRESSANTS

Sirolimus, pimecrolimus and tacrolimus are immunosuppressants that increase the *in vitro* production of melanin – Table V.15. In MNT-1 cells, the effect of sirolimus was achieved through increased protein

Table V.14. Drugs used in erectile dysfunction with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Sildenafil 	G04BE03	B16	175-200% (20 μM)	200-225% (20 μM)	[526]
Vardenafil 	G04BE09	B16	225-250% (20 μM)	250-275% (20 μM)	[526]

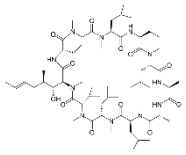
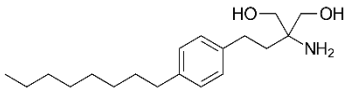
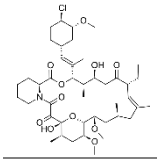
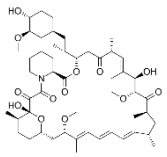
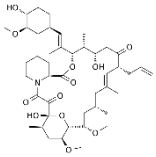
expression of MITF, TYR and TYRP1 [274]. Up-regulation of MITF and TYR was also observed in NHM treated with calcineurin inhibitors pimecrolimus [666] and tacrolimus [667,668], respectively. Furthermore, tacrolimus showed pro-melanogenic effect in A375, MNT-1 and B16F10 cells [668,669]. In the mouse cell lines, despite it did not affect the transcription levels of tyrosinase, it promoted melanosome maturation by increasing the pH, enhancing the stability of the enzyme. Tacrolimus also improved UVB-mediated secretion of melanosome and their uptake by keratinocytes [669].

Cyclosporin, another calcineurin inhibitor, showed a melanogenic effect different than that presented by pimecrolimus or tacrolimus – Table V.15. In NHM, it decrease the intracellular tyrosinase activity and melanin content of cells by downregulating the gene and protein expression of tyrosinase [670]. Another immunosuppressant with proven *in vitro* anti-melanogenic activity is fingolimod (Table V.15). In Mel-Ab, inhibition of melanogenesis by was caused by a decrease in MITF and TYR, associated with a reduction in the protein and mRNA levels of β-catenin [671].

V.2.15 PSYCHOANALEPTICS

Two anticholinesterases used in the treatment of dementia are known to inhibit the production of melanin: Compound B- (Table V.2) and galantamine (Table V.16). The latter was shown to inhibit melanogenesis in NHM [672]. The former deceased the melanin content of SK-Mel-23 and SK-Mel-1 cells (Chapter III, Section III.2.2). Notably, the treatment of human scalps with topical Compound B- caused a lightening of

Table V.15. Immunosuppressants with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. NHM: Normal human melanocytes

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Cyclosporin 	L04AD01	NHM	*Decreased* (10 μM)	35.5 (10 μM)	[670]
Fingolimod 	L04AA27	Mel-Ab	60-70% (5 μM)	60-70% (5 μM)	[671]
Pimecrolimus 	D11AH02	NHM	176% (1 μM)	125% (1 μM)	[666]
Sirolimus 	L04AA10	MNT-1	≈ 150% (100 nM)	≈ 150% (100 nM)	[274]
Tacrolimus 	L04AD02	NHM	247% (1 μM)	144% (1 μM)	[667]

hair in virtue of the decreased follicular production of melanins and incorporation into the hair shafts (Chapter III, Section III.2.3, Figure III.12).

A group of ten tricyclic antidepressants (non-selective monoamine reuptake inhibitors) were identified as compounds that inhibit melanogenesis in a screening of a library containing drugs and natural products – Table V.16. These drugs are desipramine, imipramine, clomipramine, trimipramine, amitriptyline, nortriptyline, protriptyline, doxepin, amoxapine and maprotiline. Although they had no effect on tyrosinase (activity or protein expression), they disrupted the intracellular trafficking of melanogenic proteins (TYR,

TYRP1, TYRP2), leading to their accumulation in granules distributed throughout the cytoplasm instead of melanosomes, the only organelles where melanin synthesis can take place [519].

Table V.16. Psychoanaleptics with *in vitro* effects on melanogenesis. Melanin contents and intracellular tyrosinase activities are presented as a percentage of the control. IBMX: 3-isobutyl-1-methyl-xanthine. NHM: Normal human melanocytes

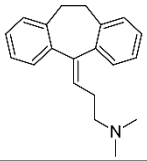
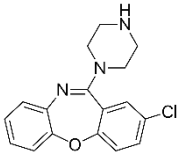
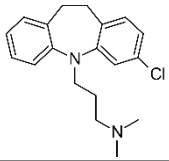
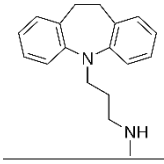
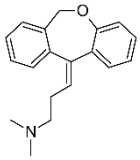
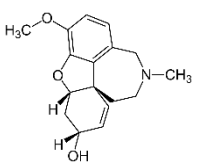
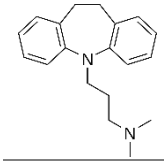
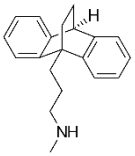
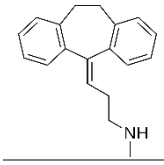
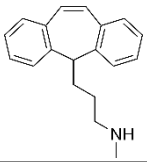
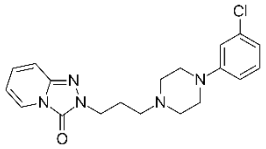
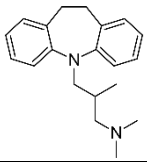
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Amitriptyline 	N06AA09	Melan-a	<20% (5 μM)	*No change* (5 μM)	[519]
Amoxapine 	N06AA17	Melan-a	20-30% (5 μM)	*No change* (5 μM)	[519]
Clomipramine 	N06AA04	Melan-a	<20% (5 μM)	*No change* (5 μM)	[519]
Desipramine 	N06AA01	Melan-a	30-40% (5 μM)	*No change* (5 μM)	[519]
Doxepin 	N06AA12	Melan-a	40-50% (5 μM)	*No change* (5 μM)	[519]
Galantamine 	N06DA04	NHM	71% (1 μM)	–	[672]

Table V.16. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Imipramine 	N06AA02	Melan-a	<20% (5 μM)	*No change* (5 μM)	[519]
Maprotiline 	N06AA21	Melan-a	30-40% (5 μM)	*No change* (5 μM)	[519]
Nortriptyline 	N06AA10	Melan-a	20-30% (5 μM)	*No change* (5 μM)	[519]
Protriptyline 	N06AA11	Melan-a	40-50% (5 μM)	*No change* (5 μM)	[519]
Trazodone 	N06AX05	B16 melanoma 4A5 (+IBMX)	50-60% (70 μM)	–	[545]
Trimipramine 	N06AA06	Melan-a	20-30% (5 μM)	*No change* (5 μM)	[519]

Modulators of the serotonergic system have also shown the ability to modulate the production of melanin. Trazodone, a serotonin antagonist and reuptake inhibitor (SARI), decreased melanin synthesis in IBMX-stimulated B16 melanoma 4A5 cells through a mechanism that do not involve the direct inhibition of tyrosinase – Table V.16 [545]. Likewise, the selective serotonin reuptake inhibitor (SSRI) Compound C-hindered melanogenesis in SK-Mel-23 and SK-Mel-1, independently of the inhibition of tyrosinase activity – Table V.2. Moreover, its topical application to the scalp of human subjects decreased follicular

melanogenesis, with the hair shafts of treated areas presenting lower melanin contents compared to the untreated ones (Chapter III, Section III.2.3, Figure III.15). On the contrary, fluoxetine increased melanin synthesis in NHM and B16F10 by increasing the expression of MITF, TYR and TYRP1 – Table V.2. The systemic application of fluoxetine to C57BL/6 mouse model also increased hair pigmentation by up-regulating the expression of those melanogenic proteins in hair follicles [538], and a stimulation of human hair follicle melanogenesis was demonstrated in *ex vivo* studies [632]. This SSRI have been proved to induce melanogenesis through serotonin (5-HT) 1A and 2A receptors [539,540].

Idebenone (Table V.1) is another psychoanaleptic with attested *in vivo* melanogenic properties, having shown to attenuate pregnancy-induced chloasma, post-inflammatory as well as post-medicinal hyperpigmentation of the skin [621].

V.2.16 PSYCHOLEPTICS

Several antipsychotics have shown to decrease the production of melanin – Table V.17. Clozapine (a dibenzodiazepine), loxapine (a dibenzoxazepine), chlorprothixene (a thioxanthene) and promazine (a phenothiazine) decreased the production of melanin in Melan-a cells [519]. Accordingly, the phenothiazines Compound K- and thioridazine inhibited melanogenesis in SK-MEL-23 (Chapter III, Section III.2.1, Table III.S1) and NHM [673], respectively; the effect of thioridazine was related to decreased protein expression of MITF.

Table V.17. Psycholeptics with *in vitro* effects on melanogenesis. Melanin contents and intracellular and tyrosinase activity are presented as a percentage of the control. NHM: Normal human melanocytes

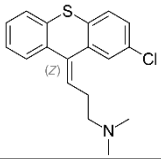
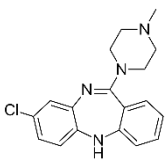
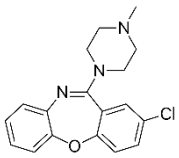
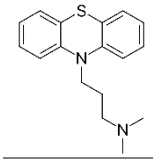
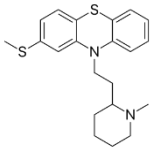
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Chlorprothixene 	N05AF03	Melan-a	29% (5 μM)	–	[519]
Clozapine 	N05AH02	Melan-a	85% (5 μM)	–	[519]

Table V.17. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound K-	–	SK-Mel-23	58% (10 μM)	–	*Current thesis*
Loxapine 	N05AH01	Melan-a	55% (5 μM)	–	[519]
Promazine 	N05AA03	Melan-a	54% (5 μM)	–	[519]
Thioridazine 	N05AC02	NHM	93% (1 μM)	94% (1 μM)	[673]

The anxiolytic diazepam (Table V.1) is a benzodiazepine with proven effects regarding the stimulation of melanogenesis in NHM, SK-Mel-2, B16F10, Melan-a and *ex vivo* cultured human skin explants. Additionally, it showed to affect melanocyte dendricity, melanosome trafficking and their capture at the dendrite tips. The effects of diazepam are attributed to the activation of peripheral benzodiazepine receptor (PBR), leading to increased intracellular levels of cAMP and phosphorylated of PKA and CREB. As a result, the expression of MITF, TYR, TYRP1 and TYRP2 as well as proteins involved in melanosome transport (RAB27A, MYO5A) and dendrite formation (RAB17 and CDC42) increases [619].

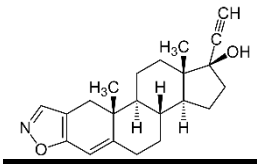
Finally, melatonin (Table V.2) decreased melanin content in MNT-1 and inhibited melanin synthesis in *ex vivo* cultured hair follicles of Siberian hamsters [635,636].

V.2.17 SEX HORMONES AND MODULATORS OF THE GENITAL SYSTEM

Compound H+, an antiandrogen, was flagged as a stimulator of melanogenesis in a screening of generic drugs in SK-Mel-23 cells (Chapter III, Section III.2.1, Table III.S1) – Table V.18 The antigonadotropin agent danazol (Table V.18) decreased unstimulated and α-MSH or IBMX-stimulated synthesis of melanin

in B16 melanoma 4A5 through downregulation of tyrosinase at the post-transcriptional level (protein was decreased but not mRNA) [674].

Table V.18. Sex hormones and modulators of the genital system with *in vitro* effects on melanogenesis. Melanin contents and tyrosinase activity are presented as a percentage of the control

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound H+	-	SK-Mel-23	170% (10 μM)	-	*Current thesis*
Danazol 	G03XA01	B16 melanoma 4A5	≈ 60% (20 μM)	-	[674]

V.2.18 OTHERS

Several drugs with proved *in vitro* melanogenic effect, singly distributed across a wide range of pharmaceutical groups other than those mentioned before, are listed in Table V.19.

Compound A- (a discontinued antigout medication), Compound F- (antitussive, antagonist of the muscarinic receptors) and Compound J- (anticholinergic experimental drug) were hit inhibitors of the production of melanin in the screening of generic performed in SK-Mel-23 in the course of this thesis work (Chapter III, Section III.2.1). Additionally, the first also showed inhibitory activity towards melanogenesis in SK-Mel-1. The effect of Compound A-, at least in SK-Mel-23 did not seem to be related to the inhibition of intracellular tyrosinase activity (Chapter III, Section III.2.2, Figure III.11). Cinnarizine, an antihistamine and calcium channel blocker used in the management of vertigo, also decreased melanin synthesis in IBMX-stimulated B16 melanoma 4A5 through a mechanism other than the inhibition of tyrosinase [545].

Cyclobenzaprine (an antagonist of 5-HT_2 receptors, used as a muscle relaxant) [519] and vincamine (a peripheral vasodilator) [675] decreased melanin contents of Melan-a and B16 cells, respectively. Auranofin, a specific antirheumatic agent, down-regulated the cAMP levels and caused an increasing in the number of immature melanosomes, leading to a diminished melanin content in B16F10 and MNT-1

cells [676]. Decreased cAMP content and further expression of MITF, TYR, TYRP1 and TYRP2 were also associated to the antimelanogenic effect of antiarrhythmic propafenone [677].

Finasteride is an inhibitor of testosterone 5α-reductase, used in the treatment of benign prostatic hypertrophy. This drug is also a successful case of drug repurposing, having a currently parallel indication in the management of male pattern baldness. In *in vitro* studies, it showed an additional antimelanogenic effect (Melan-a and B16F10 cells), by decreasing MC1R, TYR, TYRP1, TYRP2 and adenylyl cyclase (AC) expression [678].

Mesna, a detoxifying agent for antineoplastic treatment, decreased melanin synthesis in MNT-1 cells. When cells were treated with this compound, an increased activation and nuclear translocation of transcription factor forkhead box O3 (FOXO3A) was verified, resulting in the downregulation of MITF, TYR, TYRP1 and TYRP2. Additionally, mesna also increased the phosphorylation of JNK and inhibited the activity of mushroom tyrosinase [679].

Table V.19. Other drugs with *in vitro* effects on melanogenesis. Melanin contents and tyrosinase activity are presented as a percentage of the control. IBMX: 3-isobutyl-1-methyl-xanthine. NHM: Normal human melanocytes

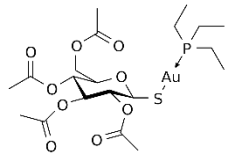
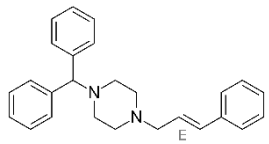
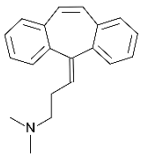
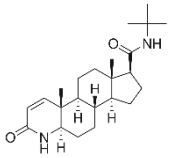
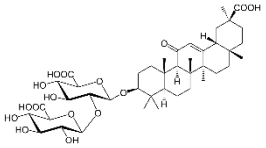
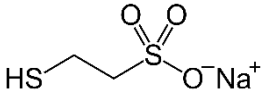
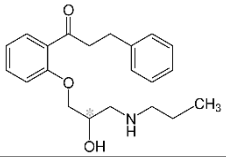
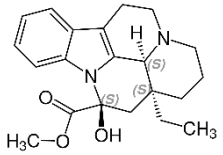
	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Auranofin 	M01CB03	MNT-1	60-70% (2 μM)	60-70% (2 μM)	[676]
Cinnarizine 	N07CA02	B16 melanoma 4A5 (+IBMX)	40-50% (70 μM)	–	[545]
Compound A-	–	SK-Mel-23	72% (10 μM)	103% (10 μM)	*Current thesis*
Compound F-	–	SK-Mel-23	54% (10 μM)	–	*Current thesis*

Table V.19. (Continued)

	ATC code	Cell line	Melanin content	Tyrosinase activity	Ref.
Compound J-	–	SK-Mel-23	57% (10 μM)	–	*Current thesis*
Cyclobenzaprine 	M03BX08	Melan-a	36% (5 μM)	–	[519]
Finasteride 	G04CB01	Melan-a	66% (10 μM)	80% (10 μM)	[678]
Glycyrrhizic acid 	A05BA08	B16F10	≈ 160% (1 mM)	220% (1 mM)	[680]
Mesna 	V03AF01	MNT-1	30-40% (1 μM)	–	[679]
Propafenone 	C01BC03	NHM	50-60% (25 μM)	–	[677]
Vincamine 	C04AX07	B16	60-70% (25 μM)	–	[675]

Omeprazole, Table V.1, is a gastric proton pump inhibitor that decreased the melanin content of *in vitro* cultured NHM, B16F10 and Melan-a cells. Regarding the mechanism of action, in Melan-a cells, omeprazole was shown to inhibit TYR and TYRP2 protein expression via activation of the p38-MAPK/JNK

pathways and further suppression of MITF. In B16F10 and NHM, a decrease in the protein level of tyrosinase was also verified without the mRNA levels being affected; it was shown that omeprazole inhibit ATP7A, a copper transporting P-type ATPase required for copper acquisition by tyrosinase, causing the degradation of the enzyme at a post-transcriptional step. Importantly, this drug showed *ex vivo* (MelanoDerm™ reconstructed human skin) and *in vivo* (zebrafish and humans) depigmenting effects; the effect in humans was related with the ability to reduce UVB-induced skin pigmentation [625,626]. The dietary supplement nicotinamide (a form of vitamin B_3) also had significant depigmenting effect in *ex vivo* (skin equivalents) and *in vivo* (women with axillary hyperpigmentation) assays – Table V.1 [623][624].

From all the drugs in this section, only glycyrrhizic acid (a lipotropic agent that help catalyse the metabolism of fat) stimulated the production of melanin (B16/BL6 and B16F10) by increasing the expression of MITF, TYR and TYRP2 through the cAMP signalling pathway [680,681].

V.3 MAJOR REMARKS AND FUTURE PERSPECTIVES

Currently, over 900 compounds are reported to have the ability to modulate melanogenesis, with 15% being recognized as inducers of the production of melanin and 85% showing to inhibit its synthesis – Figure V.1. The far higher interest in inhibitors of melanogenesis is presumed to be mostly driven by the urge to attend the consumers demands for skin lighteners, especially in the Asian markets [601]. However, over the last years, the appeal of melanogenesis inducers as a sunless tanning strategy for cancer prevention or merely aesthetic purposes have increased [603,605].

Regarding typology, the majority of compounds with melanogenic effects were isolated from natural sources. Over the last years, *clean* or *natural care* cosmetics have had a moment; in 2019, natural brands represented over 30% of total skincare, with sales growing by 14% over 2018 (The NPD Group – Market Research, NY, USA). However, contrarily to the common belief (wrongly perpetuated by the cosmetic industry), ingredients obtained from natural sources are not exempted from undesirable effects, and a significant prevalence of health problems are associated with their usage [682–685]. In this sense, extensive safety studies must always be conducted prior to the marketing of a cosmetic product based on naturally sourced ingredients.

On the opposite side, the repurposing of drugs is one of the least explored branches regarding the searching for new modulators of melanogenesis, with only 12% of documented compounds having been discovered by this approach. Since the study of available therapeutic agents is most probably the safest,

time-saving, and cost-effective tactic for uncovering new agents for pigmentation-related purposes, this review was conducted to establish the state-of-art of drugs with melanogenic effects and to serve as the foundations upon which future works can expand the knowledge in this matter.

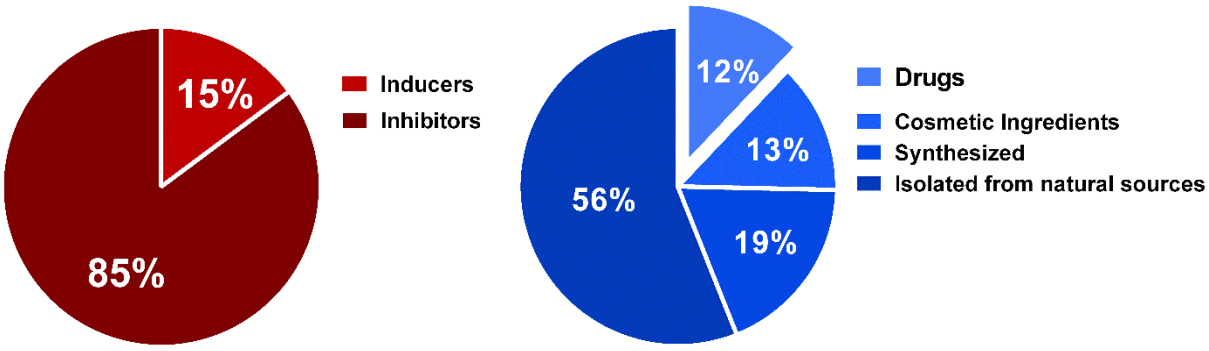


Figure V.1. Distribution of melanogenesis modulators, according to their effect (inducer or inhibitor) and typology (repurposed drugs, repurposed cosmetic ingredients, newly synthesized compounds, or compounds isolated from natural sources). The analysis took into consideration reports (scientific papers and patents) available by June 2020, and the data generated in the current PhD thesis.

In trying to ascertain a link between current clinical use and the newly discovered melanogenic effect, the drugs reviewed here were grouped according to the main anatomical group. According to Figure V.2, modulators of melanogenesis were already repurposed from drugs with known therapeutic effects on practically any organ or system. This finding is not surprising since this classification is hardly representative of the mechanism of action, with drugs sharing cellular targets being used in different systems of tissues and organs, and with different treatments within the same system of organs requiring the use of drugs with distinct cellular targets. However, the search for modulators of melanogenesis in drugs known to act on the nervous system have been remarkably meaningful. The profusion of repurposed drugs falling within this category may be related to the fact that melanocytes are derived from the embryonic neural crest and share many targetable morphological features (namely receptors and transporters) with neural cells [11,546].

A more appropriate way in trying to establish classes of drugs with melanogenic effects is grouping them according to their known cellular targets – Figure V.3. Several of the drugs reviewed here modulate the activity of cyclooxygenases, the enzymes that catalyse the biosynthesis of prostaglandins. The melanogenic role of a variety of inflammatory mediators (including prostaglandins) is widely

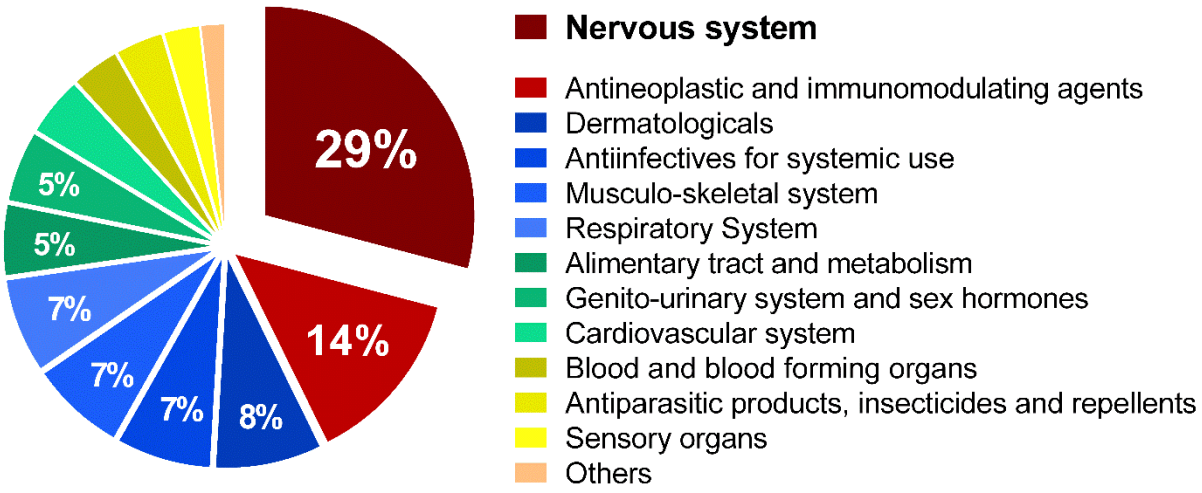


Figure V.2. Distribution of drugs with melanogenic effects, according to their anatomical main group. The analysis took into consideration reports (scientific papers and patents) available by June 2020, and data generated in the current PhD thesis.

acknowledged, with hyperpigmentation or hypopigmentation of skin being frequently reported in dermatology and cosmetology following inflammation [686]. In line with the role of inflammatory factors, some prostaglandin analogues (that target prostaglandin receptors) also showed *in vivo* pro-melanogenic effects. Alongside cyclooxygenases, the histamine receptors were the most represented clinical targets of drugs with melanogenic effects. Although the regulation of melanogenesis mediated by H_2-receptor (through the cAMP/PKA signalling pathway) has long been recognized [336,338], the fact that all drugs mentioned here are mainly antagonists of the H_1-receptor extend the scope of antihistaminergic compounds with possible implications in the down-regulation of the synthesis of melanin.

Since the control of melanin synthesis involves an intricate network of proteins which activities are dependent on their state of phosphorylation, it is not surprising that a significant number of drugs that inhibit kinases have proved ability to modulate the production of melanin. Foreseeable it is also the fact that stimulation of melanogenesis can be achieved with inhibitors of phosphodiesterases, enzymes that hydrolyse the second messenger molecules cAMP and cGMP; whereas the first takes part in one of the most important signalling pathways leading to upregulated synthesis of melanin, the latter has been recognized as a major intervenient in UVB-induced pigmentation of skin [256,525].

Finally, oestrogen receptors (ERs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), HMG-CoA reductase (that catalyses the synthesis of mevalonate, a critical

intermediate in the formation of cholesterol) and calcineurin were all identified as targets of at least two drugs with the ability to modulate the synthesis of melanin. While oestrogens/ERs [240,362–364], PPARs [261–263], HMG-CoA reductase/cholesterol [687,688] and retinoids/RARs [240,689] have been implicated in melanogenesis, a possible role of calcineurin needs to be explored.

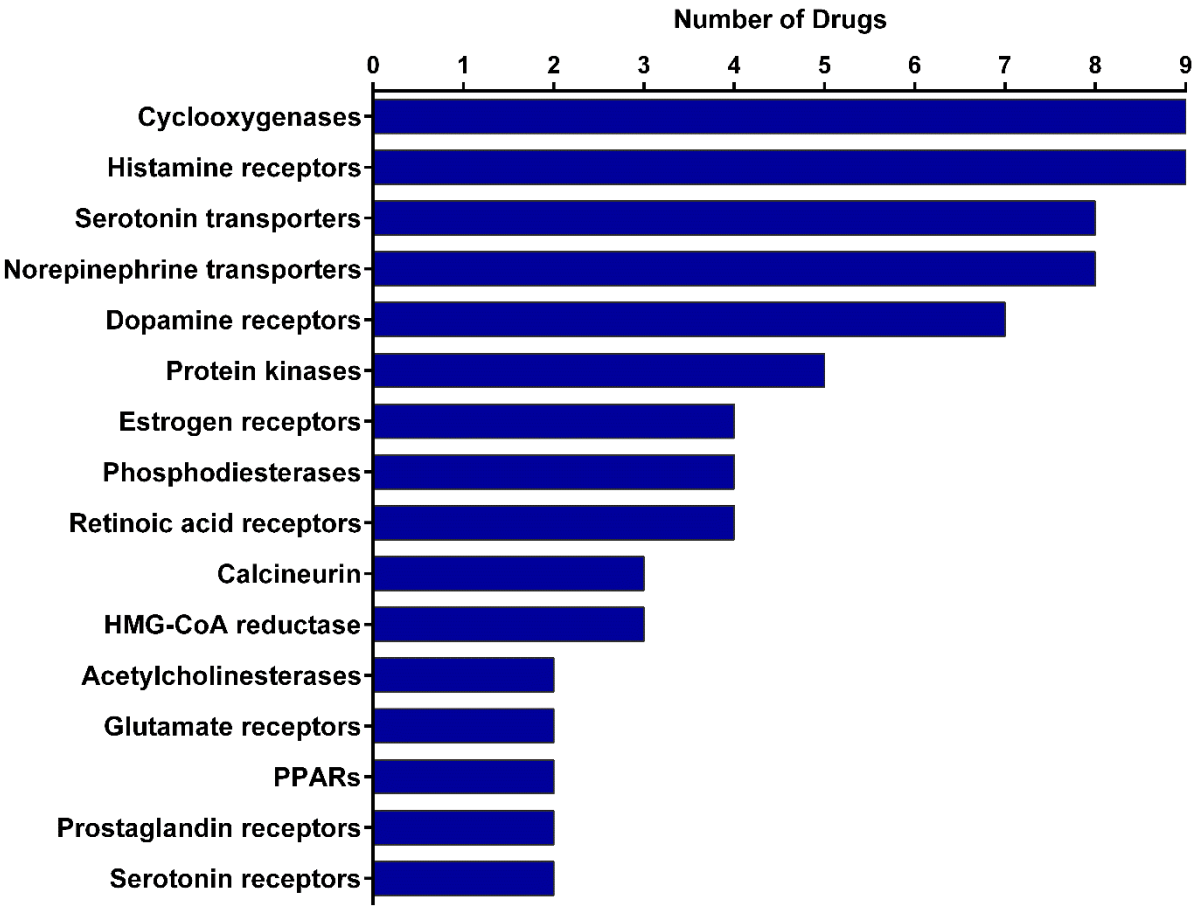


Figure V.3. Most represented molecular targets of drugs with proved melanogenic effects. The analysis took into consideration drugs reported in publications (scientific papers and patents) available by June 2020, and in the current thesis work. PPARs: Peroxisome proliferator-activated receptors.

In close association with the ability to exert actions on the nervous system, many drugs fall within one of the following therapeutic categories: inhibitors of acetylcholinesterase (an enzyme that degrade acetylcholine), modulators of dopamine receptors, antagonists of serotonin receptors, antagonists of glutamate receptors, inhibitors of the transporters of norepinephrine or serotonin. Since all the mentioned neurotransmitters are involved in the regulation of melanogenesis [69,240], a changing in their

bioavailability or in the kinetics of their binding to the respective receptors can easily explain the shift in the production of melanins induced by such drugs.

Although some insight regarding classes of drugs where it is most probable to find modulators of melanogenesis is provided, it is important to mentioned that the data generated can be constrained at different levels. First, as shown in Figure V.3, many classes were defined based on cellular targets that are shared only by two or three drugs; however, a much larger number of compounds must fit in such categories before they can be unarguably representative of drugs with melanogenic effects. Alongside, due to the lack of compounds sharing the same cellular target, there are classes of drugs neglected by this analysis (like modulators of cannabinoid [401] and sphingosine-1-phosphate [317,318] receptors) that are expected to be highly relevant in terms of melanogenesis modulation.

Second, as most works do not provide clear evidence that those drugs act on melanocytes via their canonical targets, it is always possible that their melanogenic effects are independent of their known shared mechanism of action. Consequently, other associations may be also useful for the prediction of new pharmacological modulators of melanogenesis. As a matter of fact, nine of the drugs reviewed here were uncovered by sharing chemical structural similarity to tricyclic antidepressants tested previously for their ability to inhibit melanogenesis [519]. Moreover, all anti-psoriatic drugs (with poorly explored cellular targets) are classified as furanocoumarins. Many other drugs also fit into the same chemical classes, like quinolines and retinoids. In this sense, the searching for new melanogenesis modulators based on shared functional chemical groups with the drugs presented here may also be of interest.

At last, perhaps the most critical constraint of the categorizations presented here, is the poor representativity of melanogenesis modulators identified in unbiased *in vitro* testing. To rigorously rank categories of drugs (based on cellular targets, chemical structure, or other system) by the suitability of their members to exert a melanogenic effect, it is important to assure that different types of compounds have similar probability of being tested. Nevertheless, as already revealed before, most times the compounds are evaluated based on the notion that their known functionalities have implications on the synthesis of melanin or because they are somehow related to other already stablished modulators of melanogenesis. To compensate this trend as well as to expand and homogenize the knowledge about drugs that modulate melanogenesis, the screening of libraries with high chemical and pharmacological diversity is an approach to take into consideration.

High-throughput screening (HTS) is an expedite and widespread approach to generate large scale datasheets that translate into comprehensive surveys of compounds with a specific biological or

biochemical effect. Despite that, its use for the identification of modulators of melanogenesis have been poorly explored, except for tyrosinase targeting assays. Although the direct targeting of tyrosinase is still hailed as the key to attain a changing in the production of melanin, there is increasingly evidence of compounds that change the pigmentation status of melanocytes without directly affecting the catalytic activity of the enzyme. In fact, amongst the twenty-nine drugs in this review that were tested regarding the ability to interact with mammalian and/or mushroom tyrosinase, only thioguanine and mercaptopurine were reported to exert melanogenic effects mainly through direct modulation of catalytic activity of the enzyme [654]. In this sense, for more inclusive screening of compounds, a straightforward quantification of melanin must always be preferred. Until recently, the problem with such approach was related to the impracticality of employing existing methods to large scale assays. On one hand, despite being highly sensitive and accurate, techniques as electron spin resonance spectrometry, photoacoustic spectroscopy and high-performance liquid chromatography are not easily performed in most laboratories as they require very specific equipment and expertise. On the other hand, although very easy to perform, the estimation of melanin contents by absorption spectroscopy do not provide the sensitivity and specificity required to make the simultaneous assessment of a substantial number of compounds doable. However, a recent re-examination of fluorescence spectroscopy method allowed the establishment of a protocol for quantification of melanin in biological samples (cells, zebrafish embryos, human hair) that it is both easy to perform as well as highly sensitive and accurate – Chapter II [507]. This protocol can be adapted to a format that match the necessities of large-scale screenings, having been successfully applied in testing Prestwick Chemical Library® – Chapter III. From this assay, twenty-one drugs distributed across several biological and chemical classes were targeted as new modulators of the production of melanin. Importantly, as some highly significant effects came from drugs that do not fit in any pharmacological or chemical classes of compounds known to affect melanogenesis (e.g., Compound B+, Compound C+, Compound A-), this assay reinforced the importance of screening libraries with high diversity to find drugs with potential to influence the pigmentary processes and expand the current expertise on this matter.

Whereas most modulators of pigmentation included in this review are still in early stages of discovery, a noteworthy number of drugs have by now undertaken some steps towards a successful dermatological and/or cosmetic product. Attending to Table V.1, six drugs already showed the ability to modulate the pigmentation of *ex vivo* cultured skin explants or skin equivalents and other two presented *in vivo* melanogenic effects on animal models (zidovudine and metformin). More important, Idebenone, nicotinamide, omeprazole and tranexamic acid all performed well in clinical trials regarding the treatment of hyperpigmentary conditions, making them the current upfront drugs for development of new skin

whitening/lightening products. Attending to Table V.2, seven drugs have shown the ability to shift the hair colour in *in vivo* studies. Although the tendency is not illustrated here, the searching of modulators of melanogenesis for hair-related purposes have been extensively neglected in comparison to skin-related ones. However, given the mounting evidence of health hazards associated with existing procedures, changing the colour of hair with non-toxic agents of melanogenesis modulation have the potential to revolutionize the hair cosmetic industry. In this regard, Compound A+, Compound B- and Compound C- are all in the frontline of new cosmetic ingredients for the development of such innovative class of products. In a preliminary clinical trial, the hair of volunteers become darker after topical application of Compound A+ (Chapter III, Section III.2.3, Figure III.12). In the same trial, the treatment of the scalp with Compound B- induced the lightening of the growing hair. Compound C- did not promote any visual lightening of colour, but there was a significant decreased in the melanin content of hairs (Chapter III, Section III.2.3, Figure III.15). The data generated undoubtedly provided pioneer evidence of the feasibility of topically applied products, formulated with modulators of melanogenesis, to efficiently change human hair colour, but other trials are needed to improve the effects and validate the cosmetic significance of those findings. In performing those trials, one of the main aspects to consider is the choosing of a formulation that promotes selective targeting of hair follicles, maximizing the concentration of drugs reaching the follicular melanocytes; poor follicular penetration upon topical application is the reason why many molecules used in the treatment of hair follicle-related conditions like alopecia fail to produced acceptable results. In this context, nanoparticles-based formulations seem to be the most obvious choice given the natural tendency of nanoparticles to permeate the skin along follicular ducts upon topical application. Among particulate systems suitable for follicular delivery of drugs, poly (D,L-lactide) (PLA) nanoparticles have been showing to perform amazingly, being responsible for relaunching Cyclosporin A (a compound with very low follicular permeability) as a promising treatment of alopecia – Chapter IV [690].

In conclusion, by incorporating the most up-to-date information regarding drugs with melanogenic effects, this thesis is expected to be beneficial for future discovery of novel modulators of melanogenesis, accelerate the development of skin whitening or tanning products and, above all, boost the research in the field of disruptive cosmetics for hair colour modification.

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