João Miguel Lousa Dias Ferreira

# ADVANCES ON PHOTODYNAMIC THERAPY: NEW PYRIDINE-FUSED DIPHENYLCHLORINS AS PHOTOSENSITIZERS FOR MELANOMA TREATMENT

Relatórios de Estágio e Monografia intitulada "Advances on photodynamic therapy: new pyridine-fused diphenylchlorins as photosensitizers for melanoma treatment" referentes à Unidade Curricular "Estágio", sob orientação, respetivamente, do Dr. João Manuel Baliza Santiago Maia, e dos Professores Doutores Maria Filomena Rabaça Roque Botelho e Sérgio Paulo Magalhães Simões e apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas de Mestrado Integrado em Ciências Farmacêuticas.

Setembro 2017

UNIVERSIDADE DE COIMBRA

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The experimental work described in this dissertation was conducted at Biophysics Institute and at Institute for Biomedical Imaging and Life Sciences (IBILI) - Faculty of Medicine, University of Coimbra.

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*Coimbra, 12 de setembro de 2017.*

João Miguel Lousa Dias Ferreira

*(João Miguel Lousa Dias Ferreira)*

*Recomeça…*

*Se puderes*

*Sem angústia*

*E sem pressa.*

*E os passos que deres,*

*Nesse caminho duro*

*Do futuro*

*Dá-os em liberdade.*

*Enquanto não alcances*

*Não descanses.*

*De nenhum fruto queiras só metade.*

*E, nunca saciado,*

*Vai colhendo ilusões sucessivas no pomar.*

*Sempre a sonhar e vendo*

*O logro da aventura.*

*És homem, não te esqueças!*

*Só é tua a loucura*

*Onde, com lucidez, te reconheças*

***In Sísifo, Miguel Torga, Diário XIII***

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### Works presented as Oral Communication

**1. "Cytotoxicity studies on novel 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-fused diphenylchlorins as photosensitizers in human melanoma cells."**

J. Dias Ferreira, Nelson A. M. Pereira, Mafalda Laranjo, Marta Pineiro, João Casalta-Lopes, Ana Margarida Abrantes, Teresa M. V. D. Pinho e Melo, Maria Filomena Botelho.

8th AIMS Meeting - Basic Research Plenary Session by Fundação AstraZeneca

10th to 12th of March 2017 at Faculty of Medicine of University of Lisbon

**2. "Targeting Human Melanoma Cells with Novel 4,5,6,7-tetrahydropyrazolo[1,5a]pyridine-fused diphenylchlorins as new photosensitizers"**

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**1. "P88 – Novel Ring-Fused Diarylchlorins as Very Active Photodynamic Agents Against Cancer Cells"**

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## ABBREVIATION LIST

**AA -** Acetic acid

**AIF -** Apoptosis-inducing factor

**AnV -** Annexin V

**BCA -** Bicinchoninic acid

**Bcl-2 -** B-cell lymphoma 2

**Bid -** BH3 interacting-domain death agonist

**BRAF -** v-Raf murine sarcoma viral oncogene homolog B

**CDKN2A -** Cyclin-dependent kinase Inhibitor 2A

**c-KIT/CD117 -** Tyrosine-protein kinase Kit

**DCF -** 2',7'-dichlorofluorescein

**DCFH -** 2',7'-dichlorodihydrofluorescein

**DCFH2-DA -** 2',7'-dichlorodihydrofluorescein diacetate

**DHE -** Dihydroethidium

**DMSO -** Dimethyl sulfoxide

**DNA -** Deoxyribonucleic acid

**DO7 -** Anti-p53 monoclonal antibody

**DTT -** Dithiothreitol

**EMA -** European Medicines Agency

**ERK -** Extracellular signal-regulated protein kinase

**FDA -** Food and Drug Administration

**FITC -** Fluorescein isothiocyanate

**GSH -** Reduced glutathione

**$H_2O_2$ -** Hydrogen peroxide

**$HO^{\cdot}$ -** Hydroxyl radical

**$IC_{50}$ -** Concentration that inhibits cell proliferation in 50 %

**JC-1 -** 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide

**LED -** Light emitting diodes

**MMP -** Mitochondria membrane potential

**MOMP -** Mitochondria outer membrane permeabilization

**MTT -** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**NRAS -** Neuroblastoma RAS viral oncogene homolog

**$O_2$ -** Molecular oxygen

**$O_2^{\cdot-}$ -** Superoxide anion radical

**p53 -** Tumor suppressor p53

**PBS -** Phosphate Buffer Saline

**PDT -** Photodynamic Therapy

**PE -** Plate efficiency

**PI -** Propidium iodide

**PI3K-AKT -** Phosphatidylinositol 3-kinase/protein kinase B

**PS -** Photosensitizer

**PTEN -** Phosphatase and tensin homolog

**PVDF -** Polyvinylidene difluoride

**RG -** Radial growth

**RIP1 -** Receptor interacting protein 1

**RIPA -** Radio-immunoprecipitation assay

**RNA -** Ribonucleic acid

**ROS -** Reactive oxygen species

**SF -** Surviving fraction

**Smac/DIABLO -** Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low isoelectric point

**SOD -** Superoxide dismutase

**SRB -** Sulforhodamine B

**TBST-BSA -** Tris-Buffered Saline Tween-20

**UV -** Ultraviolet

**VG -** Vertical growth

# SECTION I

DISSERTATION

## RESUMO

O melanoma constitui o mais raro tumor de pele. Não obstante, apresenta uma taxa de mortalidade extremamente elevada com inúmeras dificuldades terapêuticas no que respeita às atuais estratégias aplicadas no seu tratamento e remissão enumerando-se as de índole farmacológica e cirúrgica. Decorrente de tal facto urge a necessidade de novos tratamentos que permitam uma superior eficácia relativamente à demonstrada pelas terapêuticas anteriormente evidenciadas.

O presente projeto surge como tentativa de resposta a tal solicitação através do enquadramento de trabalhos anteriores nesta particular combinação entre patologia e terapia fotodinâmica. Assim sendo, aliando-se a experiência prévia do presente grupo de investigação no campo do conhecimento em referência, estudaram-se quatro novas moléculas fotossensibilizadoras, NAMP 103A, NAMP 103B, NAMP 263A, NAMP 263B, quimicamente do tipo clorina.

Os estudos efetuados foram exclusivamente realizados *in vitro*, tendo sido primeiramente averiguada a citotoxicidade de cada um dos fotossensibilizadores assim como a concentração que em cada caso inibia em 50 % a proliferação celular. A fim de verificar a seletividade dos compostos em trato para as células tumorais efetuaram-se também verificações através da sua aplicação em células fibroblásticas humanas (linha celular não tumoral), HFF1, não se tendo apurado diferenças estatisticamente significativas. Através da aplicação destes compostos, as células tumorais demonstraram diminuições significativas nas suas atividades metabólicas bem como de viabilidade, estando tais factos dependentes da concentração de cada fotossenssibilizador. Foi ainda demonstrado que estas ocorrências são consequência do tipo de morte celular, fase do ciclo celular em que as células se encontram, alteração da homeostase mitocondrial, e geração de espécies reativas de oxigénio a nível intracelular.

Decorrente dos resultados obtidos é possível inferir que a terapia fotodinâmica constitui uma oportunidade terapêutica cujos efeitos poderão ser diferenciadores no que respeita aos resultados terapêuticos na patologia referida.

**Palavras-chave:** Melanoma, terapia fotodinâmica, fotossensibilizadores, clorinas.

## ABSTRACT

Melanoma is the rarest skin tumor. Nevertheless, it is related with an extremely high mortality rate and abundant therapeutic difficulties concerning the current strategies applied in its treatment and remission, including those of pharmacological and surgical nature. Because of this, new treatments are required to allow a higher efficacy than the previously demonstrated therapies.

The present project emerges as an attempt to solve this problem through the framework of previous studies in this particular combination of pathology and photodynamic therapy. Therefore, merging the previous experience of the present research group in this field of knowledge, four new photosensitizer molecules, chemically of the chlorine type were studied, NAMP 103A, NAMP 103B, NAMP 263A, NAMP 263B.

The studies performed were only achieved *in vitro*, with the cytotoxicity of each of the photosensitizers first being ascertained as well as the concentration which in each case inhibited cell proliferation by 50 %. To ascertain the selectivity of each photosensitizer to melanoma cells, each molecule was applied to HFF1 human fibroblast cell line (non-tumor cell line) and results showed that there was no significant difference. Through the application of these compounds, the tumor cells demonstrated significant decreases in their metabolic activities as well as viability, being such facts dependent on the concentration of each photosensitizer and it has been shown that these occurrences are a consequence of the type of cell death, cell cycle phase, variation of mitochondrial homeostasis, and generation of reactive oxygen species at intracellular level.

From the obtained results, it is possible to infer that photodynamic therapy constitutes a therapeutic opportunity whose effects may be differentiating with respect to the therapeutic results in the mentioned pathology.



**Keywords:** Melanoma, photodynamic therapy, photosensitizers, chlorins.

# 1. INTRODUCTION

## 1.1. Melanoma

### 1.1.1. Epidemiology & Pathogenesis

Cancer is a pathology in which a multitude of genetic modifications that occur in one or several types of body cells, can give rise to an uncontrolled process of cell division and spreading. Unfortunately, and due to the underlying mechanism of tumor cell formation, every cell in the body may eventually undergo a carcinogenic process leading to tumor proliferation.

Skin is the largest organ on the human body and, being the outermost layer, it is organized to resist to harmful effects from the surrounding environment (Schadendorf *et al.*, 2015). Nevertheless, when cells become susceptible to some physical or chemical aggressions, they may accumulate mutations that, when are not fixed by intracellular mechanism of homeostasis, lead to cancer development. In the top layer of the skin, the epidermis, three main kinds of cells are present: squamous cells, basal cells and melanocytes (Dummer *et al.*, 2011). The first ones are flat-shaped and are present on the outer side of epidermis being removed by a continuous process of turnover. Basal cells are on the innermost part of the epidermis and their function is to produce new cells, which get flattened as they go to the skin surface, replacing squamous cells. Melanocytes have a protective function by synthesizing melanin, giving a darker color to the skin. This pigment absorbs ultraviolet (UV) radiation preventing important cell structures from being reached, which can lead to cell death or immortalization through carcinogenesis. The capacity to form melanoma relies on these cells (Figure 1). Melanoma and non-melanoma skin cancers' incidence increased over the past decades, occurring 2 to 3 million non-melanoma skin cancers and almost 132,000 melanoma skin cancers worldwide, per year. However, although less common than basal and squamous cell carcinoma, melanoma is deadlier. Statistically, one-third of every diagnosed cancer is a skin cancer (Figure 2).

One of the main reasons for the large number of cases may be related to depleted ozone levels in the atmosphere (Watson, Holman e Maguire-Eisen, 2016; Young, 2006). Melanoma development shows that in the key phases a radial growth (RG) and a vertical growth (VG) occurs. On an initial stage, melanoma grows horizontally within the epidermis, with a radial tendency. This period is extended and the melanoma cells do not present the ability to either metastasize or induce angiogenesis. After some time, a vertical growth phase begins. On this stage, the tumor grows on a descendant path into the deeper layers of the dermis being characterized as an expansive mass without differentiation (Wong e Ribas, 2016). Melanocytes malignant transformation into melanoma comprises mutations in proto-oncogenes, leading to

their activation, and mutations in tumor suppressor genes, with their disablement. Germline mutations on the cyclin-dependent kinase Inhibitor 2A (*CDKN2A*) gene, located on the chromosome 9p21 locus and encoding the tumor suppressor *p16*, a cyclin dependent kinase inhibitor that regulates the G1-S transition of the cell cycle, are present in almost 40 % of diseased with familial melanoma and is also silenced in infrequent tumors by a methylation process. Somatic mutations localized in the proto-oncogenes v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) or neuroblastoma RAS viral oncogene homolog (*NRAS*) promoting their activation are also observable in a high proportion of melanomas, leading to cellular proliferation and later survival by activation of the extracellular signal-regulated protein kinase (ERK) pathway. Normally, these pathways are mutually exclusive, since *BRAF* functions downstream of *RAS*. It is also frequently seen a loss of function of the tumor suppressor phosphatase and tensin homolog (*PTEN*), an important negative regulator of the phosphatidylinositol 3-kinase/protein kinase B (PI3K-AKT) pathway, which promotes growth and survival. Some melanomas, particularly those arising in acral and mucosal sites, have activating mutations in the tyrosine-protein kinase Kit (c-KIT/CD117) receptor (Figure 3). Pharmacological agents with an inhibitor role on mutant BRAF and c-KIT promoted revival responses in patients with metastatic tumors possessing these mutations (Sullivan, 2015).

### 1.1.2. Morphology

Melanoma exhibits variations in its color and can be black, brown, red, dark blue or grey, often with irregular and rough borders. On a microscopic look, malignant cells can grow from small colonies or from single transformed cell, being present at all levels of the epidermis, and in expansive dermal nodules (Liao *et al.*, 2015; Liao, Dent e McCulloch, 1975). When superficially spreading, melanomas are usually associated with a lymphocytic infiltration, a condition revealing a host's immunological response to tumor cells antigens. Melanomas evolution results from the extent and nature of the VG phase. When merged, interconnected and analyzed, these conditions are a source of accurate prognosis. Identification of melanoma is supported on cell size which is larger than nevus cells, presenting prodigious nuclei with an asymmetrical profile, a chromatin clumped on a distinctive pattern present on the nuclear membrane periphery, and an eosinophilic nucleolus. Due to that, immunohistochemical techniques are eligible to help in the process of metastasis identification (Banerjee e Harris, 2000).

### 1.1.3. Clinical features

Mucosal surfaces such as oral and anogenital, esophagus, meninges, and eye are structures, which can also be tremendously affected by melanoma development. Melanoma is usually asymptomatic being pruritus an early signal of the disease. Nonetheless, the most important clinical feature is the change of color or even size of a pigmented lesion. Some other signs must be considered when performing a clinical observation, such as the enlargement of a preexisting nevus, pain in lesion, the development of a novel pigmented lesion during adult life, irregularity of the borders of a pigmented lesion and variation of color inside a pigmented lesion (Hawryluk e Tsao, 2014). Such parameters are included on the so-called ABCDEs of melanoma - asymmetry, border, color, diameter and evolution. It is vital, then, to recognize melanoma as fast as possible since such disease has a tremendous negative prognostic when metastases occur. This process is greatly increased on tumors with high mitotic rate and on tumors with a failure by the host immune response (Baumert *et al.*, 2003). Metastases formation is deducted by observation of the overall depth of invasion - in millimeters - of the VG phase nodule, beginning on the top of the granular cell layer, being this invasiveness termed *Breslow thickness*. This process involves tumor cells intrusiveness on lymph nodes, liver, lungs, brain and in any other place that cells may be able to penetrate and settle (Figure 4). To be aware of tumor aggressiveness, a biopsy of the sentinel lymph node of primary melanoma must be performed. Nevertheless, many years after surgery with complete tumor excision, in some cases, a metastatic process may be observed signifying an extensive stage of dormancy (Landi *et al.*, 2000).

## 1.2. Photodynamic Therapy (PDT) - historical facts

The history of Photodynamic Therapy (PDT) starts three thousand years ago, when light was already focus of application for medicinal purposes. With further development along the centuries, new bids were found to light. Nevertheless, health sciences were a mark for this purpose - vitiligo, psoriasis and cancer are some examples of the extraordinary power of photonic energy used to treat illness. The year of 1900 can certainly be called an *annus mirabilis* by everyone who has had benefits from PDT application. On that year, a German medical student, Oskar Raab, noticed that *infusoria* (a Paramecium species) would experience death when exposed to light if the acridine molecule was present. Three years later, in 1903, Niels Finsen was awarded a Nobel Prize on behalf of his research studies concerning the use of red-light to eliminate smallpox abscesses and treat this disease (Figure 5).

PDT comprises three vital components, a photosensitizer (PS), light and molecular oxygen. Individually none of these is toxic, but when assembled a photochemical reaction is initiated,

culminating on the generation of singlet oxygen and other reactive species, leading to cell death by either an apoptotic or necrotic pathway. PDT exerts its effects by three non-similar routes. The first is related to direct cytotoxic effects on tumor cells. The second does respect to the damages on tumor vasculature and, the third, due to the initiation of an inflammatory response which can, ultimately, lead to a systemic immune reply. Outcomes of each route are dependent of some notable features as the total light dose and its fluence, the time between PS administration and further irradiation, the type and dose of PS administered and tumor oxygen concentration.

### 1.3. PDT mechanism - Photochemistry & Photophysics

Ground state PSs (singlet state) possess two electrons with contrary spins. When light hits the molecule, it is absorbed and one electron is transferred to an orbital with higher energy. Once such condition drives PSs to be unstable, the surplus energy is released as fluorescence or/and heat. Instead, PSs might undertake an intersystem crossing leading to the formation of a triplet state with an inverted spin of one electron (Benov, 2015). After this process, PSs in triplet state can decay to the ground (fundamental) state, releasing radiation or transferring its energy to molecular oxygen ($O_2$) - which holds the unique property of being in the ground state of electronic excitement but holding a triplet configuration state. Such reaction, called Type II, leads to the formation of $^1O_2$. A Type I reaction may also occur if PSs react with intracellular organic molecules through transfer of an electron or even a hydrogen atom, forming radical species. Ensuing autoxidation of the reduced PSs warrants formation of superoxide anion radical - $O_2^-$. Dismutation processes or reduction by one electron of $O_2$ molecule crops hydrogen peroxide - $H_2O_2$. $H_2O_2$ may experience one-electron reduction to an oxidant hydroxyl radical ($HO^\cdot$) (Figure 6 & 7) (Yoon, Li e Shim, 2013).

### 1.4. Photosensitizers evolution - a brief timeline overview

Emerging from multiple requests to achieve new PSs with refined properties, science efforts produced a vast array of molecules, which were assembled in groups labeled generations according to their characteristics. The first PS to arise belonged to the class of hematoporphyrins and it was produced by the 19th century. Firstly, used as a tool for diagnostic due to their fluorescence, this product was constituted by several structure-related porphyrin molecules, however, the required amount to produce an appreciable effect was too high. Subsequent purification processes lead to more tumor-selective derivatives and, at last, to the well-known molecule, Photofrin® (Yoon, Li e Shim, 2013). Such product gained the approval of both mandatory regulatory agencies from the US and Europe, Food and Drug

Administration (FDA) and European Medicines Agency (EMA), respectively, for purposes of cancer and pre-cancer treatment. Photofrin® was very complex in its formulation and revealed both low light absorption and tissue selectivity. Besides, it was necessary a too high quantity of product causing extreme skin sensitivity on patients. Even though it is still used on medical context, such considerations were not wholly compatible with clinical requests and a hunt for a second generation of PSs begun. This new class ambitioned innumerous tuned features as lower concentrations, better selectivity for tumors absorption, improved purity and reproducibility on its production by holding molecules with different chemical structures such as porphyrins, chlorins, pheophorbides, bacteriopheophorbides, texaphyrins and phthalocyanines (Figure 8) (Gomer, 1991; Wöhrle *et al.*, 1998).

With the advent of these compounds, adjustments on light wavelengths were also conducted. Longer wavelengths are preferentially taken by PSs and reveal a better capacity to overcome skin blockade, being eligible for treatment of unreachable tumors through reducing the requirements for invasive procedures to perform PDT. The third, and last, generation of PSs to arise, is related with biotechnological improvements on second PSs generation through their conjugation with carriers as antibodies or even liposomes, aiming improvements on their properties. Referred strategies lead to a higher tumor selectivity reducing the dosage required, side effects and allowing a better absorbance of light, resulting on improved outcomes (Josefsen e Boyle, 2008).

## 1.5. Critical features to PDT success

### 1.5.1. Molecular Oxygen

Oxygen molecule presence on tumor cells is one part of three crucial factors to guarantee PDT success. Its lack is related with lower success on both chemotherapy and radiotherapy applied to solid tumors due to the inner hypoxia (Vaupel, Thews e Hoeckel, 2001). Such condition is usually associated with low tumor stroma vasculature and, therefore, a deficit on the delivery and uptake of therapeutic molecules. Transposing such considerations to PDT context, oxygen is fundamental once cell damages are caused by reactive oxygen species (ROS) started by generation of singlet oxygen from ground state oxygen. Some other molecules as quinone methides and nitrogen mustards are prodrugs also dependent on metabolic activity and oxidative reactions to become activated and accomplish their purpose (Wilson e Hay, 2011; Xiaohua Peng, 2012).

In this context, ablative effects on tumor cells are diminished when oxygen supply is stopped. As a consequence, a resistance or inefficacy arises (Moan e Sommer, 1985; Wyld, Reed e Brown, 1998). An alternative to avoid this unwanted effect is the oxygen hyperbaric therapy

that raise blood oxygenation and so the probability of achieving better tumor-ablative results, which has already been demonstrated (Moen e Stuhr, 2012; Tomaselli *et al.*, 2001). PSs used on PDT show different responses: some molecules as Photofrin® show better tumor-killing results when gathered with hyperbaric therapy, being dependent on oxygen concentrations whereas for others, such values do not play any role on ablative consequences for tumor cells. PDT also conducts to dual results once generated ROS perform damages on tumor vasculature shutting down blood supply what prevents remaining cells to receive following administered molecules and reduces oxygen on such places. This pattern can be avoided with adjustments on light irradiation intensity and PS concentrations (Henderson *et al.*, 2000; Huang, 2005; Huang *et al.*, 2008).

### 1.5.2. Light Penetration

Efficacy of PDT is directly associated with incidence of light and its properties. Regarding a successful protocol, light should reach PS and have a precise wavelength to activate it. Light penetration depends on wavelength and tissue characteristics. On the skin it is smooth, however, other tissues hold enough heterogeneity to turn PDT difficult. When applied at short wavelengths light can be quenched by biological-derived chromophores, as hemoglobin, but at higher wavelengths, water also represents an obstacle. Nevertheless, light at wavelengths longer than 850 nm cannot supply PS with adequate radiant energy to trigger its activation (Mallidi *et al.*, 2016). Accordingly, the effective wavelength range of light is between 620 nm and 850 nm - red region of light spectrum. Nowadays, light administration is based on laser technology due to their powerfulness and availability to connect to optical fibers leading to a much more precise irradiation of the tumor. Innovative researches are looking for other possibilities for PDT purposes such as Light Emitting Diodes (LED). Additionally, we should refer that as important as light source and its properties, the technique by which light is applied is also paramount. Literature data suggests that administration rates and doses are also fundamental once higher ones can deplete oxygen concentrations inside the tumor so fast that ROS could not be available to spread by all the tumor cells (Mang, 2004).

### 1.5.3. PS Uptake & Location

Regarding the maximum reach of PDT-derived ROS, chiefly the singlet oxygen, one can deduce that PS location has a profound impact on the outcomes of this therapy. From the moment of administration, PS molecules are exposed to a complex environment ranging from serum proteins, liver metabolism, other circulating molecules, to different tissues and cells. According to the molecule structure, the interactions with all these elements will change the

pharmacokinetic parameters of each PS (Castano, Demidova e Hamblin, 2005). To reach tumor cell PS must pass through endothelium and other layers of blood vessels until they get the destination. Particular properties as overall charge and its distribution, the lipophilicity-hydrophilicity ratio and structure were demonstrated to be determining for cellular uptake and subcellular localization of PS molecules (Benov, 2015).

### 1.5.4. Charge

Another property of molecules is their overall charge. In the biological environment, this feature is responsible for interact with subcellular structures. Particularly, cellular membrane interacts with PS molecules according to its net charge. Cell membranes possess a natural occurring negative charge attracting and allowing passive diffusion of positively charged molecules (Jensen *et al.*, 2010). Nonetheless, substances with a negative charge are remarkably banned from such diffusion processes. The way the molecules go into the cell governs its intracellular location. The cationic molecules, after diffusion through membranes, attach to mitochondrial membrane whilst the anionic molecules undergo an endocytic internalization process mediated by lysosomes, being the majority of the latter molecules located in the structures underlying the endocytic process (Woodburn *et al.*, 1991). Such reality can indeed be improved by rearrangements of the hydrophilic-lipophilic relation of these molecules. When molecules with equal charge but different distributions are considered, it comes clear that charge localization has a tremendous impact on the interactions that arise. This fact is extremely important when looking for compounds with better pharmacokinetic parameters and an improved phototoxicity (Dummin, Cernay e Zimmermann, 1997).

### 1.5.5. Hydrophilic-lipophilic balance

When a drug is being designed, structure-activity relationship must be held. In addition, certain parameters as hydrophilic and lipophilic balance are of supreme importance, having a direct impact on the drug kinetics - absorption, distribution and metabolism. When the hydrophilic capacity of a drug is upstretched an increase on albumin binding capacity is verified. An intermediate, amphiphilic capacity, can lead to binding to high-density lipoproteins if more lipophilic PS molecules will distribute on the hydrophobic core of low-density lipoproteins. For instance, if the desired effect is an accumulation on the tumor site, the lipophilic characteristics are the desired ones, with the enlarged localization in the direction of mitochondria (Ruck e Steiner, 1998).

### 1.5.6. Three-dimensional profile

Additionally, to all the referred prior points, the cell uptake of PS depends also of its structure.

Although net charges are about the same and the lipophilicity almost coincident many analogs do not present equal capacity to penetrate and act in the cell. Chemical structure of a compound is always related with orbital distribution of energy being the interactions outcome patterns always different for distinct molecules. Studies conducted with homolog molecules ranging distinct lipophilicity, display results which show that molecules with the same lipophilia but different structures are taken up in unalike manners with better results for the less lipophilic. Such patterns put in evidence that much more features than hydrophilicity and lipophilicity are related to the performance of PS molecules (Akilov *et al.*, 2006).

### 1.6. Cellular toxicity

PDT leads to $^1O_2$ generation, which by its time has a very short life, approximately 10 to 320 nanoseconds. Such parameter is restrictive to diffusion of $^1O_2$ molecule, only capable of reaching a dispersion range up to 55 nm in cells under such conditions. According to this, the damage induced by PDT are nearby PS location (Castano, Demidova e Hamblin, 2005). Using PDT there are three chief pathways cell-death related: apoptosis, necrosis and autophagy-associated cell death. Subsequently to PDT, the major pathway evolved is apoptosis, starting in mitochondria outer membrane permeabilization (MOMP), later to photodynamic-induced damages, which is controlled by B-cell lymphoma 2 (Bcl-2) factors and, apparently, be tumor suppressor p53 (p53) independent. PSs mitochondria-associated apply their photodamage by activating membrane-bound Bcl, a lenient signal for MOMP and, arising from such, by the allowance of the release of caspase activators as *cytochrome c* and Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low isoelectric point) complex, or even other proapoptotic molecules as apoptosis-inducing factor (AIF) (Felsher, 2003). Is also observable a rupture of lysosomal membrane and outflow of cathepsins derived from these oxidized organelles, inducing Bid (BH3 interacting-domain death agonist) cleavage and MOMP. Intracellular toxicity is spreadable not only by these referred means but also by the involvement of other proteases as calpains and other nonapoptotic pathways. Such certainty can be deduced if caspases cascade is inhibited, or if are lacked important functionalized genes and the toxicity is only delayed or the cell is driven to death by a necrosis mechanism. Recent evidence suggests that certain forms of necrosis can be propagated through signal transduction pathways (Mroz *et al.*, 2011). Necrosis mechanism of action is not well expounded yet. Nonetheless, the occurrence of events as the activation of receptor interacting protein 1 (RIP1) kinase, disproportionate mitochondrial ROS production, lysosomal harm, and intracellular $Ca^{2+}$ overload, are frequent. Necrosis is also favored when transitory mitochondrial permeability transition is attained, generally as result

of mitochondrial membrane photodamages or $Ca^{2+}$ impairments. Another phenomenon that is able to occur is autophagy, a lysosomal survival pathway triggered in order to destroy and recycle organelles and undesired intracellular proteins (Robertson, Evans e Abrahamse, 2009). Oxidative stress is a magnificent example of an impetus that may well generate autophagic stimulus. Autophagic signals represent a sword with two cut ends - they possess both cytoprotective and prodeath effects subsequently to applied chemotherapies. Still, current studies display the autophagic process with implications on cell preservation after photodynamic treatment. The mechanism underlining such effect seems to be an incomplete autophagic process due to lysosomal defects achieved through PDT and, so, and inefficient elimination of vesicle cargo. In parallel with this occurrence, the intracellular organelles ROS-photodamaged undergo inducement of apoptosis (Straten, van *et al.*, 2017). Comprehension of the balance/unbalance between three prior described processes is a requirement to develop new and more effective strategies on PDT.

# 2. MATERIAL & METHODS

## 2.1. Cell culture procedures

The A375 human melanoma cell line was purchased from *American Type Culture Collection* (ATCC® CRL1619™). It was thawed and cultured using *Dulbecco's Modified Eagle Medium* (Sigma D5648) supplemented with 5.0 % of heat-inactivated fetal bovine serum (FBS) (Sigma F7524), 1.0 % penicillin-streptomycin (Sigma A5955; 100.0 $U.mL^{-1}$ penicillin and 10.0 $mg.mL^{-1}$ streptomycin) and 100.0 mM sodium pyruvate (Gibco 11360), kept in a humidified incubator at 37.0 °C with 5.0 % of $CO_2$. Ensuing studies required preparation of cell suspensions. The first stage was medium removal. After, cells were washed using a *Phosphate Buffer Saline (PBS)* solution (137.0 mM of sodium chloride [Sigma S7653], 2.7 mM of potassium chloride [Sigma P9333], 10.0 mM of sodium dihydrogen phosphate [Sigma S5011] and 1.8 mM of potassium dihydrogen phosphate [Sigma P0662], at a pH 7.4). Later, cells detachment was obtained with 4.0 mL of a 0.25 % trypsin-EDTA solution (Sigma T4049), being in contact with cell culture up to 5 minutes, and then medium was added to inhibit trypsin enzymatic activity. Once this step was completed an aliquot of cell suspension was picked up to determine cell concentration. For this purpose, 20.0 μL of cell suspension were diluted in a ratio of 1:1 in a trypan blue solution. Cells' number was ascertained in a hemocytometer (*Neubauer* chamber), placed on an inverted optic microscope (Motic AE31), using a 10x10 magnification. Once prior parameter was determined, the proper cell volume was collected, placed on a Falcon tube and platted in each well according to outlined concentration.

### 2.2. Photodynamic treatment

In the succeeding studies four photosensitizers were used - NAMP 103A, NAMP 103B, NAMP 263A and NAMP 263B. Each PS was solubilized in DMSO (dimethyl sulfoxide) and, therefore, the desired concentrations were obtained through successive dilutions of the main one. PS volume was optimized to equalize 1.0 % DMSO in the cell studies. Tested concentrations ranged values of 5.0 nM, 10.0 nM, 20.0 nM, 25.0 nM, 30.0 nM, 40.0 nM, 50.0 nM, 75.0 nM, 100.0 nM, 125.0 nM, 250.0 nM, 500.0 nM, 1,000.0 nM, 5,000.0 nM and 10,000.0 nM. On each treatment, cells were always incubated for a period of 24 hours with the PS. After that, the medium was removed and cells washed with PBS. This procedure assured the removal of non-internalized PS by cells. After this, a new medium was added and, at this point, cells were exposed to a light at a specific wavelength ($\lambda_{cut\ off}$ = 560 nm) with a radiant-flux of 7.5 $mW.cm^{-2}$, until an accumulated energy of 10.0 J was totalized. Further experiments with 0 J; 5.0 J and 20.0 J were also conducted.

### 2.3. Metabolic activity by MTT assay

MTT protocol was performed as it correlates metabolic activity by measuring the cell capacity to metabolize the yellow salt *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (Mosmann, 1983). The biological mechanism by which this occurs is nowadays well understood with studies pointing to a key role of intracellular dehydrogenases, which reduce the referred salt to its organic-soluble free structure (purple formazan) diminishing reducing equivalent molecules as NADH and NADPH (Stockert *et al.*, 2012). Arising from this, a correlation between formazan and cell metabolic activity can be settled.

The medium was removed from plates and wells and cells washed out with PBS, which was discarded. A MTT reagent volume of 200.0 µL was then added to the plates, to each well. After this, each plate was kept overnight in the dark at 37.0 °C. Formazan crystals were solubilized using 200.0 µL of a 0.04 M (0.5 $mg.mL^{-1}$, Sigma M2128) hydrochloric acid solution in isopropanol and solubilized content transferred to a 96-well plate. Absorbance was measured using a Biotek® Synergy HT Plate Reader on two wavelengths - 570 nm (for MTT absorption) and 620 nm as a baseline reference for this proceeding. Cytotoxicity was expressed as the inhibition percentage of cultures subjected to PDT, correlated with cultures treated only with the vehicle of PS administration.

This procedure allowed determination of dose-response curves and, thus, the concentration for each PS that inhibits cell proliferation at a 50 % value (Brites, 2016).

$$\%\ \boldsymbol{Metabolic\ Activity} = \frac{[Treated\ Cells\ (Abs\ 570 - Abs\ 620)]}{[Control\ cells\ (Abs\ 570 - Abs\ 620)]} * 100\ \%$$

### 2.4. Cell viability and protein content by SRB assay

Cell viability was determined using the SRB protocol. This procedure relies on the SRB reagent - *sulforhodamine B* - aptitude to bind to protein cell components. Cells are subjected to acetic acid (AA). Once SRB is a pink dye with a molecular structure containing two sulfonic chemical groups, this compound easily binds basic amino-acid portions when exposed to a mild acidic milieu. After this, molecules are detached using basic reagent conditions. As this compound binds to the cells on a stoichiometric proportion, the intensity of staining is directly proportional to cell mass as protein content (Vichai e Kirtikara, 2006).

To perform this assay, first, plates had the medium removed and cells washed with PBS. Once discharged, were left to dry at room temperature, by contact with the air. Next, a volume of 200.0 µL of AA 1.0 % on methanol was added to each well and the plates were kept in a dark chamber at 4.0 °C, for 2 hours. Completed this schedule, methanol was removed and wells were left to air dry at room temperature. Next, a 0.4 % of SRB reagent solution (Sigma S9012) on 1.0 % of methanol was placed on a volume of 200.0 µL in each well reacting for 1 hour without light. Later, plates were washed to remove SRB excess and dried. Regarding the referred purpose of SRB, to achieve its detachment from cells' components, a volume of 200.0 µL of TRIS-NaOH (pH=10) was added. Solubilized content was transferred to a 96-well plate which was taken to a spectrophotometer concerning absorbance at two wavelengths, 540 nm (SRB absorption wavelength) and 690 nm (reference filter), using a Biotek® Synergy HT spectrophotometer. Ultimately, results were described as the protein content of cells subjected to photodynamic therapy correlated with cultures treated only with the vehicle of administration of PS.

### 2.5. Flow Cytometry

Flow cytometry is a technique on which cells, considered in an isotonic buffer suspension, go through a laser beam, one by one. This procedure makes quantifiable measurements possible as cell size, granularity, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content, cell surface, intracellular receptors and gene expression, using fluorescence principles. The flow cytometer consists of four systems: fluidics, optics, electronics, and computer interface (Betters, PhD, RN, 2015).

### 2.6. Cell Death

Several viability tests are available regarding the use of cationic dyes - such as propidium iodide (PI) - to scrutinize plasma membrane transportation function through the cell. PI is usually applied on flow-cytometry context due to its capacity to differentiate alive and death cells. PI

is slowly incorporated by initial apoptotic cells resulting in a low degree of fluorescence. On the opposite, late apoptosis/necrosis cells take PI in a much higher ratio, having a tremendous fluorescence (Waring *et al.*, 1999). In plasma membrane of living cells an asymmetry between membrane layers can also be observed. When apoptosis event starts, phosphatidylserine, ordinarily placed on the inner side of plasma membrane, is translocated to the opposite layer breaking asymmetry on the membrane (Lee *et al.*, 2013). Annexin V (AnV) is a coagulant protein, which binds with high affinity to phosphatidylserine and, conjugated with a fluorochrome (FITC, fluorescein isothiocyanate), is frequently used as a marker of apoptosis cells in flow cytometry. The combination with PI dye turns possible the identification of living cells, in initial apoptosis, in late apoptosis/necrosis or in necrosis. Such considerations can be identified through results obtained: if both probes show negative results cells are alive; if PI is negative and AnV-FITC positive then cells are on initial apoptosis stage; both positive results point to late apoptosis or necrosis (Rieger *et al.*, 2011).

To perform this protocol, cell cultures were submitted to photodynamic treatment based on each PS on 50.0 nM and 500.0 nM concentrations. After 24 hours, wells were washed with PBS, cells trypsinized and wells' content transferred to a cytometry tube and then subjected to a 1,000 xG centrifugation for 5 minutes. Next, pellet was incubated with a buffer volume of 100.0 µL (0.01 M Hepes [Sigma, H7523]; 0.14 M NaCl [Sigma, S7653] and 0.25 mM $CaCl_2$ [Sigma, C4901]), 2.5 µL of AnV-FITC (Immunostep ANXVFKIT Immunotech) and 1.0 µL of PI (KIT Immunotech), for 15 minutes at room temperature, on a dark chamber. After this, a volume of 400.0 µL of PBS was added and flow cytometry analysis was performed using FACSCalibur cytometer (BD Biosciences) (Jiang *et al.*, 2016; Laranjo, 2014).

### 2.7. Mitochondria Membrane Potential

Mitochondria membrane potential ($\Delta\psi_M$) loss is one of the signals of eminent cell death (Hengartner, 2000). JC-1 (*5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide*), a cationic lipophilic molecule, has the capacity to selectively enter the mitochondria and then accumulate within it on the inverse proportion of $\Delta\psi_M$ according to the Nernst equation, giving rise to monomers at time $\Delta\psi_M$ is fairly low (mitochondria membrane depolarized), emitting at that point green fluorescence. When $\Delta\psi_M$ is present on high values (mitochondria membrane hyperpolarized), JC-1 dye goes on an aggregation process leading to the emission of red fluorescence. Finally, the ratio obtained between green and red fluorescence values provides an overview of $\Delta\psi_M$ which is independent of mitochondrial mass (Perry *et al.*, 2011).

The protocol performed started with the removal of the cell medium followed by cells' trypsinization. The content was centrifuged at 1000 xG for 5 minutes. Next, the obtained pellet was suspended in 1.0 mL of PBS and a volume of 1.0 µL of JC-1 (Sigma T4069), previously prepared in DMSO from 5.0 $mg.mL^{-1}$ to a final concentration of 5.0 $µg.mL^{-1}$, was added. Incubation of cells suspensions was conducted for 15 minutes in a 37.0 °C atmosphere without light. Subsequently, these suspensions were centrifuged, using 2.0 mL of PBS, at 1000 xG, for 5 minutes and, after this, a PBS volume of 400.0 µL was added. Cytometer device made the detection on an excitation wavelength of 488 nm. Obtained results are presented as the average fluorescent intensity for both aggregates and monomers. Afterwards, the ratio monomers/aggregates was calculated for each condition tested (Brites, 2016).

### 2.8. Cell Cycle

A precise arrangement of events occurs in diverse stages of mitotic cell cycle, leading ultimately to the division of a cell into two daughters. Two main phases define this cycle: first, DNA replication, occurring when the nuclear genome is duplicated, being S (synthesis) phase demarcated by this event; the second is mitosis which is the period when chromosomes are condensed, then sorted and equally distributed to each daughter cell, describing M phase. Between M and S phases stand two 'gap' phases: G1 and G2, respectively. Cells can also enter a quiescence (or G0) state, distinct by replicative dormancy, after M phase.

To evaluate cell cycle, a PI and RNase solution was handled (Hustedt e Durocher, 2017). As PI is capable to penetrate and insert itself between adjacent bases on DNA and RNA molecules a specific reaction of this compound with DNA is only possible by deletion of RNA molecules using RNase (Collection, 2016).

Followed protocol required centrifugation of cells at 1000 xG for 5 minutes and subsequent supernatant elimination. A volume of 200.0 µL of 70.0 % ethanol solution was added to each cytometer tube during agitation at vortex device. After, tubes were incubated for 30 minutes in a dark chamber at 4.0 °C. Cells were then washed with 2.0 mL of PBS, centrifuged at 1000 xG for 5 minutes and supernatant eliminated once more. Succeeding, a volume of 200.0 µL of PI/RNase solution (Immunostep, PI/RNase) was placed on each tube and incubated for 15 minutes in a dark chamber at room temperature. An excitation wavelength of 488 nm was used on FACSCalibur cytometer (BD Biosciences) to detect the probe (Brites, 2016; Laranjo, 2014).

### 2.9. Western Blot

Western blot technique allows detection of proteins within a sample of tissue or cells' extract.

In such technique, a mixture of proteins is separated based on its molecular weight, isoelectric point, electric charge or even combination of these, through gel electrophoresis. Proteins are, at that time, relocated to a membrane creating a band for each protein. The membrane is, at that time, incubated with antibodies specific to the protein of interest and their label control. Unbound antibody is washed off, leaving only the bound antibody. A second antibody, specific of the first one, is then added, possessing an associated alkaline phosphatase. Further detection is achieved by the addition of a substrate of the referred enzyme, ECF, which emits fluorescence at 540/560 nm when excited at a proper wavelength.

Cell cultures were subjected to PDT with each PS on 50.0 nM and 500.0 nM concentrations. After 24 hours, protein extracts were arranged. To do that medium was removed and cells were washed for 3 times with PBS. Following, a radio-immunoprecipitation assay (RIPA) solution with proteases inhibitors (cOmplete Mini, Roche) and 1.0 mM of dithiothreitol (DTT) was added. With a scraper, contents were released from flasks and placed inside a microtube which was subjected to a vortex agitation and subsequent sonication with 35.0 % amplitude (Sonicador VibraCell, modelo VC50 Sonic and Materials Inc. USA). Samples were then centrifuged for 15 minutes at 14,000 xG and the supernatants translated to new identified microtubes and kept at - 80 °C.

To achieve protein amount information, the BCA (bicinchoninic acid, BCA™ protein assay kit, Pierce) method was used and, then, samples were denatured at 95 °C for 5 minutes in a denaturing solution (Tris at 100.0 mM, glycine at 100.0 mM, SDS at 4.0 %; urea at 8.0 mM and bromophenol blue at 0.01 %). On the intent to perform electrophoresis, acrylamide gels were prepared and after positioned on the electrophoresis dish with adequate buffer (Bio-Rad 161-0772). Samples and molecular weights control (Precision PlusStandards, Dual Color, Bio-Rad) were also disposed on the gel. Electrophoresis was performed with a potential difference of 150.0 V and once terminated each gel was sited in direct contact with polyvinylidene difluoride (PVDF) membranes previously activated by methanol. To perform protein transfer, the Trans-Blot Turbo Transfer System® was used with an optimized buffer which is a part of the prior kit. Membranes were afterwards blocked with TBST-BSA solution at 4.0 % (Tris-Buffered Saline Tween-20) at room temperature. One hour later membranes were incubated with primary antibodies at room temperature and over agitation on a constant rate and left by one hour. To perceive P53 protein, the previously prepared monoclonal antibody anti-P53 (DO7) was used and detected a 53 kDa band. The presence of β-actin - a protein present in every cell and generally used as a control of detected protein amount - was determined using an anti-β-actin monoclonal antibody (antibody prepared in mouse model, Sigma, A5315) and the detection made through the identification of a 42 kDa band.

On the next day, TBS-T at 1.0 % was used to wash membranes. Then, they were incubated with the appropriated secondary antibody under constant agitation and at a constant temperature for 1 hour. A secondary antibody was used to react with the primary antibody (anti-mouse: RPN5781 GE Healthcare). Each wash was repeated and membranes were subsequently incubated with proper enzymatic substrate (ECF Western Blotting Reagent Pack, Amersham Biosciences, United Kingdom) for 5 minutes and later revealed using and fluorescence apparatus (Typhoon FLA 9000, Sweden) (Laranjo, 2014).

### 2.10. Quencher cytotoxicity, Singlet oxygen & Hydroxyl radical assessment

On PDT context, singlet oxygen is typically the most pertinent radical amongst ROS. An assay based on ROS inhibition was performed using as quenchers sodium azide (Sigma 71290), for singlet oxygen, on 1.0 mM and 10.0 mM concentrations; and D-mannitol (Sigma M4125), as a scavenger of hydroxyl radical, on 4.0 mM and 40.0 mM concentrations (Obata *et al.*, 2009).
To achieve the former goal, 48-well plates were prepared as previously described. Cells were incubated with each PS on 50.0 nM and 500.0 nM concentrations for 24 hours. Before irradiation, cell medium was removed, wells were washed with PBS and new medium supplemented with 5.0 mM or 50.0 mM of sodium azide, or 40.0 mM or 120.0 mM of D-mannitol was added. Irradiation occurred as described before and, 30 minutes after the prior procedure, PS and inhibitors free medium was added. Cell metabolic activity was obtained according to MTT assay (Laranjo, 2014).

### 2.11. Intracellular Peroxides Production

DCFH2-DA (*2',7'-dichlorodihydrofluorescein diacetate*) was the selected probe to assay intracellular peroxides production. Such molecule does not have fluorescent properties but is highly lipophilic. Due to this, it undergoes easy entrance on cell and deposition on cytosol being further deacetylated by intracellular esterases giving rise to 2',7'-dichlorodehydrofluorescein (DCFH), also not fluorescent, which reacts with inner peroxides conducting to 2',7'-dichlorofluorescein (DCF) formation. The latter compound is easily detected when excited with a light of 485/20 nm wavelength releasing a 528/20 nm wavelength light. Detected fluorescence is directly proportional to intracellular peroxides (Cossarizza *et al.*, 2009).
After PDT treatment, cells suspensions were subjected to a rinse with PBS and then centrifuged. The remained pellet was suspended with a volume of 1.0 mL of PBS and at that point incubated with 5.0 μM DCFH2-DA probe (Molecular probes, Invitrogen) for 45 minutes in a dark chamber at 37.0 °C. The suspension was next washed with PBS and centrifuged at 200 xG for 5 minutes and suspended in buffer solution. Following this detection was performed

on the fluorimeter, for detection purposes. Each result was figured as the fluorescence intensity average and was correlated with control cell cultures having a value of 1 (Laranjo, 2014).

### 2.12. Intracellular Superoxide Anion Production

Superoxide anion production can be accurately monitored using dihydroethidium (DHE) probe. This molecule has the capacity of easily permeate cell membranes. DHE reaction occurs upon reaction with superoxide anions and subsequent formation of a red fluorescent product, ethidium, intercalating with DNA structure. Nevertheless, recent studies were able to determine that 2-hydroxyethidium is the actual molecule that undergoes prior referred reaction (Owusu-Ansah, Yavari e Banerjee, 2008).

This assay required, at first, culture cell medium removal and wells' wash with PBS, followed by trypsinization of cells. Cells were then transferred to a microtube and centrifuged at 1000 xG for 5 minutes. Following this, the pellet was suspended in a volume of 1.0 mL of PBS, ensuing addition of a 5.0 μL of DHE probe (Sigma Aldrich, D7008) concerning a final concentration of 5.0 μM. The tube content was incubated at a temperature of 37.0 °C for 15 minutes in a dark chamber. After this, cells' suspensions were centrifuged with 2.0 mL of PBS at 1000 xG for 5 minutes and next suspension on 400.0 μL of PBS. An excitation wavelength of 530/25 nm and an emission wavelength of 465/40 nm were selected on fluorimeter for detection purposes. Each result was figured as the fluorescence intensity average and was correlated with cell cultures having a value of 1 (Brites, 2016; Laranjo, 2014).

### 2.13. Reduced Glutathione Expression

Reduced glutathione (GSH) is a vital antioxidative intracellular non-enzymatic mechanism to avoid cell damages induced by ROS (Birben *et al.*, 2012). To evaluate the expression of this molecule, mercury orange was selected. Its mechanism consists of a reaction with GSH molecule giving rise to a red fluorescent product.

The protocol comprised a first centrifugation of cells at 200 G for 5 minutes. Next, the supernatant was removed and a volume of 1.0 mL of PBS was added to the pellet suspension. Then, 4.0 μL of mercury orange on a concentration of 10.0 mM were applied (Sigma M7750). The suspension was homogenized and incubated for 15 minutes on a dark chamber at a temperature of 37.0 °C. Afterwards, the referred suspension was washed with PBS and subsequently centrifuged at 200 xG for 5 minutes and suspended again, in the buffer. For detection purposes, excitation wavelength used was 540/35 nm. Results obtained were disposed as the fluorescence intensity average and compared with cell cultures having a value of 1 for the

present assay (Brites, 2016).

### 2.14. Superoxide Dismutase

Superoxide dismutase (SOD) enzyme is responsible for catalyzing a reaction on which superoxide anion ($O_2^{\bullet -}$) dismutation into oxygen and hydrogen peroxide occurs. Accounting so, this enzyme is considered one of the most important metalloenzymes with an antioxidative activity. Its lack results in an excessive amount of reactive oxygen species (ROS) leading to overall damages on cell structure and subsequent abnormalities in its functioning (Fukai e Ushio-Fukai, 2011). SOD's activity was measured using a SOD Assay Kit-WST (Sigma, 19160, Switzerland). Initially, cell cultures were treated with each PS using 50.0 nM and 500.0 nM concentrations. Completed 24 hours after that, the medium was removed, wells washed with PBS and, next, a RIPA protein-extraction solution (radio-immunoprecipitation assay) was applied and scraped the wells using a scraper tool. Once free from wells surface, contents were transferred to a microtube. Next, were subjected to a vortex agitation and later sonication on 35.0 % amplitude. Then, centrifugation was applied for 15 minutes at 14,000 xG and the supernatants afterwards translated to new identified microtubes, ultimately frozen at -80.0 °C. Protein amount was determined by use of BCA method (BCA™ protein assay kit, Pierce). SOD determination was conducted using the referred kit according to the manufacturer's recommendations. Final results were placed as the ratio between SOD activity and total protein concentration (Brites, 2016).

### 2.15. Clonogenic Assay

Accepted as a "gold-standard", this assay intent to evaluate the overall ability of an *in vitro* cell culture to form a colony starting from one single cell. In this context becomes that a colony is defined having, at least, 50 cells. Initially, 6-well plates were seeded with 10.000 cells on each well. Cells were left by 24 hours to attach to the bottom of the wells and then each PS was administered. PDT was applied 24 hours after this stage. A final volume of 3.0 mL of medium was then added to each well and, subsequently, plates had a rest period of 12 days. Ended this stint the number of colonies was totalized. Concerning this aim, the medium was removed from each well and rinsed gently with PBS. After this, PBS was removed. The prior procedures were repeated twice. Next, a volume of 2.0 mL of methanol was added to each well - to fix the colonies on the plates - and left for 5 minutes. Ended this period, methanol was removed and plates left to dry on air at room temperature. The imminent step was adding 2.0 mL of a crystal-violet solution at 0.5 % on methanol and left plates on resting for 5 minutes. At last, the excess of dye was removed and the plates were subjected to tepid water and gently

shacked. Later, cells colonies on the plates were subjected to a counting process allowing determination of further parameters as Plate efficiency (PE) and Surviving fraction (SF) (Franken *et al.*, 2006; Marques, 2016).

$$\boldsymbol{PE} = \frac{number\ of\ colonies\ formed}{number\ of\ cells\ seeded} * 100\ \%$$

$$\boldsymbol{SF} = \frac{number\ of\ colonies\ formed\ after\ treatment}{number\ of\ cells\ seeded\ *\ PE}$$

### 2.16. Statistical Analysis

Statistical analysis was performed via IBM® SPSS® software, version 22.0 (IBM Corporation, ARMONK, New York, USA). The results of the studies are presented as the mean and standard error. The normality of the distribution of each quantitative variable was evaluated according to the Shapiro-Wilk test. The homogeneity of variance was achieved through Levene test. The comparison of the metabolic activity and protein content of the cell cultures submitted to treatment was performed with the t-student test, comparing the sample value of each group with the 100 % normalization value. Comparison between groups was performed using Kruskal-Wallis test.

In the cases of cell viability, mitochondrial membrane potential and cell cycle, the comparison between conditions was performed with the one-way *Analysis of variance* (ANOVA) test in cases where normal distribution and homogeneity of variances were verified or with the Kruskal-Wallis test in the otherwise. Between some pairs of experimental groups multiple comparisons were made which were corrected using Bonferroni method with a significance level of 5% for all comparisons.

## 3. RESULTS

### 3.1. Metabolic activity by MTT assay

The results obtained for this assay, using A375 cells, are presented in Figure 9. For NAMP 103A, 103B, 263A and 263B the IC50 and its respective confidence intervals were, respectively, 14.3[10.5, 19.5] nM; 14.5[10.2, 20.7] nM; 4.9[3.1, 7.9] nM; and 2.9[2.2, 4.0] nM. After analyzed the effect of PDT on A375 human melanoma cells, PS cytotoxic effect *per si* was determined.

#### 3.1.1. Absence of light exposure

The results obtained for this assay, using A375 cells, are presented in Figure 10. In general, PDT did not cause a significant difference in relation to control with some exceptions, having

only a significant cytotoxic effect for 1 µM, in NAMP 103B, with a value of 75.27%±2.12% (p=0.021), for 5 µM, in NAMP 263B, with a value of 19.03%±2.08% (p=0.003), for 10 µM, in NAMP 263A and NAMP 263B, with values of 45.44%±4.1% (p=0.006) and 6.43%±2.0% (p<0.001), respectively.

The results obtained for this assay, using HFF1 cells, are presented in Figure 11. In general, PDT did not cause a significant difference in relation to control with some exceptions, having only a significant cytotoxic effect for 5 µM and 10 µM, in NAMP 263B, with values of 54.48%±12.2% (p=0.042) and 29.54%±9.83% (p=0.003), respectively.

### 3.1.2. Overall energy of 5J

For total irradiation energy of 5 J, obtained values of metabolic activity are presented in Figure 12. It is possible to see significant disturbances on the values of metabolic activity for NAMP 103A, at 5 nM, 50 nM and 500 nM, with values of 106.09%±1.99% (p=0.048), 2.83%±0.3% (p<0.001), 1.37%±0.09% (p<0.001), respectively; for NAMP 103B, at 50 nM and 500 nM, with values of 1.18%±0.16% (p<0.001) 1.42%±0.15% (p<0.001), respectively; for NAMP 263A, at 50 nM and 500 nM, with values of 10.43%±1.48% (p<0.001), 1.52%±0.18% (p<0.001); for NAMP 263B, at 50 nM and 500 nM, with values of 1.39%±0.13% (p<0.001) and 1.48%±0.13% (p<0.001), respectively. For the remaining cases, PDT did not cause a significant difference in relation to control.

Regarding concentrations, it was observed that the higher the concentration the lower the metabolic activity. For NAMP 103A, 50 nM was more effective than 5 nM (p=0.041) and 500 nM more effective than 50 nM (p<0.001); for NAMP 103B, 50 nM was more effective than 5 nM (p<0.001) and 500 nM more effective than 5 nM (p=0.004); for NAMP 263A, 50 nM was more effective than 5 nM (p=0.048), 500 nM more effective than 5 nM (p<0.001) and 500 nM more effective than 50 nM (p=0.048) and, for NAMP 263B, 50 nM was more effective than 5 nM (p<0.001) and 500 nM more effective than 5 nM (p=0.002).

Concerning compounds, the concentration of 50 nM, NAMP 103B was more effective than NAMP 103A (p=0.029), NAMP 103B more effective than NAMP 263A (p<0.001) and NAMP 263B more effective than NAMP 263A (p<0.001).

### 3.1.3. Overall energy of 10J

For total irradiation energy of 10 J, obtained values of metabolic activity are presented in Figure 13. It is possible to observe significant disturbances on the values of metabolic activity for NAMP 103A, at 50 nM and 500 nM, with values of 1.76%±0.23% (p<0.001) and 0.99%±0.34%

(p<0.001), respectively; for NAMP 103B, at 50 nM and 500 nM, with values of 3.57%±0.76% (p<0.001) and 1.33%±0.32% (p<0.001), respectively; for NAMP 263A, at 5 nM, 50 nM and 500 nM, with values of 39.08%±6.75% (p<0.001), 1.43%±0.33% (p<0.001) and 0.71%±0.11% (p<0.001), respectively; for NAMP 263B, at 5 nM, 50 nM and 500 nM, with values of 0.93%±0.17% (p<0.001), 2.76%±1.06% (p<0.001) and 0.77%±0.17% (p<0.001), respectively. For the remaining cases, PDT did not cause a significant difference in relation to control.

Regarding concentrations, it was observed that the higher the concentration the lower the metabolic activity. For NAMP 103A, 50 nM was more effective than 5 nM (p=0.018) and 500 nM more effective than 5 nM (p<0.001); for NAMP 103B, 500 nM was more effective than 5 nM (p<0.001); for NAMP 263A, 50 nM was more effective than 5 nM (p=0.006), 500 nM more effective than 5 nM (p<0.001); for NAMP 263B, 50 nM there was no significant value.

Concerning compounds, for 5 nM, NAMP 263B was more effective than NAMP 103A (p=0.003), NAMP 263A more effective than NAMP 103B (p=0.038) and NAMP 263B more effective than NAMP 103B (p=0.001); for 50 nM, NAMP 263A was more effective than NAMP 103B (p=0.013) and NAMP 263B more effective than NAMP 103B (p=0.019); for 500 nM, there was no significant value.

### 3.1.4. Overall energy of 20J

For total irradiation energy of 20 J, obtained values of metabolic activity are presented in Figure 14. It is possible to see significant disturbances on the values of metabolic activity for NAMP 103A, at 5 nM and 50 nM, with values of 65.27%±9.13% (p=0.039) and 1.01%±0.11% (p<0.001), respectively; for NAMP 103B, at 5 nM and 50 nM, with values of 41.64%±8.10% (p<0.001) and 1.03%±0.1% (p<0.001), respectively; for NAMP 263A, at 50 nM, with a value of 0.94%±0.17% (p<0.001); for NAMP 263B, at 5 nM and 50 nM, with values of 51.73%±4.58% (p<0.001) and 1.53%±0.18% (p<0.001), respectively. For the remaining cases, PDT did not cause a significant difference in relation to control.

Regarding concentrations, it was observed that the higher the concentration the lower the metabolic activity. For NAMP 103A, 50 nM was more effective than 5 nM (p<0.001); for NAMP 103B, 50 nM was more effective than 5 nM (p<0.001); for NAMP 263A, 50 nM was more effective than 5 nM (p<0.001); for NAMP 263B, 50 nM was more effective than 5 nM (p<0.001).

Relating compounds, at 5 nM, NAMP 103B was more effective than NAMP 263A (p=0.003), and NAMP 263B more effective than NAMP 263A (p=0.041); at 50 nM, NAMP 263B was more effective than NAMP 263A (p=0.039); for 500 nM, there was no significant value.

### 3.1.5. Assessment of different overall irradiation energies

Regarding accumulated irradiation energies, it was perceived, for each concentration, that total radiation energy of 20 J was more deadly than 10 J and this deadliest than 5 J. For NAMP 103A, at 5 nM, 20 J was more effective than 5 J ($p<0.001$) and 20 J was more effective than 10 J ($p=0.002$); at 50 nM, 10 J was more effective than 5 J ($p=0.008$) and 20 J was more effective than 5 J ($p<0.001$). For NAMP 103B, at 5 nM, 20 J was more effective than 5 J ($p=0.004$) and 20 J was more effective than 10 J ($p=0.001$); at 50 nM, 10 J was more effective than 5 J ($p=0.004$) and 20 J was more effective than 10 J ($p<0.001$). For NAMP 263A, at 5 nM, 10 J was more effective than 5 J ($p<0.001$) and 20 J was more effective than 10 J ($p=0.003$); at 50 nM, 10 J was more effective than 5 J ($p=0.001$) and 20 J was more effective than 5 J ($p<0.001$); at 500 nM, 10 J was more effective than 5 J ($p<0.001$). For NAMP 263B, at 5 nM, 10 J was more effective than 5 J ($p<0.001$) and 20 J was more effective than 5 J ($p=0.015$); at 500 nM, 10 J was more effective than 5 J ($p=0.006$).

## 3.2. Cell viability by SRB assay

Once determined the effect of PDT on metabolic activity of A375 and HFF1 cell lines, the studies were conducted forward to analyze the effect of PDT on viability of A375 cells. For every condition presented in Figure 15, the values obtained had disturbances that constituted significant diminishments on the referred viability, predominantly for concentrations of 50 nM, 125 nM, 250 nM and 500 nM. The following values were observed for each concentration above mentioned. For NAMP 103A, the concentrations of 50 nM, 125 nM, 250 nM and 500 nM showed values of 43.2%±7.0% ($p<0.001$), 37.42%±1.94% ($p<0.001$), 32.45%±1.17% ($p<0.001$) and 34.96%±3.1% ($p<0.001$), respectively. For NAMP 103B, the concentrations of 50 nM, 125 nM, 250 nM and 500 nM showed values of 27.95%±1.27% ($p<0.001$), 33.23%±1.29% ($p<0.001$), 33.04%±1.16% ($p<0.001$) and 26.97%±2.09% ($p<0.001$), respectively. For NAMP 263A, the concentrations of 50 nM, 125 nM, 250 nM and 500 nM showed values of 15.38%±3.15% ($p<0.001$), 18.32%±2.22% ($p<0.001$), 21.03%±3.07% ($p<0.001$) and 16.56%±2.48% ($p<0.001$), respectively. For NAMP 263B, the concentrations of 5 nM, 50 nM, 125 nM, 250 nM and 500 nM showed values of 78.81%±3.79% ($p=0.005$), 16.76%±1.77% ($p<0.001$), 18.82%±3.13% ($p<0.001$), 15.2%±2.6% ($p<0.001$) and 13.73%±2.47% ($p<0.001$), respectively.

## 3.3. Cell Death

As settled in Figure 16, the overall population of alive (A) cells suffered a decrease whilst the percentage of cells at initial apoptosis (AP), late apoptosis/necrosis (AP/N) and necrosis (N)

increased. For NAMP 103A, two significant values were observed for each cells' population: for A population, a decrease both at 50 nM in comparison with control (93.13±0.44%), 87.6±1.46%, ($p=0.025$) and at 500 nM in comparison with control (93.13±0.44%), 33.11±7.35%, ($p<0.001$); for AP population, an increase both at 50 nM in comparison with control (2.79±0.25%), 7.0±1.64%, ($p=0.013$) and at 500 nM in comparison with control (2.79±0.25%), 15.33±1.8%, ($p<0.001$); for AP/N population, an increase both at 500 nM in comparison with control (1.38±0.1), 26.0±6.94, ($p<0.001$) and at 500 nM, 26.0±6.94%, in comparison with 50 nM, 1.0±0.0%, ($p<0.001$); for N population, an increase both at 500 nM in comparison with control (2.71±0.44%), 25.44±6.73%, ($p<0.001$) and at 500 nM, 25.44±6.73%, in comparison with 50 nM, 5.0±1.65%, ($p=0.028$).
For NAMP 103B, several significant values were observed. For A population and for AP population: in the A population, a decrease both at 50 nM in comparison with control (93.13±0.44%), 84.5±1.86%, ($p<0.001$) and at 500 nM in comparison with control (93.13±0.44%), 60.63±10.7%, ($p<0.001$); for the AP population, an increase both at 50 nM in comparison with control (2.79±0.25%), 6.1±1.11%, ($p=0.044$) and at 500 nM in comparison with control (2.79±0.25%), 14.75±5.36%, ($p=0.007$); for AP/N population, an increase at 500 nM in comparison with control (1.38±0.1%), 6.38±2.6% ($p=0.009$); for N population, an increase at 500 nM in comparison with control (2.71±0.44%), 18.25±5.54%, ($p=0.001$).
For NAMP 263A, two significant values were observed for each alive, AP and AP/N population and one for N. For A population: a decrease at 50 nM in comparison with control (93.13±0.44%), 87.75±0.8%, ($p=0.004$) and another one at 500 nM in comparison with control (93.13±0.44%), 32.5±4.91%, ($p<0.001$); for AP, an increase at 50 nM in comparison with control (2.79±0.25%), 6.38±0.75%, ($p=0.003$) and another one at 500 nM in comparison with control (2.79±0.25%), 23.75±9.51%, ($p<0.001$); for AP/N population, an increase at 500 nM in comparison with control (1.38±0.1%), 9.13±2.94%, ($p<0.001$) and an increase at 500 nM, 9.13±2.94%, in comparison with 50 nM, 1.63±0.18%, ($p=0.035$); for N population, an increase at 500 nM in comparison with control (2.71±0.44%), 34.75±7.32%, ($p<0.001$).

For NAMP 263B, two significant values were observed for each A population and AP population. AP/N population and N population demonstrated a single significant value, each one. For A population: a decrease at both 50 nM in comparison with control (93.13±0.44%), 78.29±5.99%, ($p=0.003$) and at 500 nM in comparison with control (93.13±0.44%), 47.88±10.10%, ($p<0.001$); for AP, an increase at both 50 nM in comparison with control (2.79±0.25%), 13.57±4.25%, ($p=0.002$) and at 500 nM in comparison with control (2.79±0.25%), 29.75±5.74%, ($p<0.001$); for AP/N population, an increase at 500 nM in

comparison with control (1.38±0.1%), 3.88±0.72%, (p<0.001); for N population, an increase at 500 nM in comparison with control (2.71±0.44%), 18.5±4.65%, (p<0.001).

### 3.4. Cell Cycle

The major events observed in A375 cell cycle after PDT are shown in Figure 17. For NAMP 103A and NAMP 103B, the most relevant events were a significant increase on pre-G0 peak at 500 nM in comparison with control (0.62±0.37%), in both cases, 9.8±3.15% and 5.0±0.95% (p=0.002 and p=0.005), respectively. For NAMP 263A, other events were observed, an increase on pre-G0 peak at 500 nM in comparison with control (0.62±0.37%), 17.4±6.51%, (p<0.001); a diminishment on G0/G1 peak at 500 nM in comparison with control (71.31±0.66%), 47.8±4.12%, (p=0.003); an increase on G2/M peak at 500 nM in comparison with control (6.85±0.63%), 18.8±2.6%, (p=0.001) and at 500 nM (18.8±2.6%), in comparison with 50 nM, 9.83±1.85%, (p=0.008). For NAMP 263 B, it was only observed an increase on pre-G0 peak at 500 nM in comparison with control (0.62±0.37%), 15.0±7.51% (p=0.019).

### 3.5. Mitochondria Membrane Potential

MMP values obtained for every condition represented in Figure 18 had significant disturbances and revealed a diminishment on MMP, which values of the ratio monomers/aggregates are respectively denoted after the following comparisons: for NAMP 103A, the assessment between control (0.63±0.04) and 500 nM was 4.54±1.16 (p<0.001); for NAMP 103B, the assessment between control (0.63±0.04) and 50 nM was 1.27±0.28 (p<0.05), and control (0.63±0.04) and 500 nM was 8.57±4.03 (p<0.05); for NAMP 263A, the assessment between control (0.63±0.04) and 500 nM was 8.23±2.2 (p<0.001); for NAMP 263B, the assessment between control (0.63±0.04) and 50 nM was 1.11±0.18 (p<0.05), and control (0.63±0.04) and 500 nM was 2.49±0.5 (p<0.001). For NAMP 103A, the assessment between 50 nM (1.0±0.12) and 500 nM (4.54±1.16) (p<0.05) also revealed a diminishment on MMP.

### 3.6. Western Blot

Regarding this assay, for every condition presented in Figure 19, P53 expression values obtained had increments relative to control. Within these results, only NAMP 263A 50 nM presented for the referred expression a significant increased value relative to control, of 2.45±0.07 (p=0.012). On the remaining cases, PDT did not cause a significant difference in relation to control. However, it is notable a propensity to an increment in the expression values of P53 along the referred PS. Relating compounds, there was any association with significance.

### 3.7. Quencher cytotoxicity, Singlet oxygen & Hydroxyl radical assessment

In Figure 20 are accessible the values of metabolic activity used to determine the cytotoxicity of each quencher. Within these results, for mannitol, 80 nM, 500 nM, 700 nM and 1 μM presented significant alterations on the referred activity with values of 122.62%±6.89% ($p=0.02$), 21.54%±4.38% ($p<0.001$), 14.17%±3.4% ($p<0.001$), 6.38%±1.62% ($p<0.001$); for sodium azide, 50 nM, 250 nM, 500 nM and 1 μM presented significant alterations on the referred activity with values of 136.27%±10.19% ($p=0.032$), 28.23%±4.6% ($p<0.001$), 2.74%±1.13% ($p<0.001$), 2.33%±0.55% ($p<0.001$). On the remaining cases, PDT did not cause a significant difference in relation to control. Overall, for sodium azide, metabolic activity inhibition was not observed for the concentration of 100 nM or below; for mannitol, metabolic activity inhibition was not noticeable for the concentration of 200 nM or below.

In Figure 21 are settled the results regarding the comparison of metabolic activity of cells exposed to PS alone and to cell in which was also administered a quencher. Overall, it can be observed that when both the PS and inhibitor are present the metabolic activity of cells is raised in comparison to the cells were PS alone is present. Hence, for NAMP 103A 50 nM, 10 J MTT was more effective than 10 J MTT with sodium azide at 50 mM ($p<0.01$) in generate an effective photodynamic reaction. For NAMP 103B 50 nM, 10 J MTT was more effective than 10 J MTT with mannitol at 120 mM ($p=0.019$). For NAMP 263A 50 nM, 10 J MTT was more effective than 10 J MTT with sodium azide at 50 mM ($p<0.001$) and, for NAMP 263B 500 nM, 10 J MTT was more effective than 10 J MTT with mannitol at 40 mM ($p=0.004$).

### 3.8. Intracellular Peroxides Production

Peroxides values obtained had disturbances as it is presented for every condition in Figure 22. Within these results, for NAMP 103A 500 nM, NAMP 103B 500 nM, NAMP 263A 500 nM, NAMP 263B 50 nM and NAMP 263B 500 nM a significant diminishment on peroxides in comparison with control was observed with values of 0.44±0.09 ($p<0.001$), 0.26±0.04 ($p<0.001$), 0.46±0.05 ($p<0.001$), 0.6±0.05 ($p<0.001$) and 0.46±0.03 ($p<0.001$), respectively.

Comparing compounds, at 50 nM, only two associations presented significance. NAMP 103A generated more peroxides than NAMP 263B ($p=0.017$) registered a decrease in these values, and NAMP 103B was also capable to generate more peroxides than NAMP 263B ($p=0.004$) kept the decreased generated peroxides values. At 500 nM, only an association arose with significance. NAMP 263B triggered a smaller diminishment in the peroxides than NAMP103B ($p=0.039$) did.

### 3.9. Intracellular Superoxide Anion Production

For every condition presented in Figure 23, superoxide values obtained had disturbances. Within these results, for NAMP 103A 500 nM, NAMP 103B 50 nM, NAMP 103B 500 nM, NAMP 263A 500 nM, NAMP 263B 500 nM a significant diminishment on superoxide anion in comparison with control was observed with values of 0.32±0.02 ($p<0.001$), 0.83±0.04 ($p=0.012$), 0.26±0.01 ($p<0.001$), 0.33±0.04 ($p<0.001$) and 0.38±0.07 ($p<0.001$), respectively.
Regarding compounds, at 50 nM, only one association presented significance. NAMP 103A generated more superoxide anions relative to control while NAMP 263B ($p=0.01$) presented a decreased in this relative value of superoxide anions. At 500 nM, there was any association with significance.

### 3.10. Reduced Glutathione Expression

GSH expression results are displayed in the Figure 24 where is possible to realize that NAMP 103A 500 nM, NAMP 263A 50 nM and NAMP 263A 500 nM have significantly disturbances on the values of GSH. In all these cases, a diminishment on the GSH was observed in comparison with control, with a value of 0.84±0.01 ($p<0.001$), 0.91±0.02 ($p<0.05$) and 0.86±0.02 ($p<0.001$), respectively.
Regarding compounds, for 50 nM, there was any association with significance. For 500 nM, only one association presented significance. NAMP 103A induced a superior decrease in the values of GSH than NAMP 103B ($p<0.05$), relative to control.

### 3.11. Superoxide Dismutase

Superoxide dismutase (SOD) activity was determined as an important intracellular defense against ROS once its behavior can influence the therapeutic results after PDT. According to the displayed in the Figure 25, only NAMP 103A at 50 nM revealed a significant disturbance for the value of SOD activity, which decreased, of 0.84±0.05 ($p<0.05$). For the remaining cases, PDT did not cause a significant difference in relation to control.

### 3.12. Clonogenic Assay

Aiming to determine cell proliferation/survival profile after PDT, concentrations of PSs of 50 nM and 500 nM were used and clonogenic assay was performed. According to data stated on Figure 26, a decrease on survival was noticed for both values of concentration with the exception of NAMP 103B which demonstrated an increase on the cells' proliferation value when exposed at 500 nM in comparison to 50 nM. However, must be noticed that cells

subjected to NAMP 263B at 50 nM did not reveal any survival. Cells subjected to NAMP 103A, NAMP 263A and NAMP 263B at 500nM were not also able to reveal any kind of survival.

## 4. DISCUSSION

PDT is a therapeutic approach which takes on use a drug activated by light, PS, and light itself on a proper wavelength. Being a minimally-invasive approach, this therapeutic method targets cancer cells through the generation of ROS, namely the most important among all of them, singlet oxygen, which is the effector of the cell damages (Castano, Mroz e Hamblin, 2006; Dongen, Visser e Vrouenraets, 2004). For that purpose, PS shall achieve high concentrations on the tumor cell and, then, the irradiation light should be pointed to the area where malignant cells are located to avoid further activation of PS that is not in the desired place. Several PS compounds are already approved for clinical use but some problems are still faced, such as the aggregation of these molecules, due to some hydrophobic character, resulting in low reaction yield and selectivity for cancer cells. The combination of these two more common problems lead to clinical issues as side effects (Babilas et al., 2010). Attempting to reach successful outcomes, a rational design is applied to synthesize molecules capable to surpass these problems (Bastien, Schneider e Dumas, 2015).

Arising from previous research work on this matter, new stable PS compounds were explored (Laranjo et al., 2013). Once skin melanin possesses an absorbance between 500 nm to 600 nm it can compete with shorter-wavelength-absorber compounds. It also comprehends a notable antioxidant capacity which might induce critical diminishments on the concentrations of ROS generated during PDT (Garg e Agostinis, 2014). To avoid such regressive circumstances, chlorins - which are chemically dihydroporphyrins, members of porphyrin family achieved by its hydrogenation (Yoon, Li e Shim, 2013) - were chosen to be functionalized. These molecules guaranteed the physiological homeostasis and significant tumor accumulation, holding a phototherapeutic window with a range of 600 nm to 800 nm and being capable to generate satisfactory amounts of $^1O_2$. To circumvent the problem of oxidation and turn back to porphyrin structure, the functionalization helped also the purpose to block this event as well as to attain better hydrophilic/lipophilic character regarding a better pharmacokinetic profile. The chemical process of development of such compounds involves a series of complex steps and, lately, a *de novo* synthesis thru [8π + 2π] cycloaddition of diazafulvenium methides using porphyrins and chlorins which lead to four new molecules - NAMP 103A, NAMP 103B, NAMP 263A, NAMP 263B - with absorption bands at 650 nm and 730 nm and a very good stability (Figure 27) (Pereira et al., 2015).

The MTT assay constitutes a broad test used very often to measure cytotoxicity in cell lines. As already described, their bases show that for viable cells exist a stable mitochondrial activity and, so, variations in the number of such cells are directly linked with that activity. Being the predominant, but not the only mechanism, mitochondrial activity makes the conversion of MTT to formazan crystals, which can be measured through this method (Liu et al., 1997; Stockert et al., 2012). In our study, we assessed four new compounds: NAMP 103A, NAMP 103B - tetraphenylchlorins monoesters - NAMP 263A and NAMP 263B - tetraphenylchlorins alcohols. Some previous studies performed with similar-structure molecules lead to setting the suitable wavelength of irradiation light at 570 nm ($\lambda$>570 nm) confined to an area that the overall accumulated energy would be 10 J, with auspicious cytotoxic effects (Laranjo et al., 2013; Pereira et al., 2015). First, the IC50 was determined using a range of concentration for each PS from 5 nM to 5 μM and the MTT assay as the final technique to reveal these results. Facts exposed that NAMP 263B presented the minor value for $IC_{50}$ (the most potent), being followed by NAMP 263A, NAMP 103A, and NAMP 103B. These values are quite interesting when comparing potencies of drugs in, for example, oncology, as mTOR inhibitors, tyrosine kinase inhibitors, intercalating agents, ROS generating chemotherapeutics. Melanoma cells and human fibroblast cells, were submitted to similar conditions, but without radiation exposure, to measure the inherent toxicity of these compounds.

On melanoma cells, a significant decrease was observed on metabolic activity starting at 1 μM, with exception of NAMP 263A (10 μM). On human fibroblast cells, it was observed a significant decrease on metabolic activity starting at 5 μM with exception of NAMP 263B (1 μM). The use of a fibroblast cell line allowed to verify the intrinsic toxicity of the PS molecules, confirming dependency on the irradiation process, and serves the purpose of indicating that molecules scattered on various places than tumor do not demonstrate cytotoxicity. With a cautious analysis, it is possible to observe that melanoma cells are more susceptible than human fibroblast cells to the referred compounds. It is clearly noticeable that referred concentrations for which intrinsic cytotoxicity was demonstrated are very high so, the effects are very likely potentiated by these higher values of concentrations.

Ensuing studies, the compounds were submitted to three different overall irradiation energies: 5 J, 10 J, and 20 J. For 5 J and 10 J, concentrations of 5 nM, 50 nM, and 500 nM were used, whilst for 20 J only 5 nM and 50 nM were used.

For 5 J, every compound on concentration equal or higher to 50 nM presented a significant diminishment on the metabolic activity which is even more reinforced by consecutive significant depletions along higher concentrations for almost every single compound. For 10 J, the previous referred significant diminishments were even more drastic starting the cytotoxicity at concentrations equal or higher to 5 nM. Intragroup significant diminishments were also verified at 50 nM. Nevertheless, the most abundant comparisons are established between 5 nM and 500 nM with abrupt diminishments, being salient the values for NAMP 263B which demonstrated, at 5 nM, almost the same values as at 50 nM and at 500 nM. Due to such scenario, 20 J accumulated radiation energy plan was not even attempting to reach 500 nM. All compounds presented significant diminishments relative to control and each compound verified significant decrease on its value at 500 nM relatively to 5 nM.

Overlooking this amount of information there are simple but direct conclusions that are possible to be achieved. For above described concentrations of PS, the cytotoxic effect increases with the increases of power irradiation. Also, NAMP 263B is the most potent PS. Therefore, 10 J overall radiation energy seemed to be a proper value to apply on during the following studies in this dissertation. Other studies also demonstrated similar results as, for example, Sherifa et al. proved that in hepatocellular carcinoma treated with Fospeg, a PS, the presence of this compound without irradiation lead to no cytotoxic effect whilst for same concentrations of PS, increasing radiation energies significantly proved to be more lethal, whereas, for the same radiation energy, the increase of concentrations was not proved to be more deathly (Sherifa et al., 2013). Weijer and collaborators demonstrated using liposomes with encapsulated zinc-phthalocyanine that low radiation potency was able to predominantly activate inflammatory response and consequently achieve a major survival behavior with less lethal results (Weijer et al., 2015). Chung and coworkers took on use 9-hydroxypheophorbide-a and realized that cytotoxicity was increased as the PS concentration and the radiation energy increased (Chung et al., 2003). Other studies followed the same pattern with similar demonstrated results (Laranjo et al., 2013; Li, 2015; Sano et al., 2005; Triesscheijn et al., 2004).

Following MTT assay cell survival was assessed. SRB assay was then used as it holds the protein content as a measure of viable cells (Vichai e Kirtikara, 2006). Comparing MTT and SRB data it becomes distinct that tested compounds can induce a significant cytostatic effect after 50 nM, inclusively, once the metabolic activity is far decreased but cell protein content does not get appreciable variation.

Once observed the effects of PDT applied at different energies on the metabolic activity of melanoma cells, further insightful studies were conducted concerning the mechanisms by which these compounds take an action at a subcellular level. To achieve such purpose, the predominant cell death types and cell cycle phases evolved in the cytotoxicity outcomes of PDT were assessed. In the context of cell death, apoptosis and necrosis events display different molecular signatures: the first is positive for the presence of phosphatidylserine residues on the external side of the membrane and the second positive for PI internalization. Overall, the PS compounds dictated a significant decrease on the values of alive/viable cells in relation to control at 50 nM and 500 nM. For apoptosis events, all PS compounds verified significant increments both at 50 nM and 500 nM. The necrotic events appeared with significant values for all the PS compounds but only at 500 nM.

Shortly, at both concentrations studied, NAMP 103A and NAMP 263B presented the highest population values for cells in alive condition, for apoptosis condition and the minor values for necrotic condition; NAMP 103B and NAMP 263A presented the minor population values for cells in alive condition, for apoptosis condition and the highest values for necrotic condition. Thus, cell death by apoptotic events shall be related much more to NAMP 103A and NAMP while NAMP 103B and NAMP 263A shall be related much more to necrotic events. A study concerning the use of chlorin e6 on human vascular smooth muscle cells verified that after PDT being applied the cells subjected to it possessed typical properties of cells in apoptosis. Although, time was found as a critical factor once some hours later cell membrane was found more permeable to PI, indicating an upstretched level of necrotic event (Wawrzyn, 2010). Another research group demonstrated that PDT with chlorin e6-polyvinylpyrrolidone (Photolon) lead to an increased population of bladder cancer cells entering into apoptosis (Ewelina Szliszka et al., 2012). An investigation carried with chlorin e6 on human colon cancer cells discovered that, once more, after PDT, these cells underwent predominantly apoptosis and, additionally, this event was increased by the rise of the concentration of PS (Li et al., 2014). Sasnauskiene headed a study on which was attempted to direct the PDT damages to the outer mitochondrial membrane making use of a cationic photosensitizer, 7-diamino-2,8-dimethyl-5-phenylphenazinium chloride, which revealed, at high concentrations, cell death by apoptosis through the release of cytochrome c followed by caspase-3 triggering (Sasnauskiene et al., 2009). Another study, however, settled with a chlorin-based photosensitizer, meso-tetra(3-morphlinomethyl-4-methoxyphenyl) chlorin (TMMC), demonstrated a different potential, one to induce higher values of necrosis than apoptosis in human esophageal cancer cells (Jun et al., 2014), revealing that slight chemical modifications on PS molecules may lead

to very distinct cell death types. Such conclusion is far important as it shows that PS molecules shall be designed, synthesized and applied specifically to tumors depending on the death mechanisms and physiological properties held.

As soon as the kind of cell death subsequently to PDT was determined, cell cycle phase was then assessed for cells to which each PS was applied. Following to this procedure it was observed that both NAMP 103A and NAMP 103B presented an almost identical profile with a significant pre-G0 peak rising at 500 nM which indicates cell death by apoptosis; NAMP 263A demonstrated significant increase at 500 nM also, namely for pre-G0 peak, G0/G1 peak and G2/M peak, indicating also an apoptotic trend and blockage before and after nucleic acid replication; NAMP 263B assumed a significant increase on the pre-G0 peak indicating also a predisposal for apoptosis. Chung and collaborators used a PS molecule, 9-hydroxypheophorbide-a on human hypopharyngeal squamous cell carcinoma to perform PDT and realized that cell cycle was arrested in G2/M phase (Chung et al., 2003). Liang et al conducted another study using a chlorophyll derivative, pyropheophorbidea methyl ester, and deducted by achieved results that cell cycle was arrested at S phase due to p21WAF1-activation-Cyclin D1-diminishment and consequent cell apoptosis (Liang, Bi e Tian, 2016). Sano and associates also developed a research with a water-soluble PS, ATX-S10(Na), and applied it to synchronized HeLa S3 cells. Their results proved that those cells were sensitive to ATX-S10(Na) when they were at S and G2/M phases in comparison to G1 being the concentrations of PS also increased in the S, G2/M cycle cells when also compared to G1(Sano et al., 2005). Based on these studies and on the obtained results it is clear that cell cycle shows the efficacy of PDT being a crucial factor for its success.

Taking a leading role in the apoptosis process, MMP identifies this kind of cell course due to a mitochondrial electron transporter chain homeostasis loss. The mitochondrial membrane depolarization might be a factor of highlight in the process of photosensitizing by the tested compounds (Hengartner, 2000). Such fact is supported by the characteristic hallmarks of apoptosis such as exodus of cytochrome c from the inner mitochondrial membrane, universal feature of apoptotic processes (although a late event) probably caused by caspase induction, as well as AIF (apoptotic flavoprotein), Smac/DIABLO and procaspase-2, -3 and -9 induced by death receptors (Ito e Matsui, 2016). Considering this, the referred potential was evaluated after PDT. At a concentration of 50 nM, only NAMP 103B and NAMP 263B demonstrated a

significant increase on the ratio of monomers/aggregates; at 500 nM, all the compounds presented an advanced significant increase, more than the verified at 50 nM. That suggests a direct effect of the tested compounds on the disruption of MMP, fact proposed by additional studies (Chen et al., 2008; Laranjo et al., 2013; Li et al., 2014; Li, 2015; Pereira et al., 2015; Wawrzyn, 2010).

Western blot is a very consistent and versatile technique which allows separation and identification of specific proteins through their molecular-weight. In the present study, the protein codified by the tumor-suppressor gene P53 was targeted to appreciate its expression. The expression of such protein can dictate the continuous cell division or the stoppage of this process. Analyzing the data, it was confirmed an increase on the expression at 50 nM along NAMP 103A, NAMP 103B, NAMP 263A and NAMP 263B, the third the only one with a significant value. Yet, the outlook undoubtedly points to a succeeding potentiation of P53 expression which is indirectly driven by PDT effects (Laranjo, 2014). Thus, can be inferred that NAMP 263B is the most prominent compound generating ROS and, thus, in the initiation of this expression. Sherifa and associates performed PDT with Temoporfin, meta-tetra(hydroxyphenyl)chlorin, attempting to verify the effects of this compound in human hepatoma cells. With help of a P53-luciferase vector, they proved that P53 protein was upregulated after PDT in comparison to control (Sherifa et al., 2013).

Searching for a more detailed description about the mechanism of action and role of ROS in the cell damage, ROS quenchers - sodium azide and mannitol - were used on A375 cells in different concentrations (Laranjo, 2014). First, the cytotoxicity of each quencher for this cell line was tested to observe the critical concentration above all the quencher would apply a damage for itself. For this situation was detected that sodium azide presented no cytotoxicity below 100 nM (inclusively) and either mannitol below 200 nM (inclusively). The next phase consisted in combine each quencher, at a precise concentration, with each PS, at different concentrations, with a final MTT assay to reveal to results.

For mannitol, 40 mM and 120 mM were the chosen concentrations and, for sodium azide, 5 mM and 50 mM. The results obtained in the presence of each quencher were suitably compared with PDT with each PS but without inhibitors. For the comparison with the quencher sodium azide, at 50 mM, it was revealed that NAMP 103A 50 nM and NAMP 263A 50 nM were significantly less lethal to cells. For the comparison with the quencher mannitol,

at 120 mM, it was observed that NAMP 103B 50 nM and, for mannitol at 40 nM, that NAMP 263B 500 nM were also significantly less lethal to cells. As $^1O_2$ is the principal effector species of the cell damages induced by PDT and, as sodium azide is a quencher of this species (Bancirova, 2011), it is clear from these results the importance that such compound possesses in the effectiveness of PDT (Spikes e Bommer, 1993). The results displayed from mannitol use, a hydroxyl quencher, demonstrated that this species also represents an effective role on the process of cell damage by ROS (Castano, Demidova e Hamblin, 2005). Daniell and associates demonstrated that both sodium azide and mannitol decreased phototoxicity after PDT employment with chlorin e6-conjugates on ovarian cancer cells. Nevertheless, they also realized that sodium azide was the only quencher that significantly decreased these species, in comparison with PS alone, by almost 25 % while mannitol only decreased by 4 %. Sodium azide had been recognized also as principal mechanism of protection the quenching of ROS generated from type II photodynamic reaction but was also capable of quenching other species (Daniell, 2012). Another study, this time executed by Cheng et al, used sodium azide and mannitol to prove the quenching of ROS generated from PDT reaction of type II and I, respectively. They used as PS the compound pyropheophorbide-a and submitted HeLa cervical cancer cells to it. The results presented indicate that cytotoxicity arising from PDT was diminished proving once more the potential of both $^1O_2$ and hydroxyl species in trigger inner cell damages (Cheng et al., 2017). However, a remarkable study had an opposite outcome. Huang and collaborators, in the attempt of quenching the singlet oxygen with 10 mM of sodium azide, during a PDT session on Staphylococcus aureus and Escherichia coli cultures in suspension - which had been incubated with methylene blue as PS - observed that the specie $N_3$. was formed and induced even more damages than PS alone. This instable specie killed similarly in the absence of oxygen demonstrating that quencher concentration is also a critical feature in order to avoid miscarried conclusions (Huang et al., 2012).

Tumorigenesis, tumor metabolism and homeostasis, and ROS are strong-linked concepts. ROS are directly responsible for several damages in the DNA structure as well as in other fundamental intracellular assemblies, being responsible either for tumor development or for trigger cell death (Karantza, 2012; Kim, Kim e Bae, 2016; Traverso et al., 2013). Nonetheless, PDT effect is corroborated in the production of higher concentrations of ROS, so these labile substances are indeed a powerful tool to induce a therapeutic effect (Castano, Mroz e Hamblin, 2006; Zhou, Song e Chen, 2016).

Peroxides are a kind of ROS responsible for several damages in the cell. After PDT treatment peroxides production was assessed with DCF probe. DCF is produced by an oxidative

reaction of DCFH2(-DA) undergone in the presence of $H_2O_2$ (Chekulayeva et al., 2007). This probe constitutes a specific mark for tracking the formation of the latter compound due to its fluorescence being proportional to intracellular peroxides concentration. Throughout the current study, this assay was performed and revealed disturbances at both concentrations in every compound. At 50 nM, NAMP 103A and NAMP 103B revealed a significant increase in the referred species in comparison to NAMP 263B and this one revealed a significant decrease in comparison to control; NAMP 263A, although not revealing any significant value, presented diminished values in comparison to control. At 500 nM, all compounds revealed a significant decrease in comparison to control. A study conducted by Li and collaborators demonstrated that chlorin e6, 1 hour after applied sono-photodynamic therapy, produced an increment of 83.83 % on intracellular ROS (Li et al., 2014). Additionally, Pereira and associates presented a study on which was proved that 24 hours after PDT with a chlorin-derivative, the ROS showed disturbances pointing to a decrease on the values of peroxides (Pereira et al., 2015). Arising from this it is noticeable that, very likely, some hours after PDT, peroxides are intensively produced and their concentrations kept on higher values. Although, for longer times it is denoted that the same concentrations are decreased when compared to normal values, indicating their consumption by reacting with intracellular structures.

Our study revealed disturbances in the production of superoxide anion. In general, all the compounds but NAMP 263A and NAMP 263B revealed significant diminishment in the values of superoxide anion for both 50 nM and 500 nM with one exception - at 50 nM, NAMP 103A had a significant rise in the value in comparison to NAMP 263B.

A careful analysis of peroxides and superoxide anion reveals the critical function of these ROS in the process of cytotoxicity, corroborating the literature knowledge which is on basis of PDT success. Ascending from the analysis of several studies a homogeneous conclusion validates the previous results that evidence the requirement of ROS subversion to achieve therapeutic outcomes (Chen et al., 2008; Halliwell, 2006; Li et al., 2014, 2014; Li, 2015; Wawrzyn, 2010).

ROS occur naturally inside the cell due to the activity of the entire inner cell machinery. Nonetheless, the increase on their values may lead to pernicious or deathly effects. To avoid such scenario, cells developed strategies to annul strategically these dangerous products - ascorbic acid (vitamin C), α-tocoferol (vitamin E), GSH, inner peroxidases and superoxide dismutase are the main among others (Mascio, Di et al., 1990; Patrice et al., 2016; Wang e

Quinn, 1999). Although, when dealing with cancer cells, the expression levels of these defenses are much more elevated than in normal cells. This phenomenon occurs due to the high metabolic rates inside a cancer cell, with expanded production of such species. Arising from that, it will be necessary to reach higher concentrations of ROS to promote critical damages on the housekeeping mechanisms and to interfere in the division, proliferation, and differentiation of cells (Laranjo et al., 2013; Schumacker, 2006; Shi et al., 2012).

GSH is a prevailing antioxidant in the cell milieu with demonstrated capacity to eradicate peroxides and to achieve the reduction of many oxidant species inside the cell (Gamcsik et al., 2012; Ortega, Mena e Estrela, 2011). During the present study, it was verified a significant diminishment on GSH, at 50 nM, for NAMP 263A and, at 500 nM, for NAMP 103A and NAMP 263A, and a signifying decrease on NAMP 103A in comparison to NAMP 103B. The decrease on GSH values was already proved in several cell lines and for different PS to be time-dependent as there is an obligatory time to cell to turnover its antioxidant provisions (Brites, 2016; Chen et al., 2008; Laranjo et al., 2013; Soares et al., 2016).

SOD catalyzes the reaction which produces $H_2O_2$ and $O_2$ by dismutation of $O_2^-$, being considered one of the most prominent antioxidant intracellular defenses. In the present study, it was demonstrated, at 50 nM, a significant diminishment on the value of SOD activity for NAMP 103A and, without significance, a notable raise for NAMP 263B. Nevertheless, a raise on concentration to a value of 500 nM demonstrated an overall increase in the referred value for NAMP 103A, NAMP 103B and NAMP 263A, although any of these values significant. Given this information, it can be observable that PDT does not affect significantly the activity of SOD. Due to this, is taken full advantage of the PDT potential as the therapy effect is not weakened by this intracellular antioxidative defense. Other studies also suggested a detachment from this enzyme actuation in the context (Baldea et al., 2015; Laranjo, 2014; Soares et al., 2016).

After PDT performance, part of the remaining alive cells present damages which will not allow them further proliferation while others might be able to go on their continuous division cycle or even gain new properties as therapeutic resistance (Li *et al.*, 2015; Wakita, Tanabe e Toida, 2011; Zamarrón *et al.*, 2015). To evaluate the capacity of the remaining cells to proliferate and to form colonies, the clonogenic assay was performed. The preliminary obtained results demonstrated that at 50 nM only NAMP 263B did not allow any kind of proliferation while for NAMP 103A, NAMP 103B and NAMP 263A had a crescent proliferation values. At 500 nM

only NAMP 103B allowed cancer cells to proliferate. A dose-effect outcome is observable but, overall, it is clear according to the available data that NAMP 263B demonstrated the strongest inhibitory effect over cells proliferation. Studies concerning chlorin-related molecules were able to collect data that disposed similar results, demonstrating a pronounced decrease dependent on the concentration of the photosensitizer molecule (Chen *et al.*, 2016; Li *et al.*, 2014, 2014; Sherifa *et al.*, 2013) as well as light dose (Triesscheijn *et al.*, 2004).

## 5. CONCLUSION & FUTURE PERSPECTIVES

Along the performance of this project many outcomes were collected. First, the vulnerability of A375 cell line to each one of the four PSs was verified with reliable results. It was also proved that these molecules presented optimized properties concerning the photodynamic ability and selective cytotoxicity to cancer cells. Their administration on a range of concentrations in A375 cells evidenced that decrease on metabolic activity is directly dependent on the concentration of PS administered, being also a consequence of the kind of cell death evidenced, the phase when cell cycle is stopped, the damages produced by ROS generation and impairment of critical intracellular structures as mitochondria. With such considerations, the considered therapy in strict relation with these four PSs is undoubtedly effective, presenting promising applications to treat such aggressive tumor with clear efficacy and diminished side effects.

To achieve a more complete description about the action mechanisms and possibilities for application of these four new molecules, some studies would be interesting to be performed such as comet assay, to evaluate DNA damage, WB regarding the possible presence of VEGF and Caspase-3, use of nanobodies to increase specificity to tumor cells, and subcellular localization of these PSs. In a further phase, in vivo studies would be essential to simulate the effects of these molecules in a living organism with possibility to transpose the achieved knowledge to clinical situations and improve the therapeutics in this illness.

# SECTION II

CURRICULAR INTERNSHIP

REPORT

## 6. COMMUNITY PHARMACY - AN OVERVIEW

The community pharmacy is nowadays a fascinating center of services that can be provided to the population. From the old pharmacies to the modern, sophisticated pharmacies, the national and international panorama evolved tremendously. The figure of the pharmacy and the pharmacist have trailed the needs of the populations and are now an extremely positive factor as far as the assistance to customers is concerned. Due to the inefficiency of the National Health Service, current customers often consider situations where nothing more is done than a diagnosis and a prescription in which little is explained more than the pathology that they suffer. The pharmacist is a highly qualified professional in the field of Health and, more specifically, in the subdomain of the medication. Equipped with astonishing technical and scientific abilities and a recognized professional independence, he presents himself as a public health agent and, consequently, is the professional who, due to all the logistical possibilities, is in greater contact with the regular custommers, thereby enabling them to convey confidence, security and clarification. Much more than a commercial perspective, a pharmacy is an establishment in which people who attend it feel as if they were in their own home. They entrust their daily lives to this health agents, respect the knowledge they offer, listen carefully to their advice and, more than that, recognize their profession, their talent and their workforce.

It is therefore in these basic pillars that the pharmacy of the future must be founded. For decades pharmacies served an unusual purpose, profit. The lack of deep knowledge in management coupled with the misfortune of economic crises conveyed the community pharmaceutical sector to a gap. Nevertheless, the process is reversible and, above all, it is necessary to reverse the progression through a path different from that which has led to such realities. It is time for pharmacies to commit themselves to truly providing fully functional services, to bet on the dynamism, to open horizons and to encourage their users to much more than the simple lifting of prescription or counseling. The community pharmacy space is a hub full of unexplored potentialities, waiting for a chance to thrive. It is up to the current and future pharmacists to take responsibility and change the current scenario that they experience. After attending a course such as the Integrated Master in Pharmaceutical Sciences, which is so full of rich and important training, we find ourselves fit, with the characteristics, skills and dynamics necessary to be able to prosper in this noble profession. The course opens doors to numerous opportunities, not only community pharmacy, and, once the legally obligatory internship is carried out in this final stretch, we get full notion of what awaits us in the job market, what expectations and fears. Faced with this critical reasoning, there is a need for change, revolution and momentum in daily efforts. And this requires skills, knowledge and,

above all, constant study, so that we can always be better professionals and dignify such an honorable vocation.

# 7. SWOT ANALYSIS

The model SWOT (Strengths, Weaknesses, Opportunities and Threats) analysis was applied in the current internship report. This method consists in the strategic analysis through basilar factors as strengths, weaknesses, opportunities and threats regarding an individual description about the experience concerning the curricular internship on community pharmacy. With such process, it is proposed to describe the application of theoretical knowledge on the daily labor and, also, new aptitudes acquired.

SWOT analysis comprehends two subcategories: internal analysis - strengths and weaknesses - and external analysis - opportunities and threats (Figure 27). For the first, it is intended to clarify the positive and negative aspects during all the course of the internship as a personal perspective based on the experience and own overview. For the latter, it is required to perform a critical description about the role played by the pharmacist in the current society as well as the opportunities that emerge from the curricular internship and the threats that arise in everyday life and may imperil the duties of the pharmacist.

# 8. STRENGHTS

## 8.1. Pleasant reception of trainees

The reception on the first day of my internship at Pharmacy Machado could not have been warmer. The entire technical team received me and immediately put me in touch with my duties. The feeling of unity and friendship from side to side allowed to me an easy integration and strong nature bonding with all professionals, which was undoubtedly a differentiating factor.

## 8.2. Appropriate number of employees

Pharmacy Machado is composed by four pharmacists, one pharmacy technician and a manager:

- Dr. João Santigo Maia - pharmacy technical director and proprietor;
- Dr. Graziela Grade - substitute pharmacist;
- Dr. Ana Gaspar - pharmacist;
- Dr. Rita Garrett - pharmacist;
- Dr. Maria João Santiago - manager

- Mr. Eduardo - pharmacy technician.

This team and the number of employees are appropriate to the pharmacy services. They are devoted to their jobs offering a tailored and rational service to the community, developing strong relations with the frequent customers, learning their behaviors and providing the best possible advice on each situation. They accomplish totally the role of the pharmacist in the community.

### 8.3. Location and facilities

Pharmacy Machado is located at Bernardo de Albuquerque Street, number 20 at Santo António dos Olivais, Coimbra. Due to its remarkable placement, this pharmacy holds a faithful group of customers which represent almost 85.0 % of this establishment revenue. Nevertheless, according to the nearby position to every single public service in the city, many other customers are welcomed in its modern installations, indicative of a pleasant and decent service. This pharmacy performs a schedule from Monday to Friday opening at 8am and closing at 9pm. At Saturday the schedule is shorter, starting its activity at 9am and concluding at 1pm. During Government established holidays the Pharmacy remains closed. Due to these advantages, the team must be always up-to-date to offer the best services to its customers. Pharmacy Machado is a very ancient establishment, up to 100 years old. Although this, it was continually subjected to reformulations of its space to keep pace with the evolving needs of its customers. The pharmacy area is relatively small. However, the products are arranged in an organized way so that it is easy to perceive at the entrance of the establishment where they are located. This is due to the constant rotation of linear shelfs so as not to generate a constant image of the interior of the pharmacy as well as the remarkable effort of all the professionals who work there and who put their best in the work they do.

### 8.4. Defined internship plan

The internship plan was neatly organized when I arrived at the pharmacy. The technical director maintains an up-to-date list with critical topics to be given to trainees and according to a specific order. First, I started to organize the medicines in the respective drawers of the shelf to be familiar with their trade names and their division according to the type of formulation. Procedures for the receipt of medicines as well as returns and stock adjustments followed. Afterwards, I began the public attendance, a much higher responsibility task, and in which the internship was exclusively oriented for a few weeks. In this period, I received internal training on how to cross-sell, advise on products, use SIFARMA2000® software and to revise prescriptions. After a period of about two months I was perfectly able to have work

independence and, therefore, I made daily rotations regarding the functions within the pharmacy. In short, I reiterate all the previously extant topics by stating that the internship plan has met my expectations and is highly suitable for a Pharmaceutical Sciences trainee.

### 8.5. Pharmaceutical counseling

Counseling is a fundamental act in the context of community pharmacy. Often overlooked, taken as a simple selling action, it is there that rests all the ability to capture the attention and confidence from a customer. During the counseling process, it is possible to generate proximity to the person that is on the other side of the service desk. Using the vast knowledge, typical of a pharmacist, the professional manages to capture the necessary initial attention, a fundamental step in the communication process. Then focus on the cause of concern or doubt, explaining the underlying pathology and treatment that was proposed by the doctor. It's time to go into more detail. Here all efforts are engrossed. It is extremely important that the user understand the usefulness of the medication and the dosage of the medication. It is important to ask the patient to repeat what was reported by the pharmacist to see if the information was well assimilated or not. It is in these small but enormous details that it is formed a part of a whole pharmaceutical act. In everyday life, we find ourselves with people in different favored states, with completely different health conditions and with disparate levels of education. Despite these vicissitudes they all have the right to equal treatment and, as such, it is up to the pharmaceutical professional to do so in its entirety, reiterating their competence, wisdom and, above all, human relations. It is extremely gratifying to receive a smile after a demand and knowing that at the end of each interaction there was a contribution on our part so that someone else's life might be a bit better, however small. But this is already meritorious. Professional ethics should always prevail over all prejudices and subjective opinions. After all this explanation, I can consider this the act with the greatest impact and, personally, of greater importance during my internship.

### 8.6. Electronic prescription

The electronic prescription became an innovation well embraced by pharmacists. It is much more profitable in every single way. It takes less resources, less time to analyze the prescription due to the uniformity of the font of the prescription, minimizes errors which could occur during the delivery of the medicine and the selection of the reimbursement program and allows the user to go to any pharmacy and take the medicine and amount desired within the limit of the period of validity and, of course, the prescribed amount. The advantages

and potential are immense. However, due to the technological basis that electronic prescription requires, a certain discomfort between some users is verified, who cannot fully understand the method according to which prescription works, since for the older population, *sensu lato*, for example, exists an added difficulty in handling mobile phones. The pharmacist also has a key role to play in this situation, since it serves the community as a public health agent and assists in clarifying the user's doubts.

### 8.7. Saturdays and service night

During my internship, I attended almost all the Saturdays and every service nights. These periods are fulfilled of different situations, many times far different from daily routine. The attendances mainly cover situations from people who came from the near Hospital, or another circumstance as emergency contraception or failure of critical medicines. It is the role of pharmacist to provide to these customers a tranquilizing explanation of the situation, clarifying prescription if some breaches are present and even advise more perceptively. Such periods are an asset for trainees in the way they can contact a wider range of conditions, providing them a better know-how on difficult tasks.

## 9. WEAKNESSES

### 9.1. Pharmacy workspace

Pharmacy Machado is a very pleasant space, with attractive colors, good disposure of products and a family atmosphere. Despite this, the pharmacy area is too small, revealing some problems when the number of customers entering the space ascends. To feed the flow inside the establishment a single door is available, which is difficult to conciliate when customers are going in and out and medicines providers are arriving. There are five desks for attendance and two cozy chairs in the hall for those who may want to take a short rest. Behind the desks the seasonal medicines, veterinary products, vitamins, dietetic products and oral hygiene products can be found. The cosmetics, childcare products and contraceptives are placed at disposal of the customers.

Recently, by the past month of March, INFARMED gave to Pharmacy Machado the allowance to start the procedures to relocate into a larger space, which will allow a better gathering of all the offered services.

### 9.2. Lack of information for external training

Further updates are critical for the pharmacist everyday, either by their need to be aware of the last offers or by an indirect requirement of their customers. This kind of events also allows the entanglement between professionals and discussion of prominent issues related with the products that make the quotidian of pharmacies. During my internship, I only attended one external training session. In my opinion, it is insufficient because these sorts of events permit to achieve a varied knowledge in less explorer fields such as medical devices, childcare products and to be updated also in matters as cosmetics and vitaminic supplements and, for interns as I was, it is a considerable help.

### 9.3. Initial barriers and difficulties

Due to an extracurricular internship at Pharmacy Machado that I realized in the period of July of 2016, I knew the employees and the internal organization of this establishment. Everyone on this team helped me and gave me strengths to continue this everyday work and, so, I improved my self-confidence. The lack of information about commercial medicines names is one concerning aspect because all the customers ask for it, once it is easier to remember than the active substance name. The cross-sells and up-sells were a difficult point at the beginning due to the lack of practice in relating products that can complement each other and also due to a certain difficulty in persuade the customer, respectively. The software SIFARMA2000® is somehow intuitive and the anterior experience gave me enough knowledge to be aware of some tools. However, I recognize that the short formation given at faculty is not even enough and practical classes with this software should be implemented.

## 10. OPPORTUNITIES

### 10.1. Application and reinforcement of academic knowledge

The presence on the real context of a pharmacy is a tremendous responsibility. As a future pharmacist, one must be always aware of his duties and know to integrate the learned knowledge in the daily situations. The pharmacist is, proudly, one of the professionals in the Health field with the most complete academic achievements. Because of this, it is much easier to arbitrate on everyday situations and to correlate the learnings at faculty with the practical necessities. Along my internship I started with frequent questions about all the processes which concerned the counselling, retails, medication inspection and evolved until I reached a state of complete independence. With practice, I could reinforce my knowledge and apply it to people requirements, contributing to their better life quality - the main goal.

### 10.2. Developed skills

Interaction with friends and family is evolved in a shell of confidence and amiability. It is very different when one stands before a customer, with a respectable posture and an impeccable flow of work. All of this are required skills to succeed in a pharmacy. I do not consider myself as an introverted person. I really appreciate to socialize, to talk and relate with others. During my internship, I developed even more these abilities, when trying to make someone perceive a difficult information, when inferring about an issue, trying to use the systematic and logic reasoning, or even when someone was with an unsettled state-of-mind and trying to calm down the situation, helping the person to relax, demonstrating sympathy and kindness. I have undoubtedly improved my skills at a daily stride, achieving a level of total confidence in my abilities, being able to interact with extreme ease with customers, being at the level of their demands and always presenting an impeccable and honorable posture of the profession. It was a privilege to be taught at Pharmacy Machado.

### 10.3. Ongoing instruction

The daily routine often sets up a barrier to updates in several areas related to Health, especially around medicines. It is imperative that the pharmaceutical professional keeps up to date to always accomplish the expectations of his customers and satisfy them. During my internship, I could attend various training sessions on cosmetic products, deepening and acquiring knowledge in this area that I had not known until then. I also took a daily reading of books related to my daily exercise, such as pharmacology and physiology books. I also kept me updated at the INFARMED page daily, to be abreast of constant changes made to therapeutic indications of medicinal products, their side effects and, still, circulating withdrawal orders from the market of certain products. I would therefore reiterate that these opportunities were added values to my curricular development.

## 11. THREATS

### 11.1. Portugal and its current economic *status quo*

Portugal is a very ancient country which experienced a lot of cultural and social revolutions. Through the centuries, the Portuguese people could redesign themselves to better fit the requirements of society and attend the development of the overwhelming world around. Nevertheless, the 2008 worldwide crisis hit this nation by means of its deepest structure leading to a financial, economic and social massive quake that affected all under-systems ranging from education to health care. The pharmaceutical sector was one of these damaged organizations which felt indeed on its existence a vast, penurious and disconcerting rhombus,

particularly in matters of community pharmacy. In this field, many pharmacies which were accustomed to keep poor internal financial balances and, in parallel, relevant amounts of debts to their product-providers and to financial institutions (banks), suddenly faced a tragic fate: insolvency, bankruptcy and collapse. More than six hundred pharmacies were, since 2008, in this precarious situation. Such reality lead to disastrous consequences starting from pending payments to every entity that furnished pharmacies with financial supplements and products, going to a reduced number of employed pharmacists, due to the increase in firing and fewer hiring, and a declining on the salary amount which also induces a decrease on the appeal for this career. But this constitutes a small part of the hole critical mass. On the past '90 decade, the world observed an explosion of the pharmaceutical market with exponential number of patents being registered and a multitude of therapeutic needs being fulfilled. Due to patent lifetime, now, over 20 years passed, we have been assisting to an increasing number of medicines with expiring exclusive license, giving rise to what are called the generic medicines. The latter are State-reimbursed and, in consequence of the freedom of production by any pharmaceutical industry who possesses a Marketing Authorization, their prices are much lower than the originals. Hence, the overall feed which every single pharmacy receives for sold medicine, is scanty, and so the earnings. During my curricular internship, this situation was very clear to me in everyday situations. The stock of pharmacy was kept in the lower volume as possible regarding the prevention of an accumulated value in slow-selling-medicines. Related with this, many pharmacy customers shall return in the same day or the next days to achieve the product which was readily ordered to the provider or even to the laboratory. Other situations can be reported such as products out of market due to cessation of production by industry because of a low ratio earning/price of production. But something even more important is in discussion - with the reduced economical capacity of Portugal, the incomes for the retired people were also lowered and so their capacity to buy simple but essential things as their monthly medicines. Afterward, those people try to get only the "more essential" products to sustain their health on a steady-state but, unfortunately, are not enough and, without complying with the prescription results become visible. This represents a decrease on the degree of therapeutic outcomes and, so, a decline on the health level of those persons. With this, many of them end again on the assistance of medical doctors trying to reestablish the therapeutic or adjusting it to supported levels by their owners but, then, with irreversible conditions. In these situations, the State must dispend more money to assure life-sustaining conditions, contributing this to a more expensive situation. A disturbing cyclic situation.

### 11.2. Homeopathy - the dark side of medicines

Should or should I not be pleased to introduce such extraordinary, fascinate and mind-depleted issue? This was the questions which I have debated with myself for a long time until I grasp the conclusion - the true must always triumph! Is it a singular notice (is it not?) that after almost 200 years of atomic theory development, a simple proposal shall abrogate the conclusions of so many brilliant minds which worked hardly for achieving new statements on what were the believes without fundament that Humanity possessed. But the facts are in front of us. Today we live in a century, in a world holding human beings who claims for a miraculous medicine - homeopathy. A healthy, "naturally-based" and without chemicals medicine that can absorb all kinds of evil that may cause sickness. Better! This medicine takes almost nothing to be prepared. Just a scarce protocol that child could also perform. Imagine this…you take a natural extract from a plant. You have a cup of water and place 1 μL of that wonderful extract and complete the volume with 1 L of water. Shake it well and then take another 1 μL from that dilution and perform the same procedure more 20 times. Finally, take 1 μL of the remaining solution, add 1 L of water and you have it! A "fantastic" medicine which you can give to anyone to juice and assure, that whatever kind of disease that person may possess, it will be treated and cured! What sublimity, what passion in such formulation. It is only a pity that the described scheme is part of a gigantic and obscene scheme to obliviate the scientific truth from the vision of those who really need help, inducing them to dispend their money on worthless products, giving them a false hope and may even contributing to worsen their state. Recently, on March of 2015, a press article published on *The Guardian* gave a final hit to a logical assumption: there is no evidence for the efficacy or even safety related to homeopathy. Resulting from the analysis of two hundred and thirty published clinical reports, that was the conclusion more reliable that ever came in that matter. Conducted trials demonstrated a tremendous failure trying to prove that homeopathy is more than a placebo and, worst, was verified that these astonishing preparations can be hazardous. So far, is with sadness that I state that a considerable number of this ambush is still requested at pharmacy. People are committed to buy medication which is advised by their pharmacist by having confidence in that is much more safe, cheap and the results are incredibly better. What a false statement! When economic arguments are placed first, instead of true facts, in such delicate issues, we can contemplate a dark behavior of what should be a right conduct of health professionals and, *stricto sensu*, pharmacists. Ethics are frequently not held in consideration when handle this kind of matter due to some arguments of metallic sort (read money) which are raised in contemplation above everything else. Nonetheless, corruption must be thwarted by every one of us and situations

like these censured to prevent further traps. At last, in my humble opinion, the pharmaceutical market shall duel between what is, ethically and scientifically, right and the most appealing argument, money, which should never prevail over the former, once the critical need is the well-being of diseased people and, between all these concerns, the pharmacist reputation.

### 11.3. Academic milieu *vs* daily work environment

The Academy is, today, a multifocal center of knowledge. Universities are seized and recognized as the source of an integral formation that ensure future professionals ready to perform a multitude of tasks in a determined area. Nonetheless, nowadays society requires much more than that. A fresh graduated from a University must be able to achieve another purpose: be in the intersection between Sciences and Humanities. Today, the necessities are ranked much higher than ten years ago and, hence, professionals shall demonstrate capacity and plasticity to constantly adapt to new circumstances. A bachelor degree gives to its owner the capacity and freedom to study by himself, without a teaching silhouette around. Although this, the knowledge ministered at college must be carefully selected and transmitted according to what should be the best pedagogical rules and the primary necessities when entering the labor market. Nevertheless, when finished the academic course, pharmacists face a different reality in which they feel a lack of knowledge in certain aspects that are fundamental on the daily community pharmacy environment as Phytotherapy, Dermopharmacy and Cosmetics, Medical devices, Childcare and even on certain informatic skills as the software SIFARMA2000®. Frequently, students and graduated professional are committed to frequent postgraduate courses to complement their academic formation. Holding such reality, I realize that it is urgent to change some critical aspects on the curricula of Integrated Master in Pharmaceutical Sciences course as the introduction of curricular internships during the course, starting immediately after the second year of it, regarding a better consolidation of the learnings and a much more sophisticated capacity to react to pharmacists' everyday dilemmas. In the same line of thought, should also be reformulated the program of some disciplines which are fundamental and ought transmit much more knowledge to the students than what is really transmitted. At last, I denote that students themselves shall also be more independent, creative and search more detailed sources of information than only the contents of classes. Only with continuous study a skilled professional can arise and stand. And that is imperative once pharmaceutical profession must be honored in every act.

### 11.4. Over-the-counter medicines - a spreading truth outside the pharmacies

The incessant creation of legislation to assure a much more reliable and safer system of marketing medicines leads to innumerous variations throughout the last decades on the perspective of society about this activity. Because of intensive scientific, as basic as clinical, research, many drugs are now considered safe enough to be settled in the supermarket rack, free to be taken by any customer. At the first glance, it seems interesting because it opens the possibility to market medicines not only in pharmacies. Yet, a deep glance with much more careful makes to emerge a bleak outlook. People at stores buy medications without any kind of advice because there are any professional with correct background formation to help them to solve their doubts. Therefore, the choice goes to the option which is conducted by "heard by others or friends" or "read somewhere in the internet or social networks" or even "heard by someone who took and experienced amazing results". It is not necessary to explain how dangerous and harmful can be such choices. Each person is an individual being with specific characteristics and medicines, as well as their doses, shall be adapted according to this. This point is clear. But the problem goes even further. The pharmacist is the "professional of the medicine" and, so, it falls on him the responsibility to advice the right prescription, the right medication (free of prescription) for a certain illness and often to relate to physician some mistaken prescriptions. Due to this, medicines should only be present at the proper place for their selling, and that is where the pharmacist is, at pharmacy. The described reality of over-the-count medicines out of pharmacy is a crescent danger for populations and even an unfair way of competing with pharmacies, which lose customers and gains due to the competitive prices offered by supermarkets. However, it is necessary to sensitize people to the setback that this reality represents and inform them that the difference in price does not pay the disastrous consequences that can come from such a choice. It is necessary to pharmacists to impose!

# 12. CLINICAL CASES

## 12.1. Case number 1 - Emergency Contraception

FB, 19 years old, went to the pharmacy and request emergency contraception. At that moment, I asked the patient about the period of sexual intercourse, if she used any contraceptive method, whether she was taking any medication or if she had any health problem, and if she had ever taken emergency oral contraception. Following these questions, the answers have all a negative nature, and indicated that the relationship would have occurred about 3 days ago. The patient correspondingly pointed out, after a question from me, that the last menstruation would have occurred about two weeks ago. Through this information I could conclude that the patient would be near the end of the fertile period and that normal

emergency contraception could not have an effect. In this way, I gave ellaOne® (30 mg of ulipristal acetate) and informed the patient that it should be taken immediately and about possible side effects such as nausea, vomiting and future menstrual changes. I also indicated that in the event of vomiting or diarrhea occurring within 3 hours, the effectiveness of emergency contraception would be impaired, so it would be necessary to take another pill at the time. I also informed the patient about the need to use contraception during sexual intercourse, and the emergency contraception is not at any moment an alternative to the above, and does not even protect against sexually transmitted diseases. In order to avoid future similar situations, the patient was counselled to go to a medical professional with the intention of initiating oral hormonal contraception, and during the rest of the period between emergency contraception and the onset of future contraception she would not be protected and the use of contraceptive methods would be necessary.

### 12.2. Case number 2 - Hair Lices

RS, father of a 10 years-old child, went to the pharmacy, a little desperately, and questioned about what might have been a complaint from his son for a week already. He reported that his son had a lot of itching and that he was not sure about the origin but he thought it might be due to lice because he looked at the scalp and checked for white spots that would almost certainly be lice and nits. After requesting confirmation, I put on gloves, put the child in an appropriate place and analyzed the appearance of the scalp. The suspicions were confirmed. In the case, I recommended to the father the product Quitoso®, constituted with the base of permethrin to 1.0 %. Following the indication of the product I explained how to use it to be effective: first shake the package, after that should cover the scalp and the hair itself with the product, including the back of the ear, having That should not contact with the child's eyes. The scalp is then massaged for a period of up to 5 minutes for moistening the hair throughout its length. After a working period of about 10 minutes the product is removed with the aid of abundant water. After this, the scalp is then washed with a shampoo to avoid irritations of the same. I have pointed out that care with hand hygiene after this procedure is fundamental since it is a factor that promotes parasitizes. I also indicated the use of a comb made up of fine teeth to remove dead parasites, an operation that should be performed with damp hair and, once again, across the length of hair. The combs should be disinfected by immersing them in hot water (temperature above 50 °C) for at least 5 minutes. The procedure for application of the product and subsequent passage of the comb should be repeated within 8 days, if necessary.

I also paid special attention to school friends, family members and people with whom I

contacted them soon, to verify if they are already infected with the parasite and, if they are not, they can prevent it. I warned that it would be advisable to disinfect clothes with which the infant contacts via washing at temperatures above 60 °C or in contact with a disinfectant solution in a plastic bag for a period of two weeks and so that there is no sharing of Objects that the child uses, namely those that are more susceptible such as combs, cushions, clothes and props.

# SECTION III

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# 14. APPENDIX

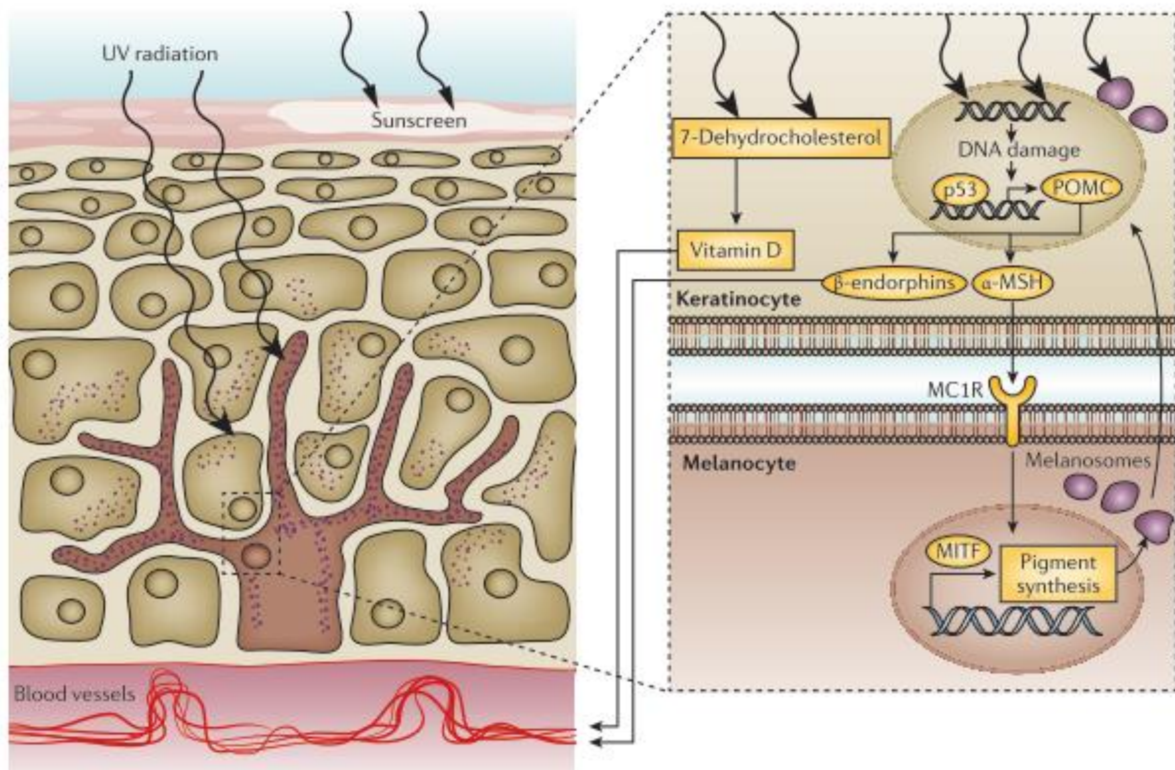



**Figure 1.** UV radiation and its interaction with skin structures: the role of vitamin D and tanning on the prevention of carcinogenesis process. (Schadendorf *et al.*, 2015)

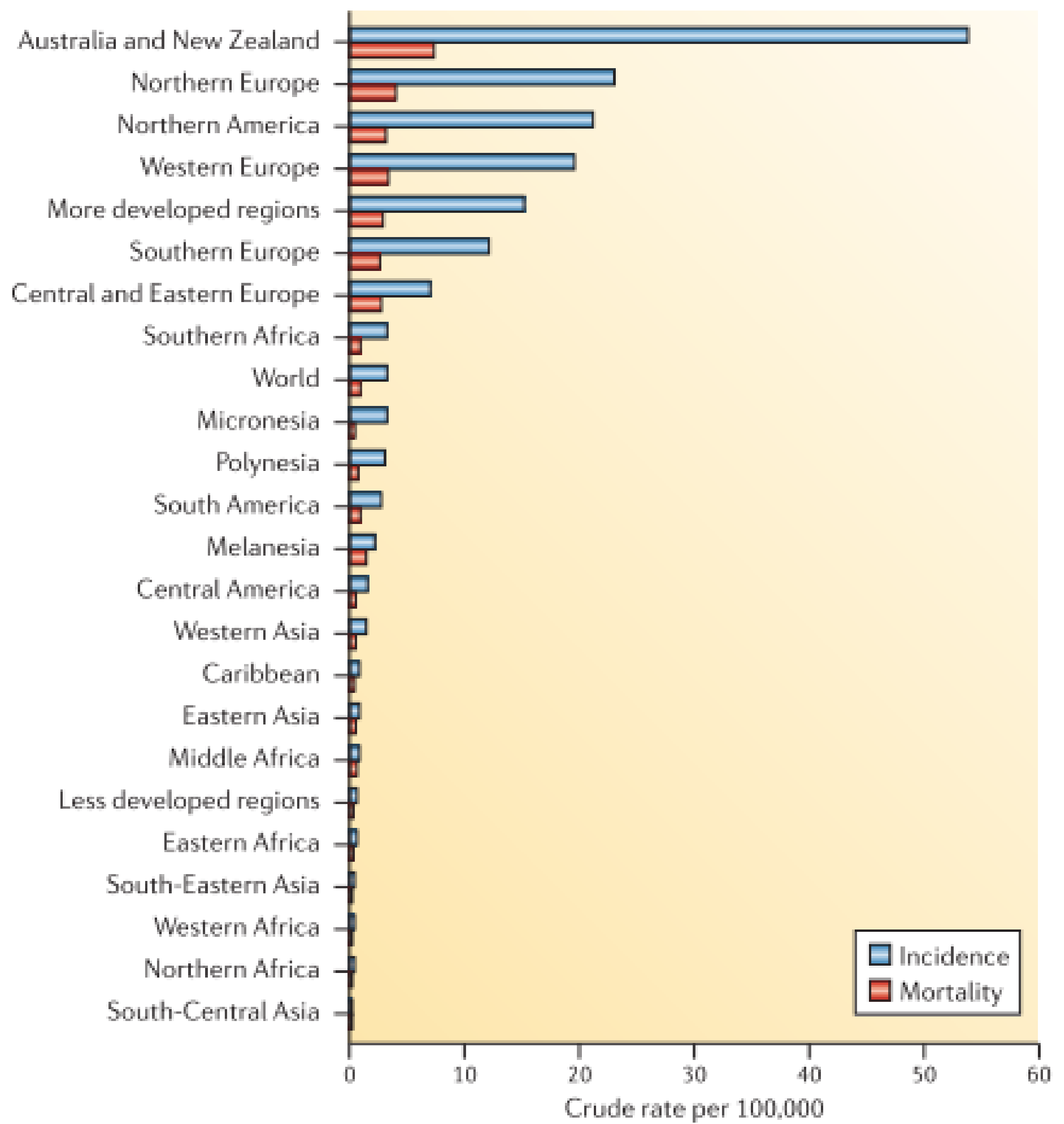



**Figure 2.** Cutaneous melanoma: mortality and incidence. (Schadendorf *et al.*, 2015)

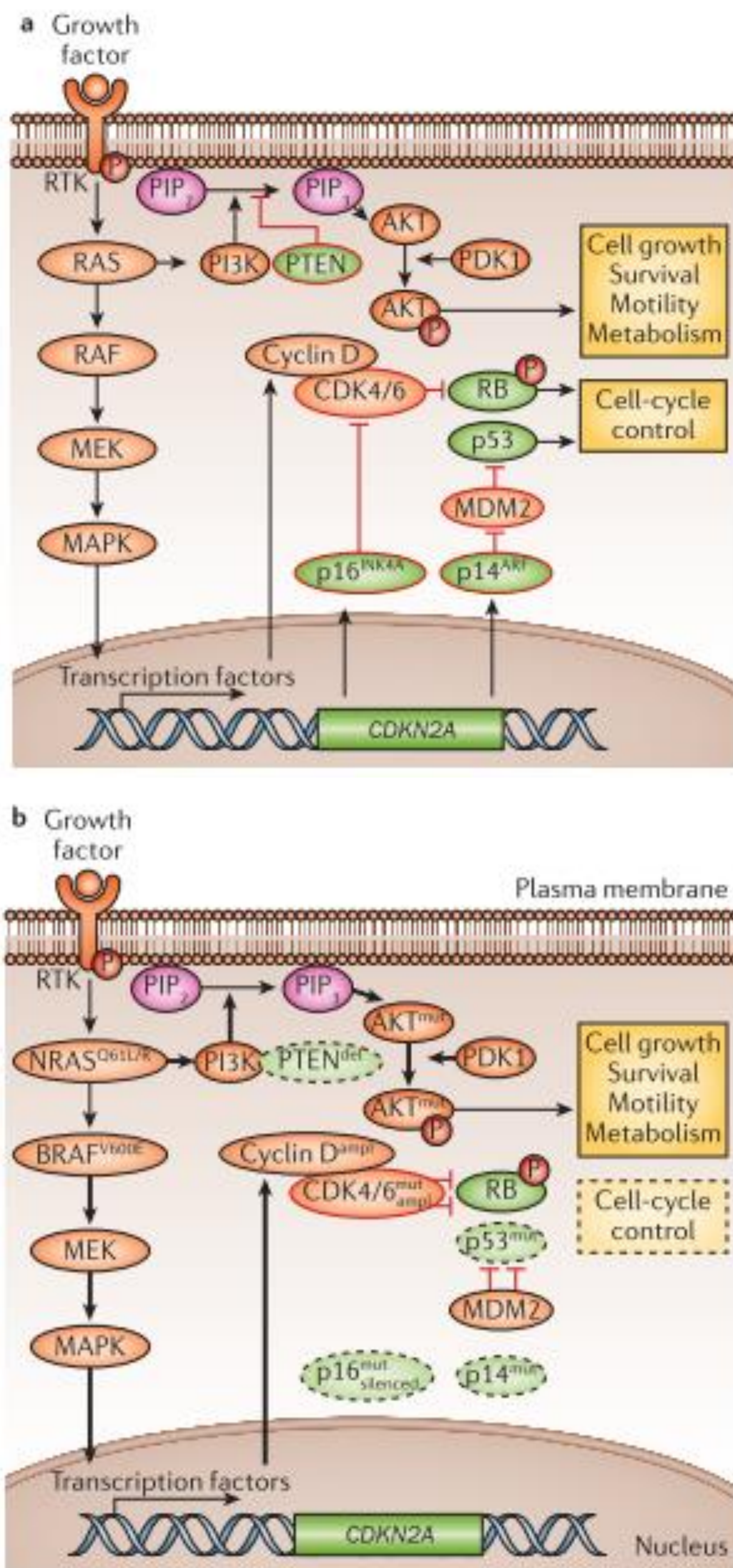



**Figure 3.** Signaling pathways: (a) normal conditions; (b) melanoma. (Schadendorf *et al.*, 2015)

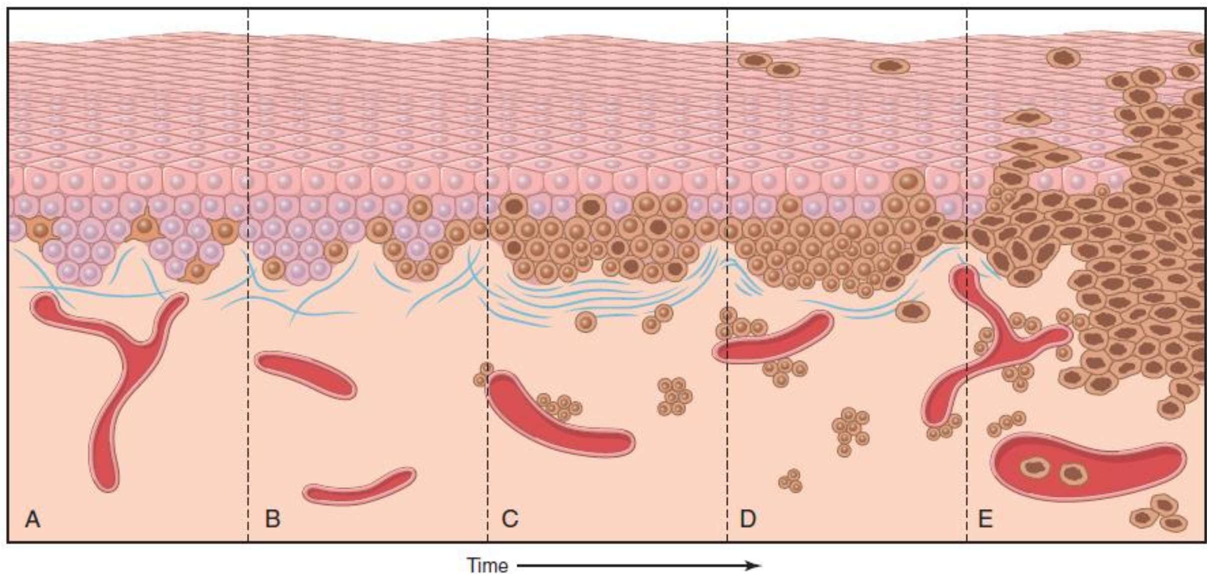



**Figure 4.** Stages of melanoma progress. **A,** Regular skin presenting dispersed melanocytes. **B,** Lentiginous melanocytic hyperplasia. **C,** Lentiginous nevus presenting erroneous shape. **D,** Radial growth phase of melanoma. **E,** Vertical growth phase of melanoma demonstrating metastatic features. (Kumar, Vinay; K.Abbas, Abul; C. Aster, 2013)

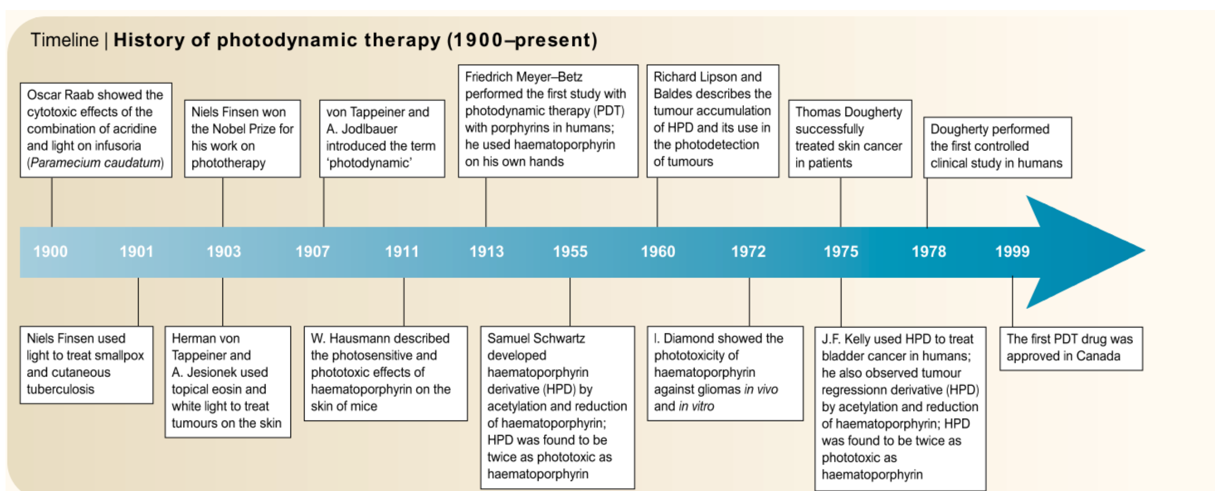



**Figure 5.** An overview of the most important developments in Photodynamic Therapy during the past century. (Felsher, 2003)

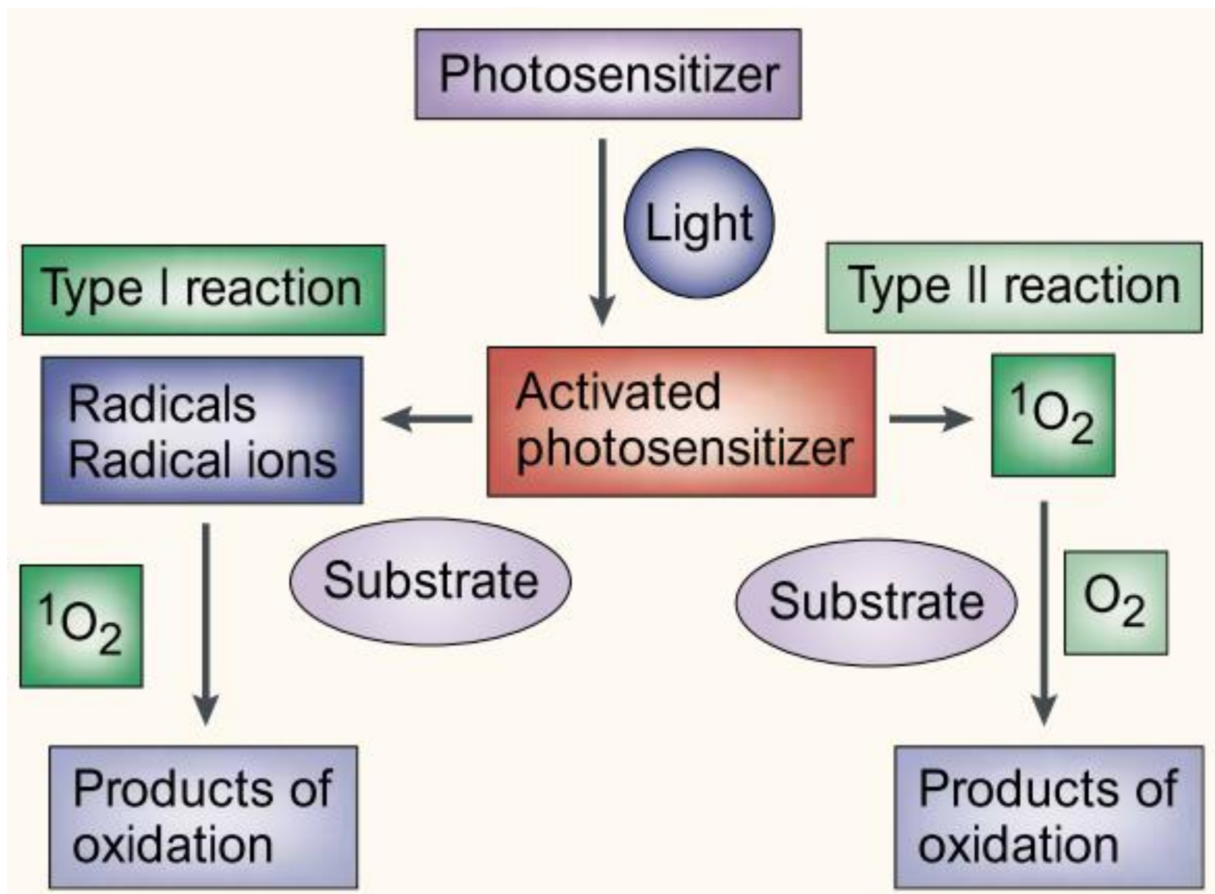



**Figure 6.** Type I and type II reactions during PDT. (Felsher, 2003)

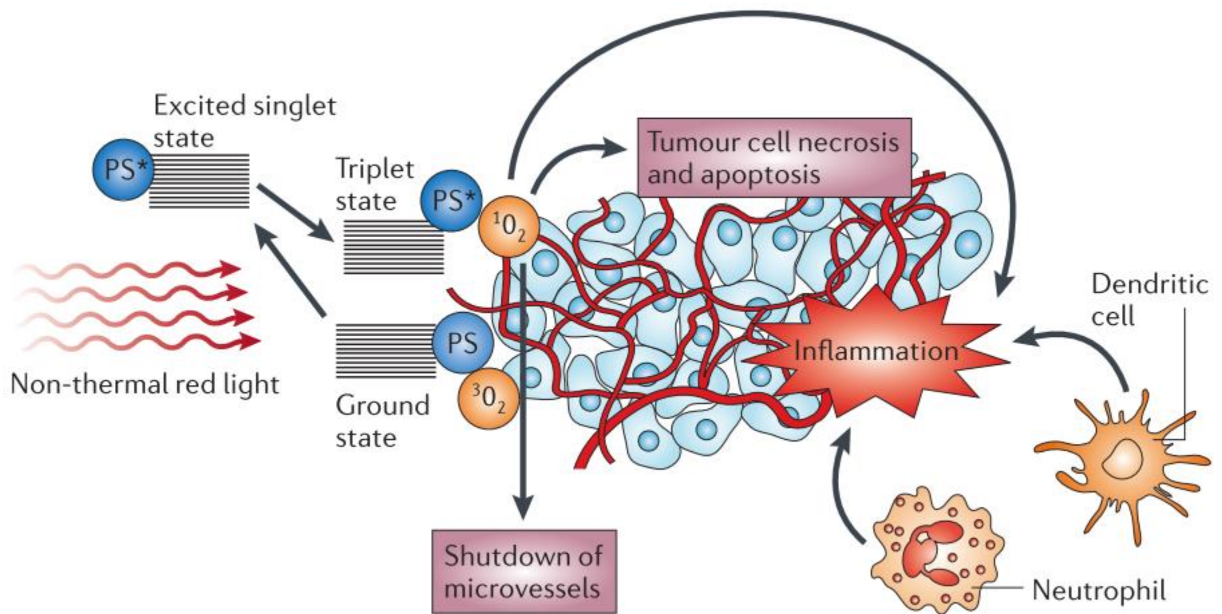



**Figure 7.** PDT background mechanisms and targets. (Castano, Mroz e Hamblin, 2006)

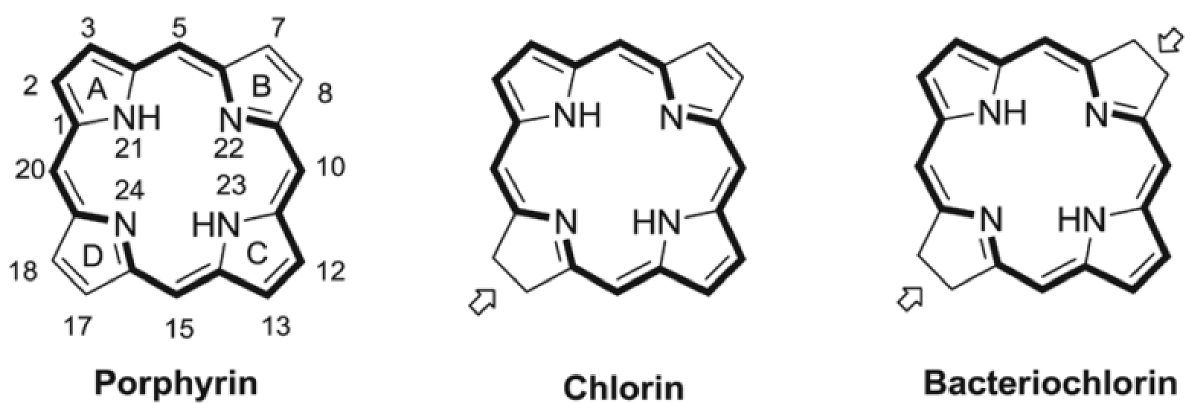



**Figure 8.** Structures of porphyrin, chlorin and bacteriochlorin. (Yoon, Li e Shim, 2013)

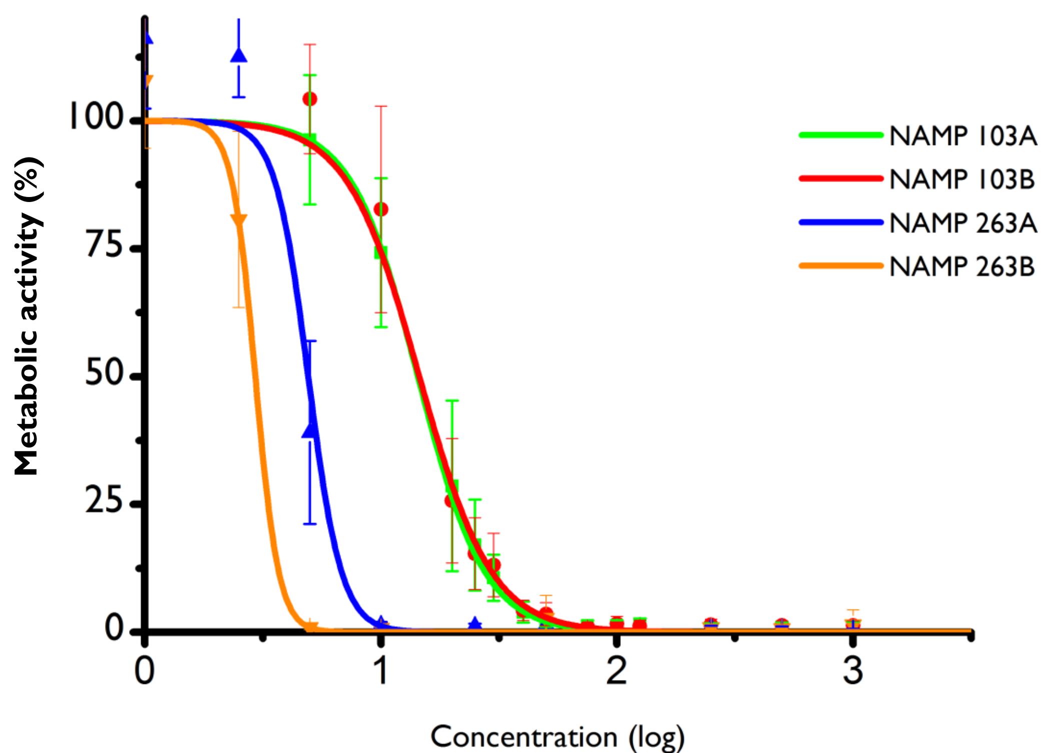



**Figure 9.** Metabolic activity on human melanoma A375 cells after PDT with PS in a set of concentrations ranging from 5 nM to 5 μM. The logarithmic curve was settled using Origin®, on a sigmoidal approach, to obtain the $IC_{50}$. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate.

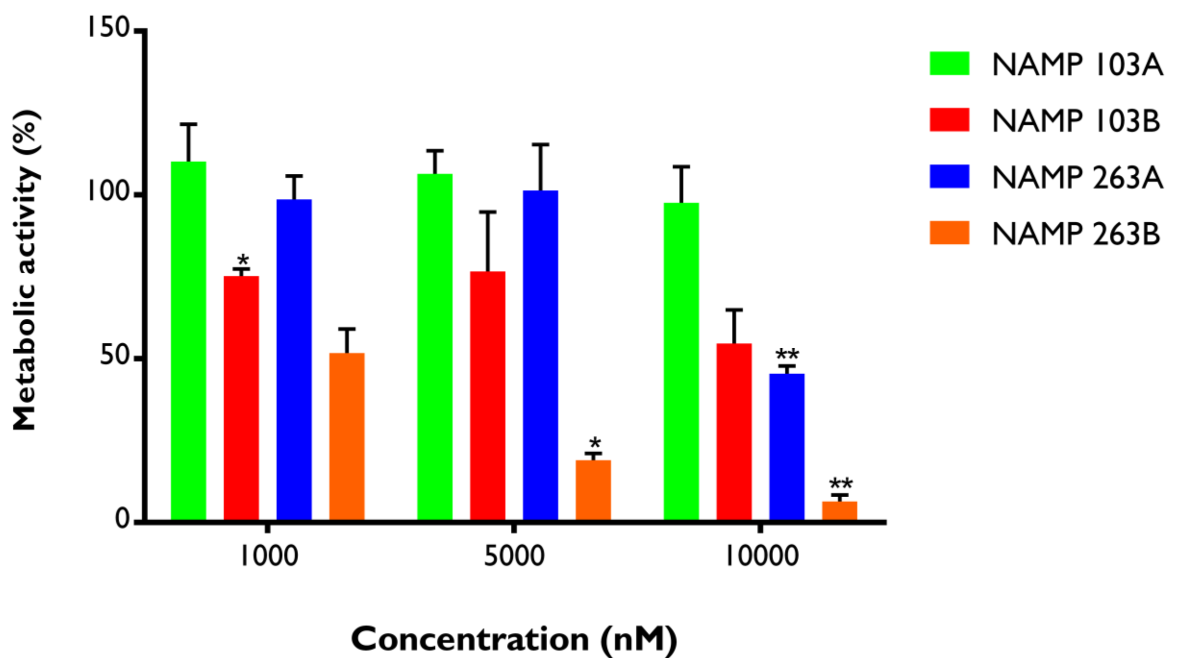



**Figure 10.** Metabolic activity on human melanoma A375 cells after exposure to the PS concentrations of 1 μM, 5 μM and 10 μM (without light exposure). The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

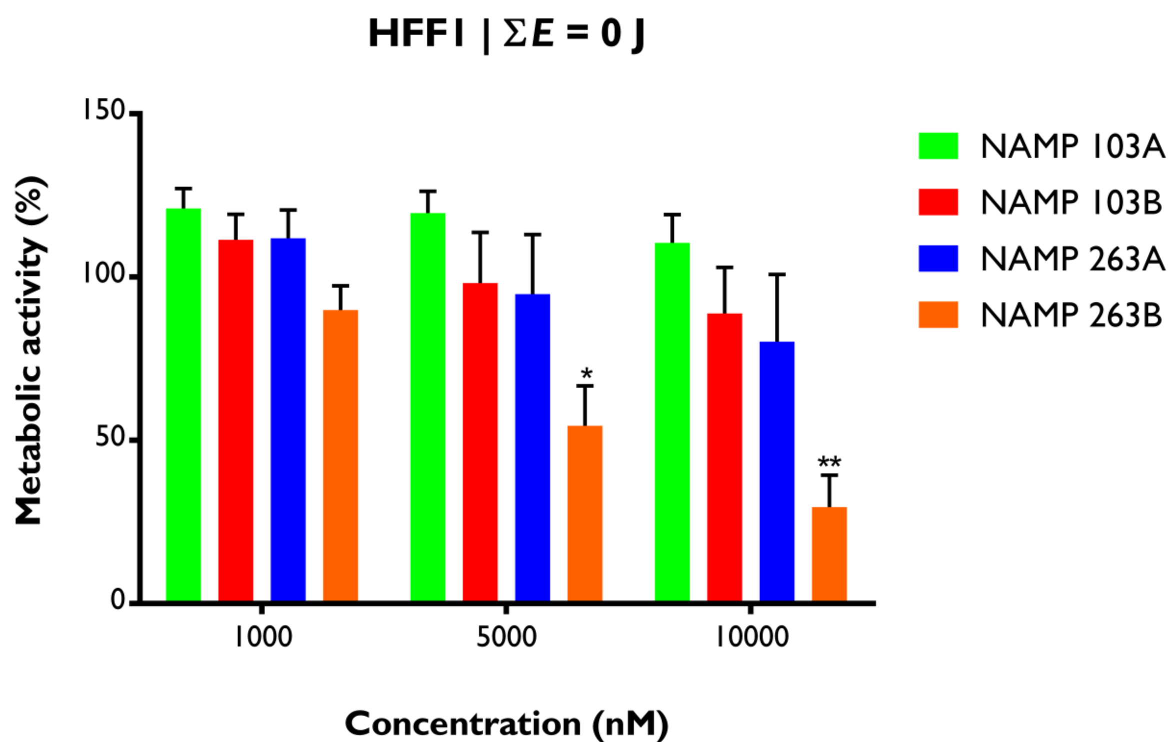



**Figure 11.** Metabolic activity on human foreskin fibroblast HFF1 cells after exposure to PS concentrations of 1 μM, 5 μM and 10 μM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

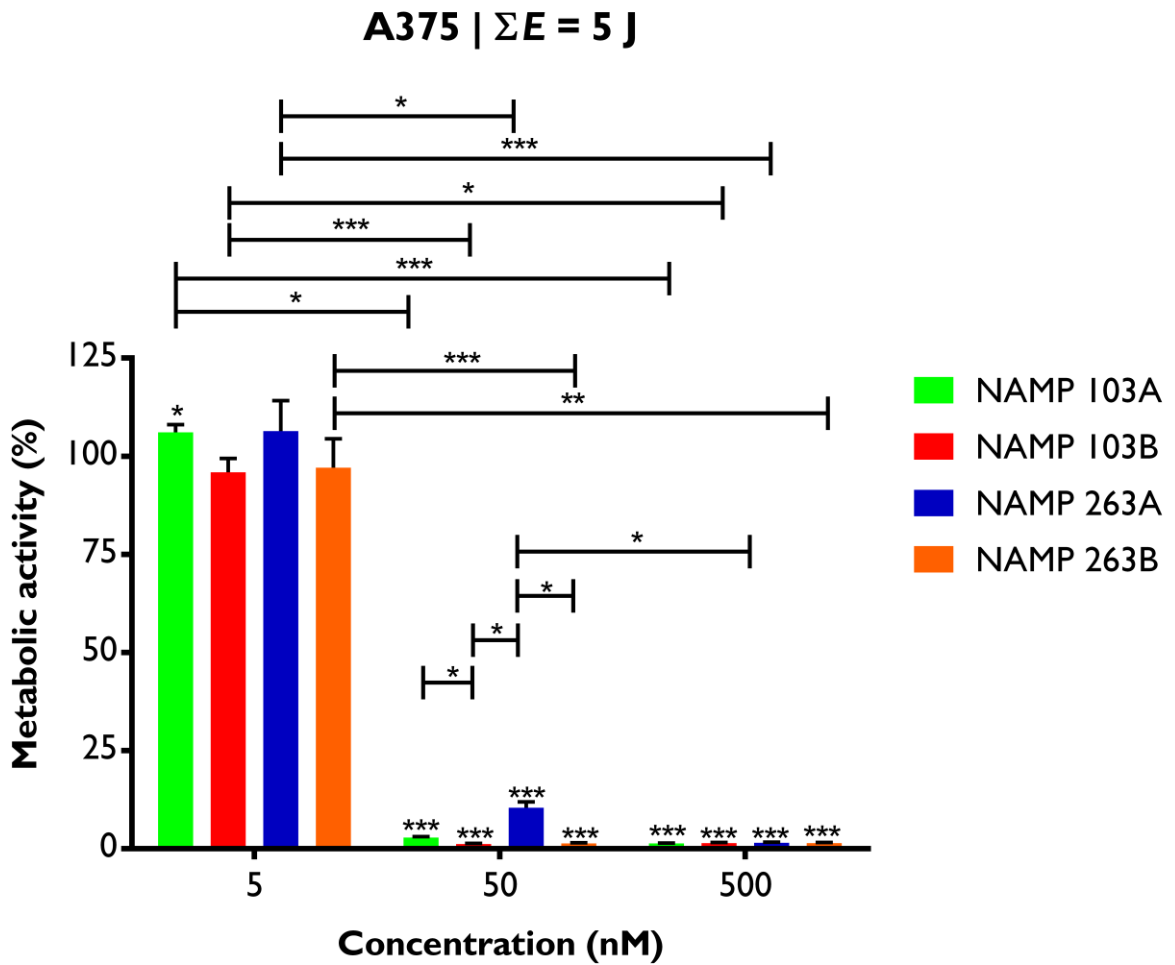



**Figure 12.** Metabolic activity on A375 cells after PDT with PS concentrations of 5 nM, 50 nM and 500 nM. Accumulated energy was 5 J. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

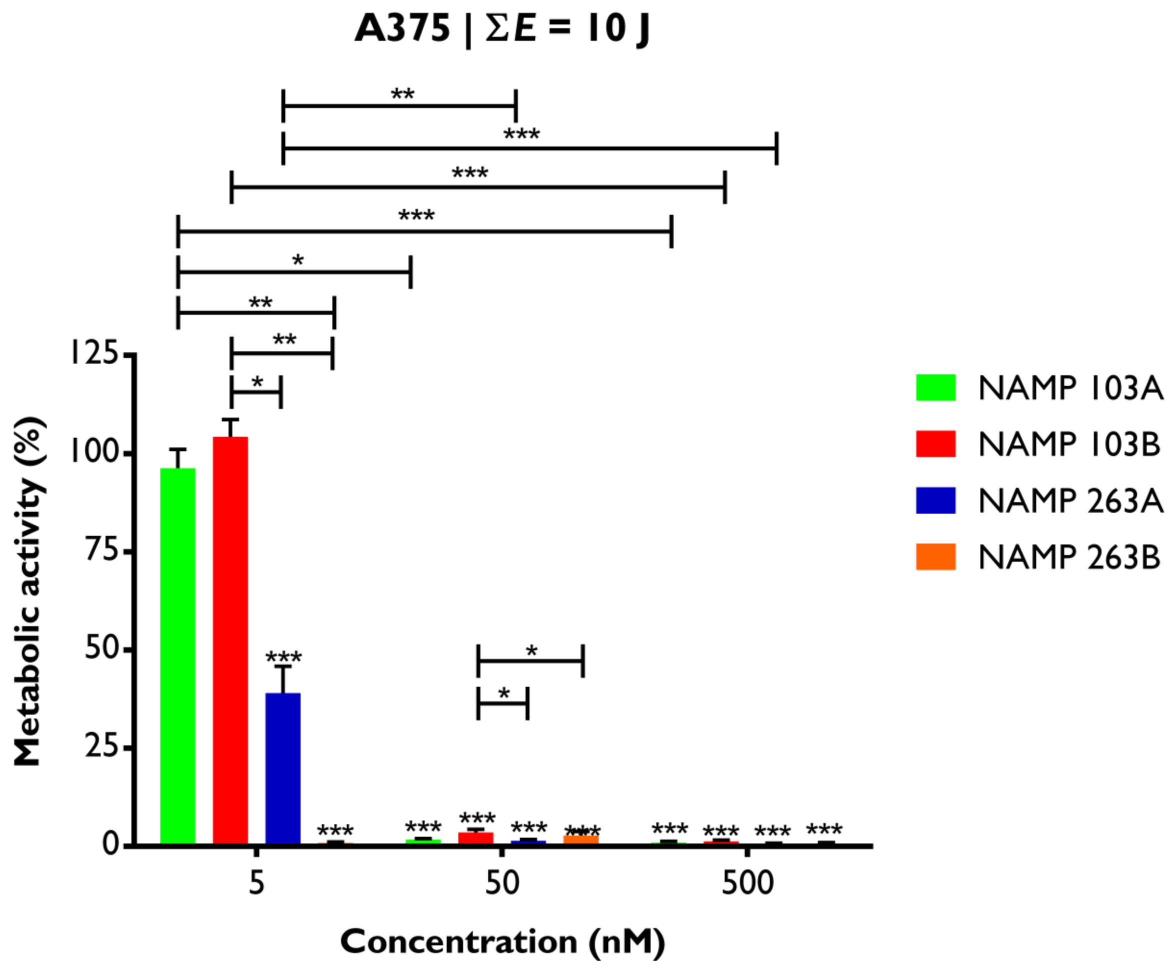



**Figure 13.** Metabolic activity on A375 cells after PDT with PS concentrations of 5 nM, 50 nM and 500 nM. Accumulated energy was 10 J. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

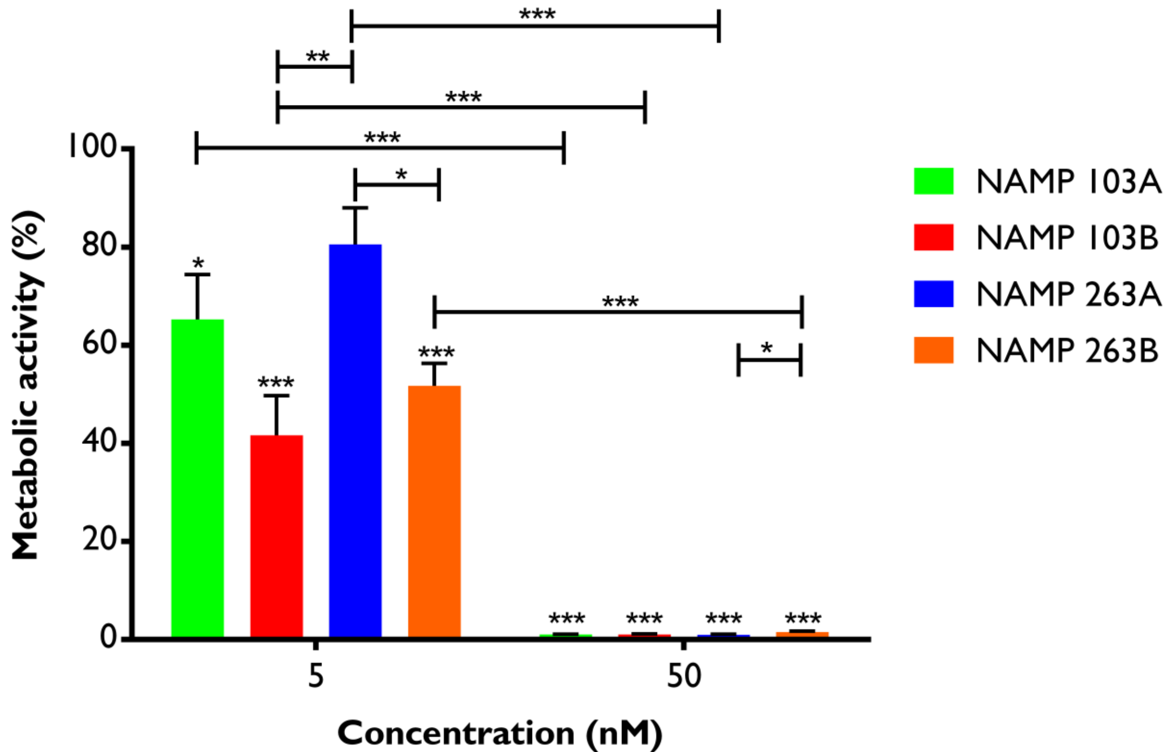



**Figure 14.** Metabolic activity on A375 cells after PDT with PS concentrations of 5 nM, 50 nM and 500 nM. Accumulated energy was 20 J. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

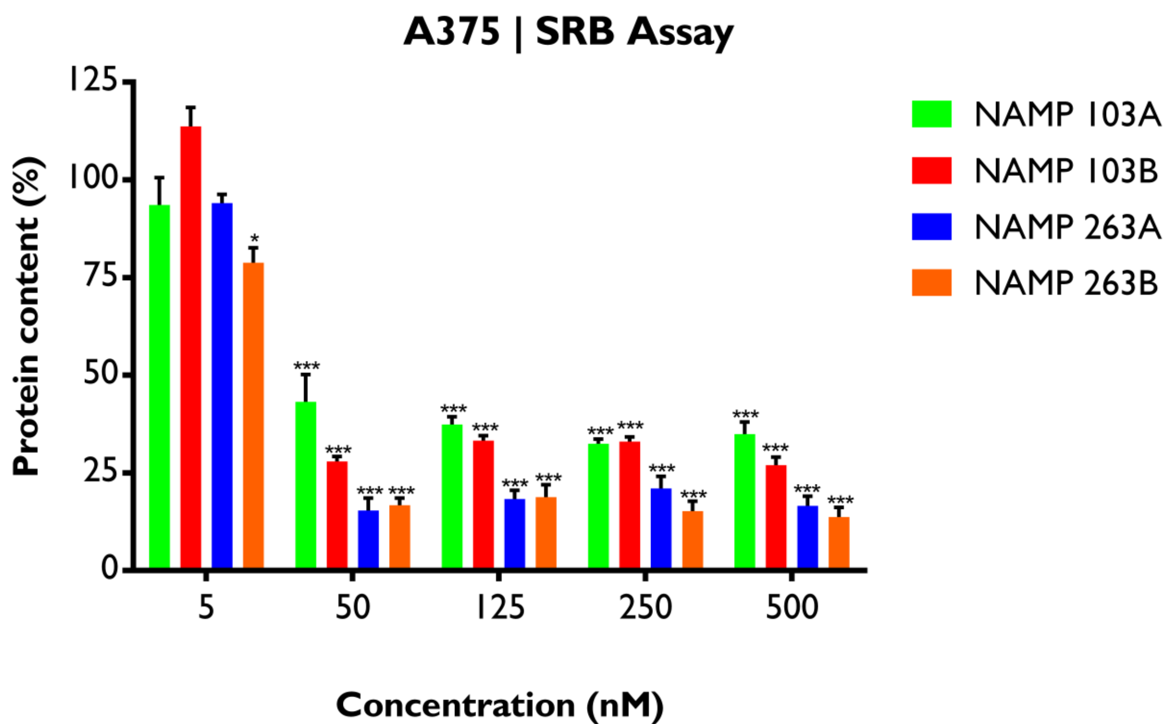
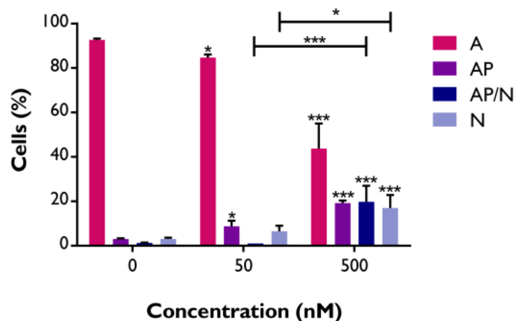
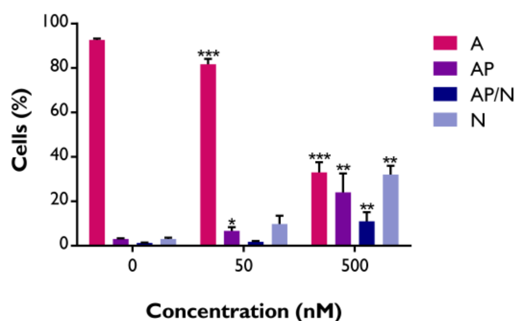
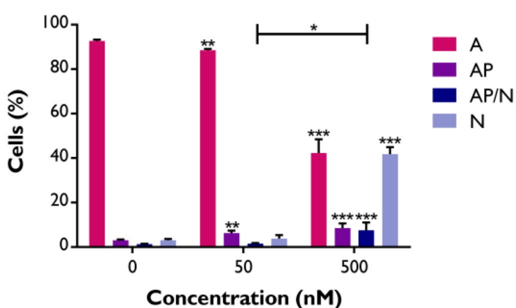



**Figure 15.** Cell viability on human melanoma A375 cells after PDT treatment with PS concentrations of 5 nM, 50 nM, 125 nM, 250 nM and 500 nM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.



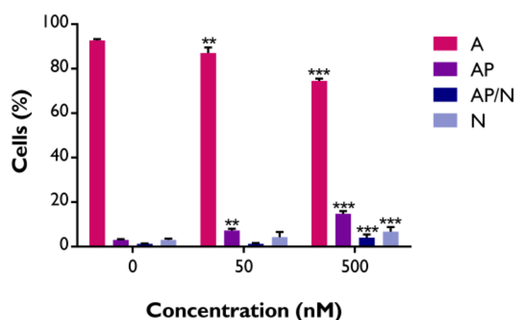
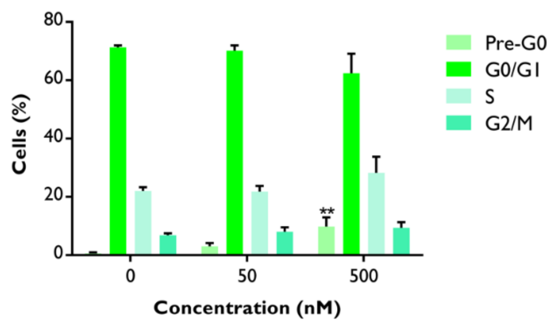
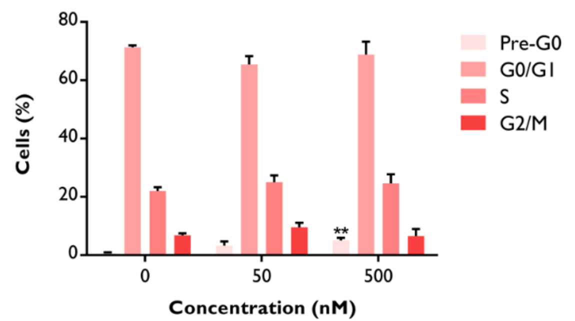
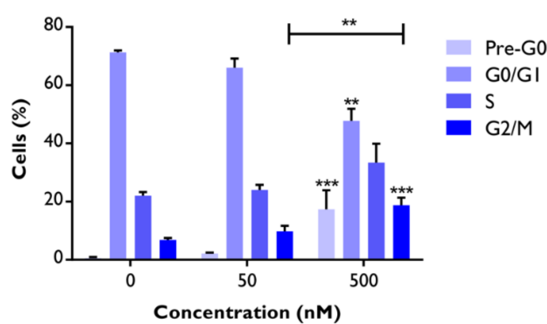




**Figure 16.** Cell death on A375 cells after PDT treatment with PS concentrations of 50 nM and 500 nM. The values on graphics represent the percentage of cells alive, in apoptosis, in late apoptosis/necrosis and in necrosis. The results presented match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.







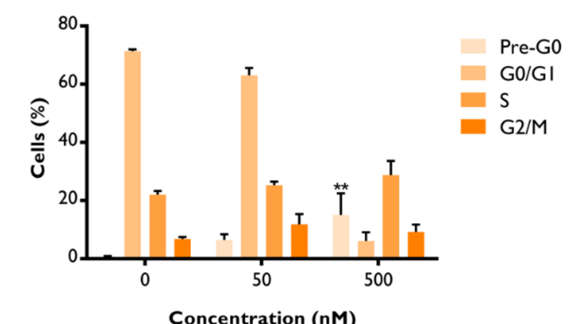



**Figure 17.** Cell cycle of A375 cells after PDT treatment with PS concentrations of 50 nM and 500 nM. The results presented match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

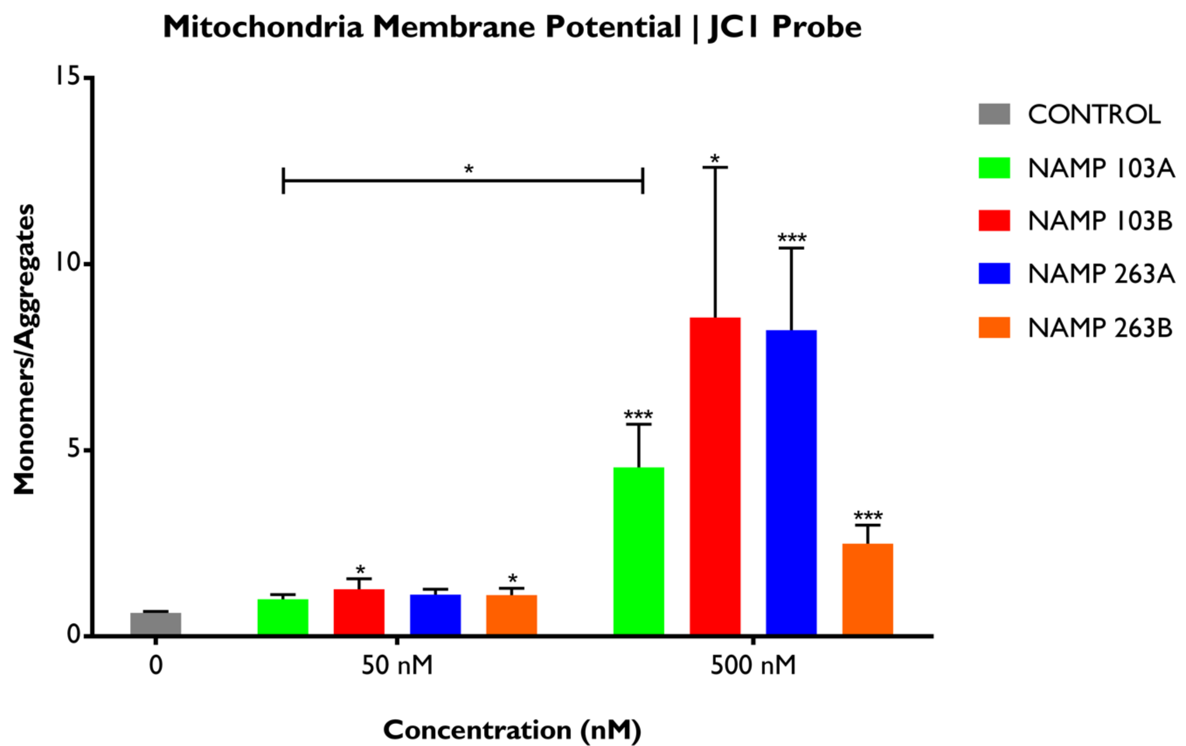



**Figure 18.** Mitochondria membrane potential on A375 cells after PDT treatment with PS concentrations of 50 nM and 500 nM. A higher monomers/aggregates ratio indicates a diminishment on the MMP. The results presented match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

## WESTERN BLOT

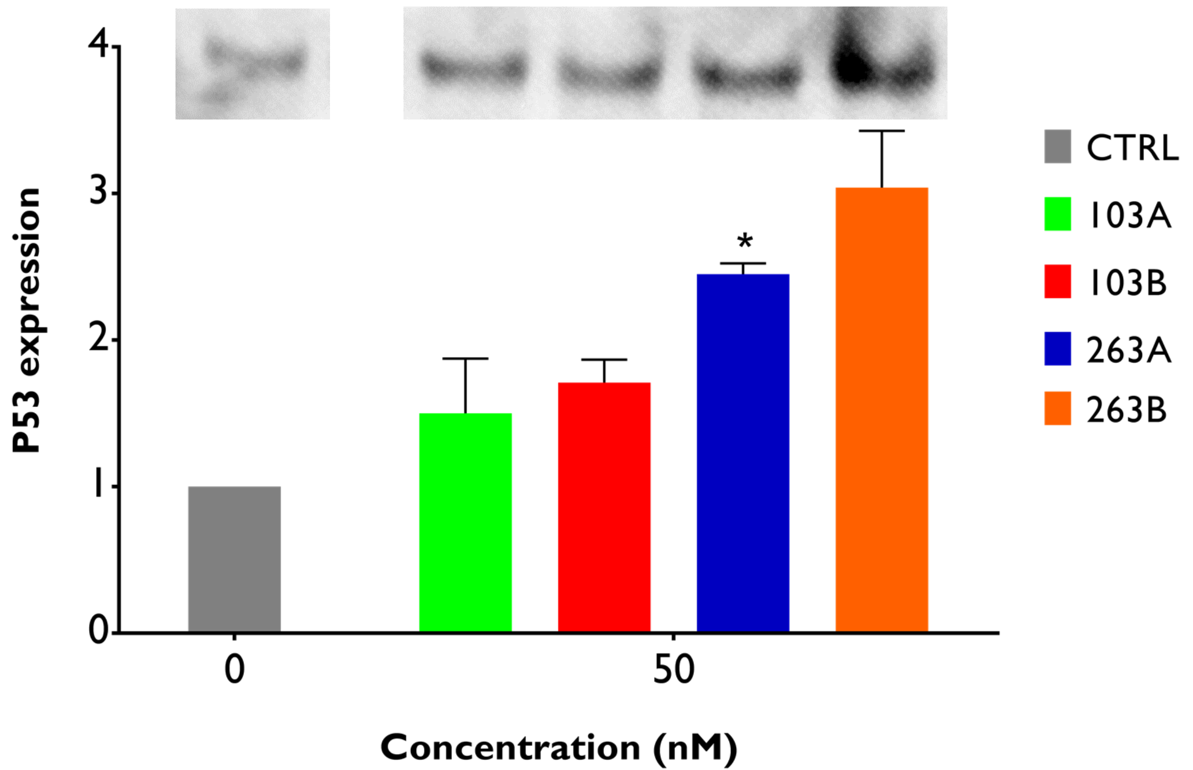
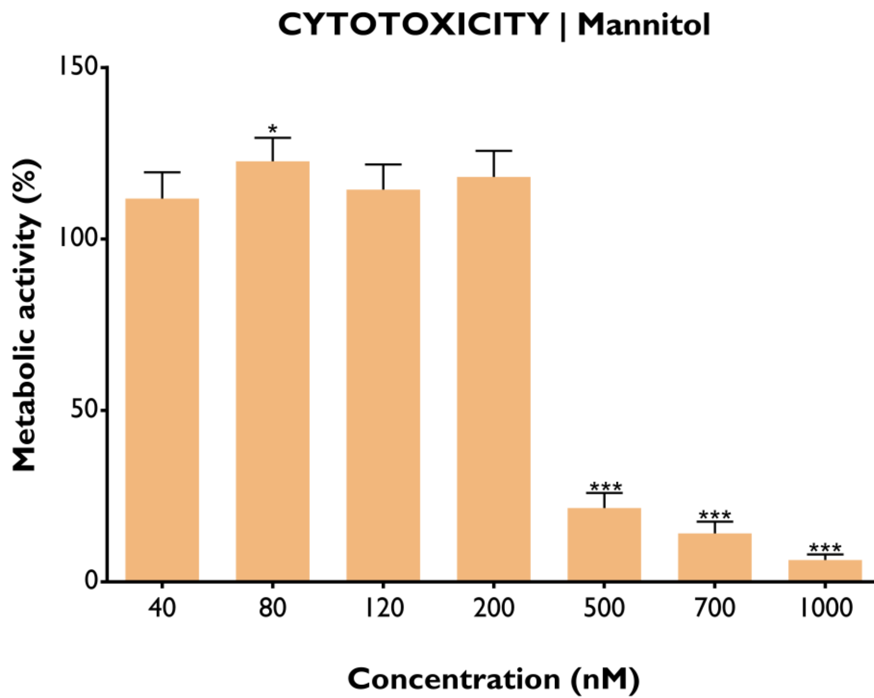



**Figure 19.** Expression of P53 protein after PDT treatment with PS concentration of 50 nM. The image is representative result of a Western Blot procedure. The results presented match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences, relative to control, are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.



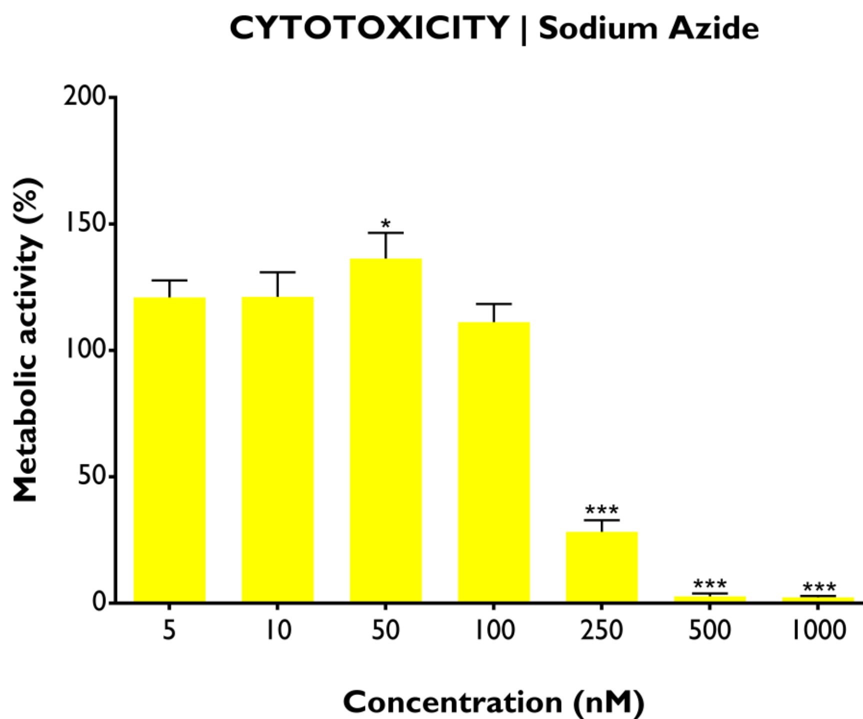




**Figure 20.** Metabolic activity on human melanoma A375 cells after incubation with ROS-quencher in a set of concentrations ranging from 5 nM to 1 μM for sodium azide and 40 nM to 1 μM for mannitol. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences, relative to control, are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

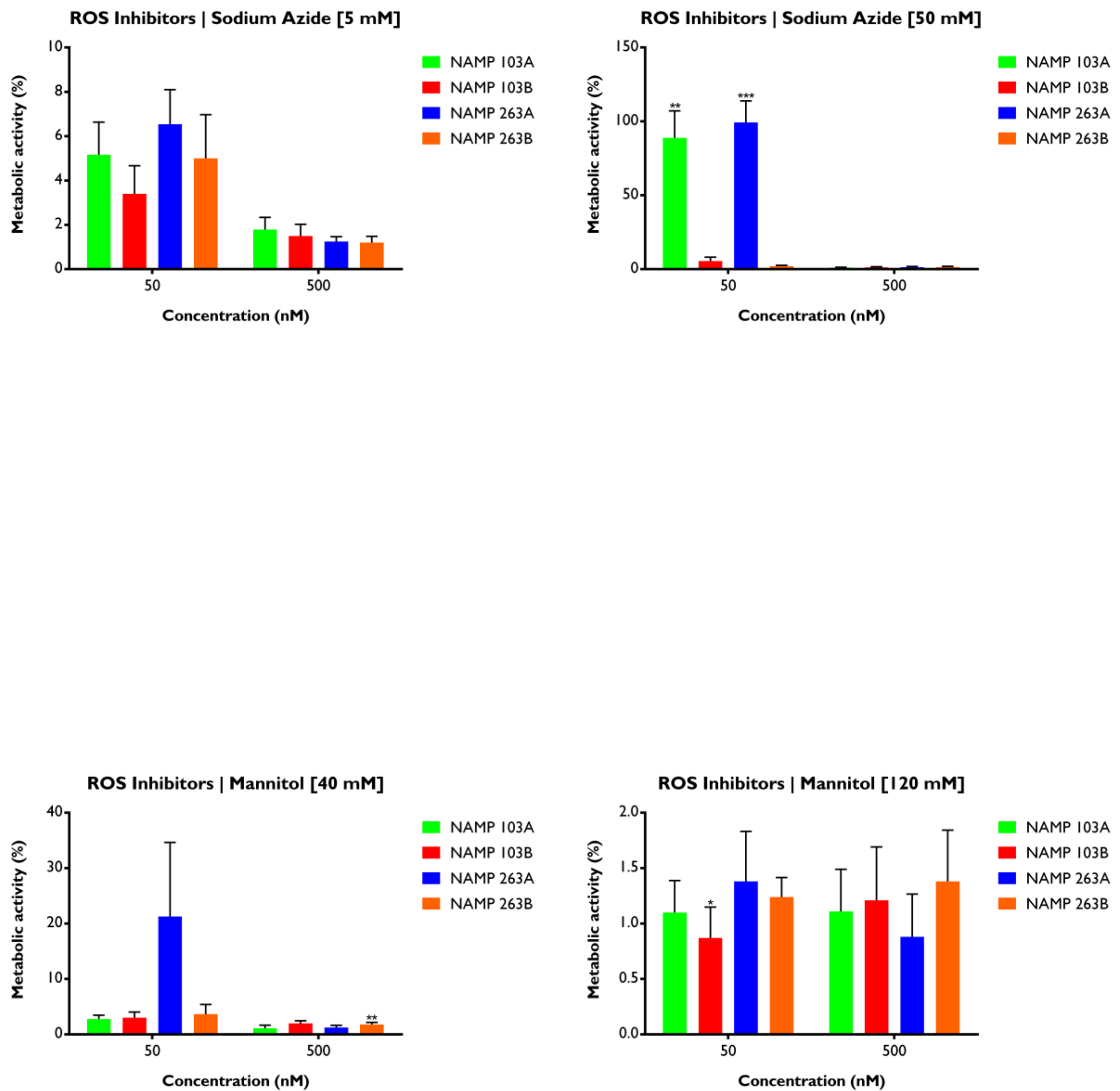



**Figure 21.** Metabolic activity on human melanoma A375 cells after treatment with ROS-quencher sodium azide and mannitol at concentrations of 5 mM and 50 mM; 40 mM and 120 mM, respectively. In each graphic, the comparisons are stablished between the condition (with the quencher) and the same condition but without the quencher (simple 10 J MTT assay). The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

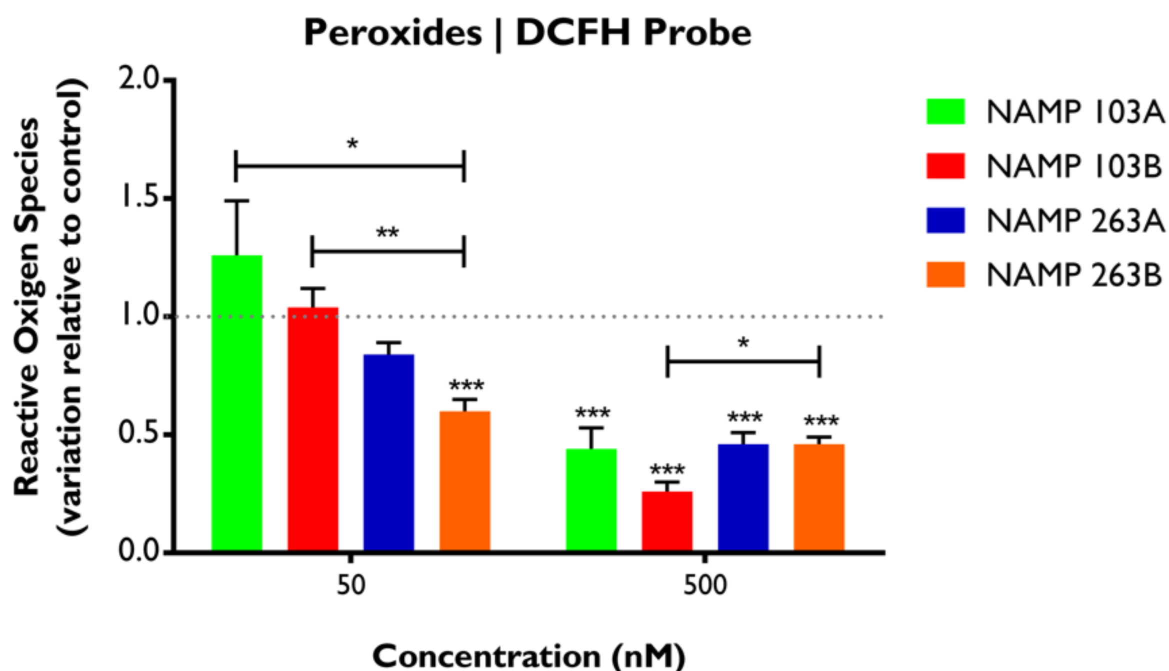



**Figure 22.** Intracellular generation of peroxides on human melanoma A375 cells after PDT with PS concentrations of 50 nM and 500 nM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

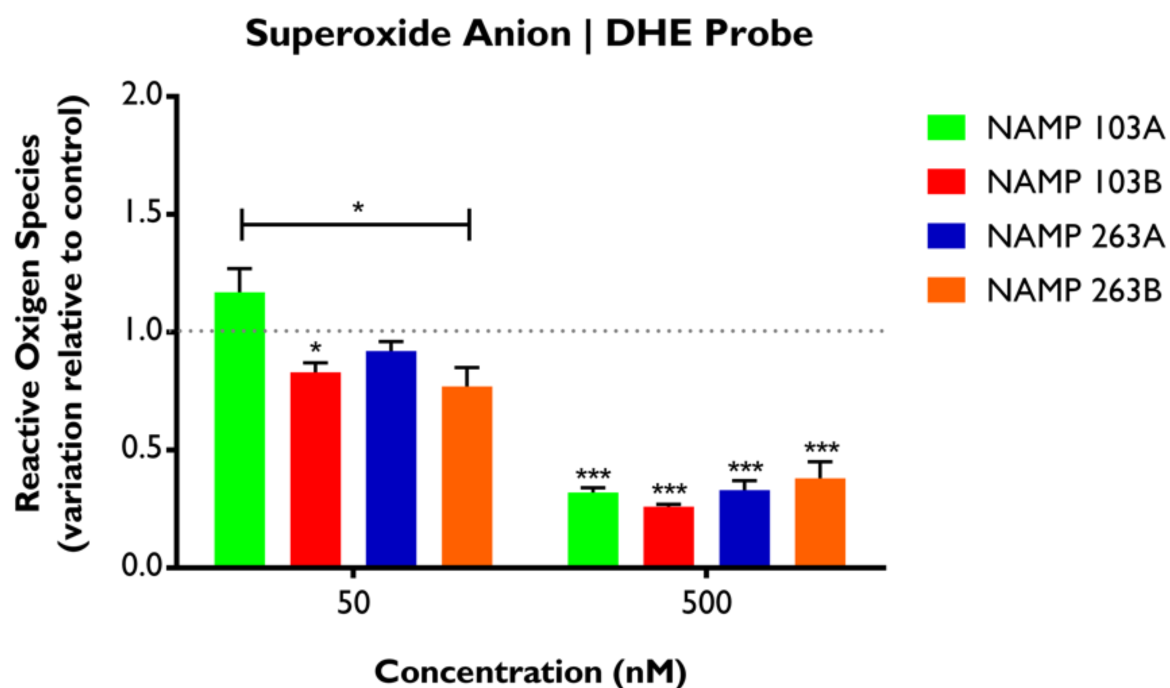



**Figure 23.** Intracellular generation of superoxide anion on human melanoma A375 cells after PDT with PS concentrations of 50 nM and 500 nM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

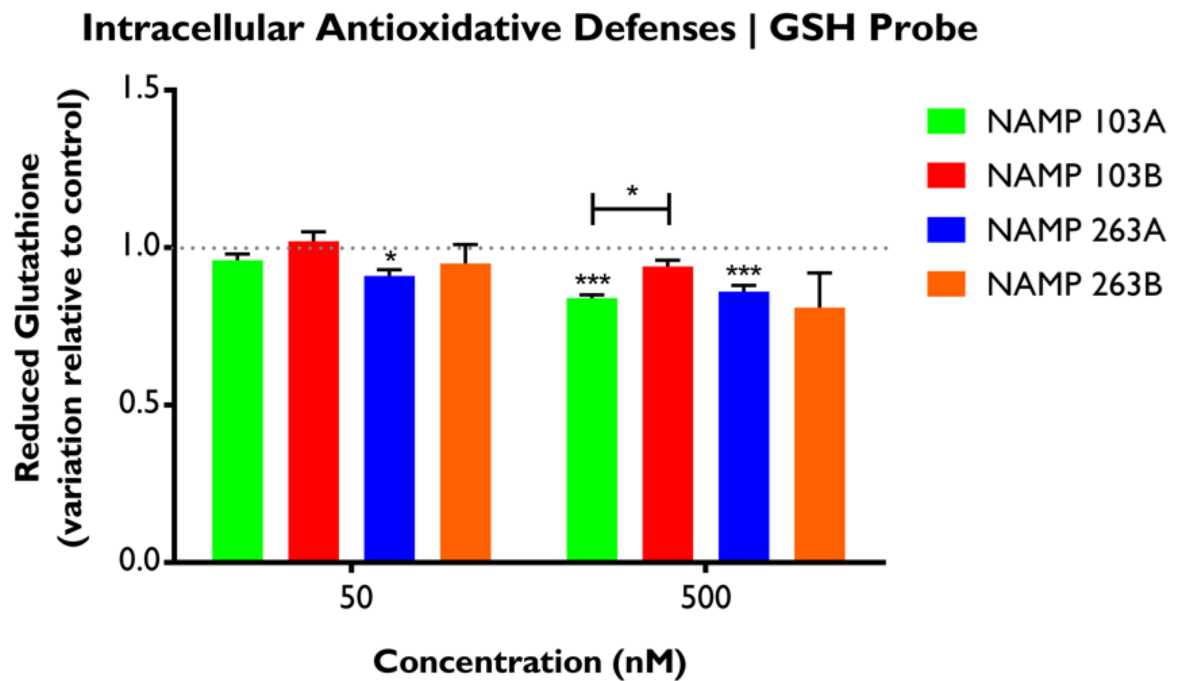



**Figure 24.** Activity of GSH on human melanoma A375 cells after PDT with PS concentrations of 50 nM and 500 nM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

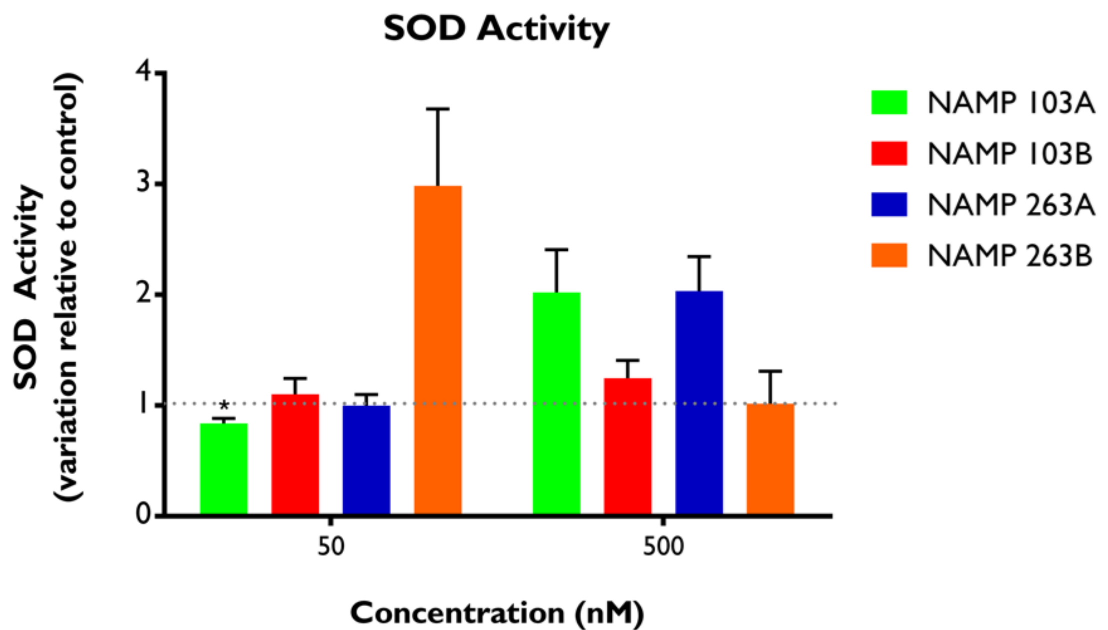



**Figure 25.** Activity of SOD on human melanoma A375 cells after PDT with PS concentrations of 50 nM and 500 nM. The results are presented as the variation relatively to control cell cultures and match to the average and standard error obtained in three independent experiments performed in triplicate. Settled significant differences, relative to control, are described with the symbol *, meaning $p<0.05$, **, meaning $p<0.01$ and ***, $p<0.001$.

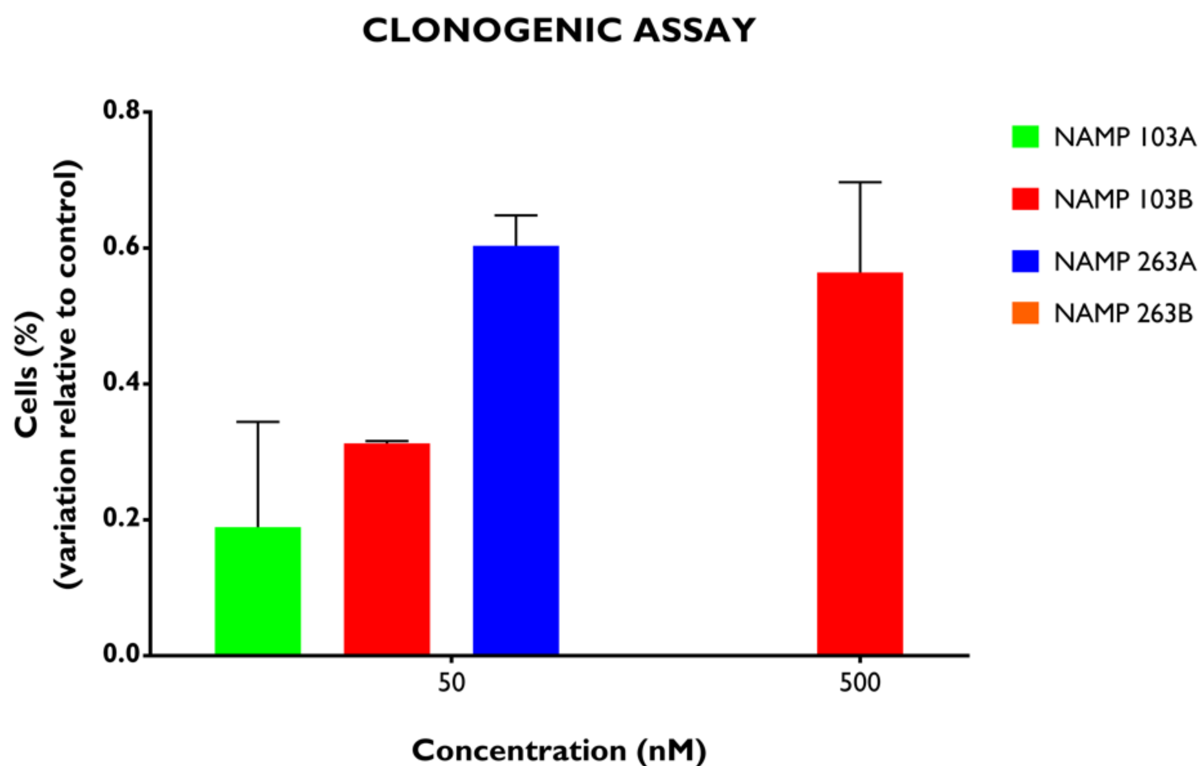
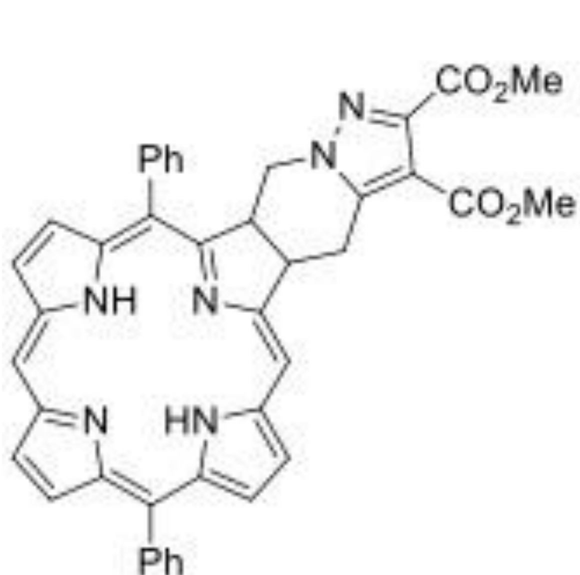
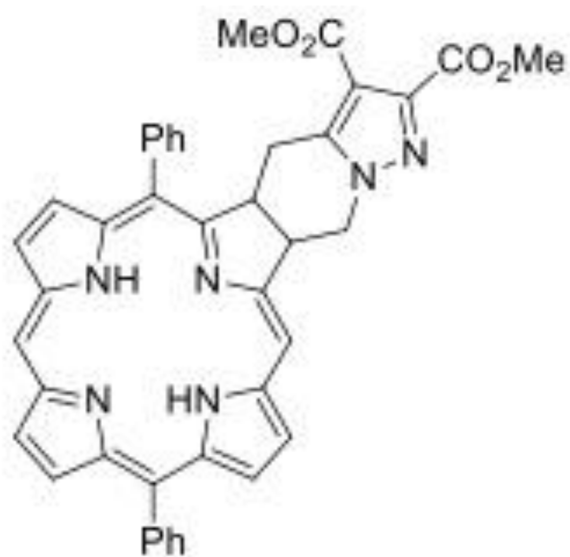
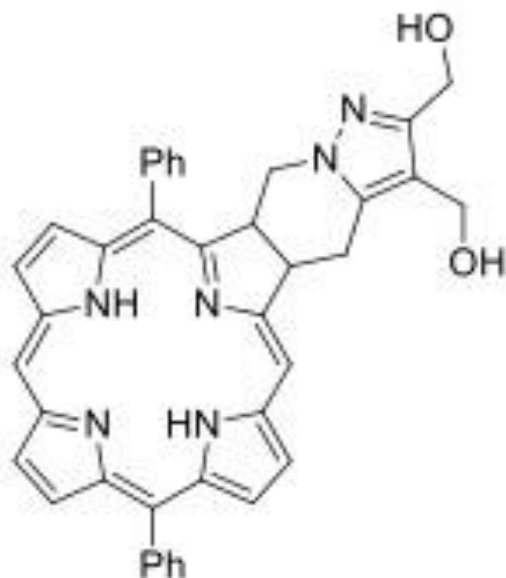



**Figure 26.** Survival profile of human melanoma A375 cells after PDT using PS concentrations of 50 nM and 500 nM. The results presented are pilot and match to the average and standard error obtained in one independent experiment performed in triplicate.



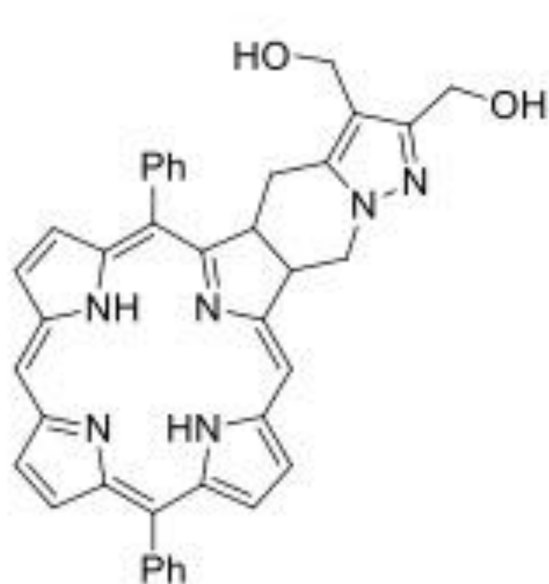




**Figure 27.** Overall structures of used photosensitizers: NAMP 103A, NAMP 103B, NAMP 263A and NAMP 263B.

**Figure 28.** Analysis of Strengths, Weaknesses, Opportunities and Threats (SWOT) relative to Curricular Internship in Pharmacy Machado.

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