



St John's Wort Photomedicine For Melanoma

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(((♥)))

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To

THE LIGHT OF LOVE

((♥))

I call forth the Light of Love

From within YOU

((♥))

Don't fear my Dear

Just LOVE

((♥))

There is a light

That shines so bright

Through the many facets of your Soul

((♥))

You will find the Light of Love

On the Path of Heart

((♥))

Through the Heart into the Light

We DANCE

((♥))

Dance Dance Dance my child

Celebrate this Gift of Life

Your heart beats

JOY LOVE JOY

((♥))

((♥)) A Life just for YOU ((♥))

((♥))

I see

I weave

I sparkle

I weave the Light of Love

((♥))

((♥)) Britta LightWeaver LoveSparkles ((♥))

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LoveSparkles

Britta

The Fairy Scientist

DECLARATION

I, Britta Kleemann

hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Signed by candidate

Signature:

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ABSTRACT

The use of photomedicine in ancient civilizations dates back 4000 years ago but it wasn't until the beginning of the 20th century that photodynamic therapy was discovered by man.

The “trinity” of photodynamic therapy (PDT) comprises a photosensitizer, light and molecular oxygen. Following cellular uptake of the photosensitizer, its activation by light produces reactive oxygen species in the presence of oxygen. The resulting cytotoxic oxidative stress elicits cancer cell death by various mechanisms including apoptosis, necrosis and autophagy. Hypericin, an extract from St John's Wort, is a promising photosensitizer in the context of clinical photodynamic therapy due to its excellent photosensitizing properties and tumortropic characteristics. However, limited reports on the efficacy of this photomedicine for the treatment of melanoma have been published. South Africa has the second highest incidence of malignant melanoma skin cancer in the world; a highly aggressive tumor due to its metastasizing potential and resistance to conventional cancer therapies.

The aim of this study was to investigate the response mechanisms of melanoma cells to hypericin-PDT in an *in vitro* tissue culture model. This investigation was three-fold. Firstly, the susceptibility of melanoma cells to the treatment was determined using cell viability assays. We found a dose of 3 μ M light-activated hypericin was effective in reducing cell viability to 50 % or less than the control, for all melanoma cells employed in this study. We therefore used this killing-dose for further experiments.

Next, hypericin uptake and its specific association with intracellular organelles was characterized using organelle-specific fluorescent-fusion proteins and dyes, in conjunction with the red fluorescent nature of hypericin and visualization by live confocal fluorescent microscopy. The intracellular localization of a photosensitizer directly influences its cytotoxic action and is thus crucial for effective cell death induction. Hypericin was taken up by all melanoma cells and co-localized with lysosomes and variably with melanosomes, the pigment producing organelles. No co-localization with the cell membrane, mitochondria, endoplasmic reticulum or nucleus was found. Investigating intracellular hypericin after treatment revealed a time-dependent decrease in all melanoma cells.

Finally, melanoma cell death mechanisms were elucidated in response to the killing-dose of light-activated hypericin. Ultrastructural examination of the cells with transmission electron microscopy

revealed extensive cytoplasmic vacuolisation, at 4 hours after treatment. In pigmented melanoma cells, the treatment furthermore induced the formation of glycogen aggregations. Fluorescent activated cell sorting analyses revealed a time-dependent increase in phosphatidylserine exposure, indicating apoptosis, in conjunction with a loss of cell membrane integrity, indicating necrosis, in all melanoma cells. An initial early necrotic population was found which decreased with time after treatment, whereas the late apoptotic/necrotic population increased. Minimal early apoptotic populations were found in all cell lines. In addition, melanoma cells showed a decrease in cellular size accompanied by an increase in granularity/pigmentation after treatment. Western blot analyses of proteins involved in specific cell death cascades furthermore verified the induction of apoptosis in melanoma cells by hypericin-PDT. The extrinsic apoptotic cascade was initiated in unpigmented A375 melanoma cells at 24 hours after treatment, mediated by activation of the suicidal proteases caspase 8 and caspase 3. Intrinsic apoptosis was found in pigmented UCT Mel-1 cells at 4 and 7 hours, mediated by activation of caspase 3 and cleavage of poly(ADP-ribose)polymerase 1 (PARP1). Induction of apoptosis by cleavage of PARP1 was furthermore evident in 501mel cells at 7 hours after treatment; however this cleavage was not mediated by caspase 3. Apoptosis inducing factor was found in its vital form in all melanoma cells, indicating that caspase-independent apoptosis or regulated necrosis by parthanatos were not induced by hypericin-PDT.

In summary, this study demonstrated the effectiveness of hypericin-PDT in killing both unpigmented and pigmented melanoma cells by the induction of apoptosis. Further investigations into the exact mechanisms of the cell death response, including the observed loss of cell membrane integrity and the involvement of lysosomes and melanosomes are interesting avenues to explore in future studies. Translation of hypericin-PDT into a three-dimensional skin model with melanoma invasion is of particular interest, to further simulate the natural environment of this aggressive cancer and thereby enable the identification of enhanced treatment options.

CHAPTER 1:
INTRODUCTION
& LITERATURE REVIEW

1.1 INTRODUCTION

The use of photomedicine dates back 4000 years ago with the ancient Egyptians, Indians, Greeks and Chinese using the synergy of sunlight and plants such as *Amni majus* and *Psoralea corylifolia* for the treatment of the skin diseases vitiligo and psoriasis¹. With the advent of the 20th century, the father of modern photomedicine, Neils Finsen, was awarded one of the first Nobel Prizes for Medicine in 1903 for his work on using ultraviolet light to cure the facial sores (lupus vulgaris) of tuberculosis patients; igniting the potential medical uses of light in the modern world¹. Around the same time, Oscaar Raab, a student of Hermann von Tappeiner at the University of Munich, discovered the toxic photodynamic effect of acridine orange and light on protozoan paramecia cultures². Paramecia treated with the same dose of acridine orange survived for 1.5 hours in one experiment and 15 hours in another, under the same conditions, except for a heavy thunderstorm during one of the experiments². Raab thus started to wonder what role the light might have played in these results. The group subsequently performed a large amount of work on photosensitization, discovered that oxygen was needed for the photodynamic effect and treated a range of skin diseases and cancers with favorable results^{1,3}. Despite these promising early findings, the first large series of cancer patients successfully treated with photodynamic therapy (PDT) was only reported in 1978 by Dougherty et al.^{4,5}. Since then, there have been over 200 clinical cancer trials for PDT⁶.

1.2 PHOTODYNAMIC THERAPY

1.2.1 ANTICANCER EFFECTS

Photodynamic therapy (PDT) for cancer involves 2 stages. First the photosensitizer is administered topically or intravenously, followed by irradiation of the tumour site with light of a specific wavelength⁶. Following cellular uptake of the photosensitizer, its activation by light produces reactive oxygen species (ROS) in the presence of molecular oxygen. These ROS have short half-lives and small radii of diffusion and thus exert their action in the vicinity of their production^{7,8}. Accordingly, the intracellular localization of a photosensitizer directly impacts its cytotoxic action⁹. PDT-induced cytotoxicity elicits tumor cell death by various mechanisms including apoptosis, necrosis and autophagy-related cell death⁹⁻¹¹. Antitumor effects of PDT are three-fold: cytotoxicity via cell death, damage to the tumor vasculature and induction of a robust inflammatory reaction that can lead to the development of systemic immunity^{6,12} (Fig. 1.1). The relative contribution of each of these three inter-related anti-tumor effects depends on various variables, including the type and dose of photosensitizer, the time between its administration and activation with light, the total light dose and fluence rate and the oxygen concentration in the tumor⁶. In addition, resistance

mechanisms to PDT affect the treatment outcome^{9,13,14}. These include altered drug uptake and efflux rates or altered intracellular trafficking, inactivation of ROS by antioxidant detoxifying enzymes, activation of heat shock proteins, induction of stress response genes and survival pathways and repair of induced damage to proteins, membranes and DNA¹³.

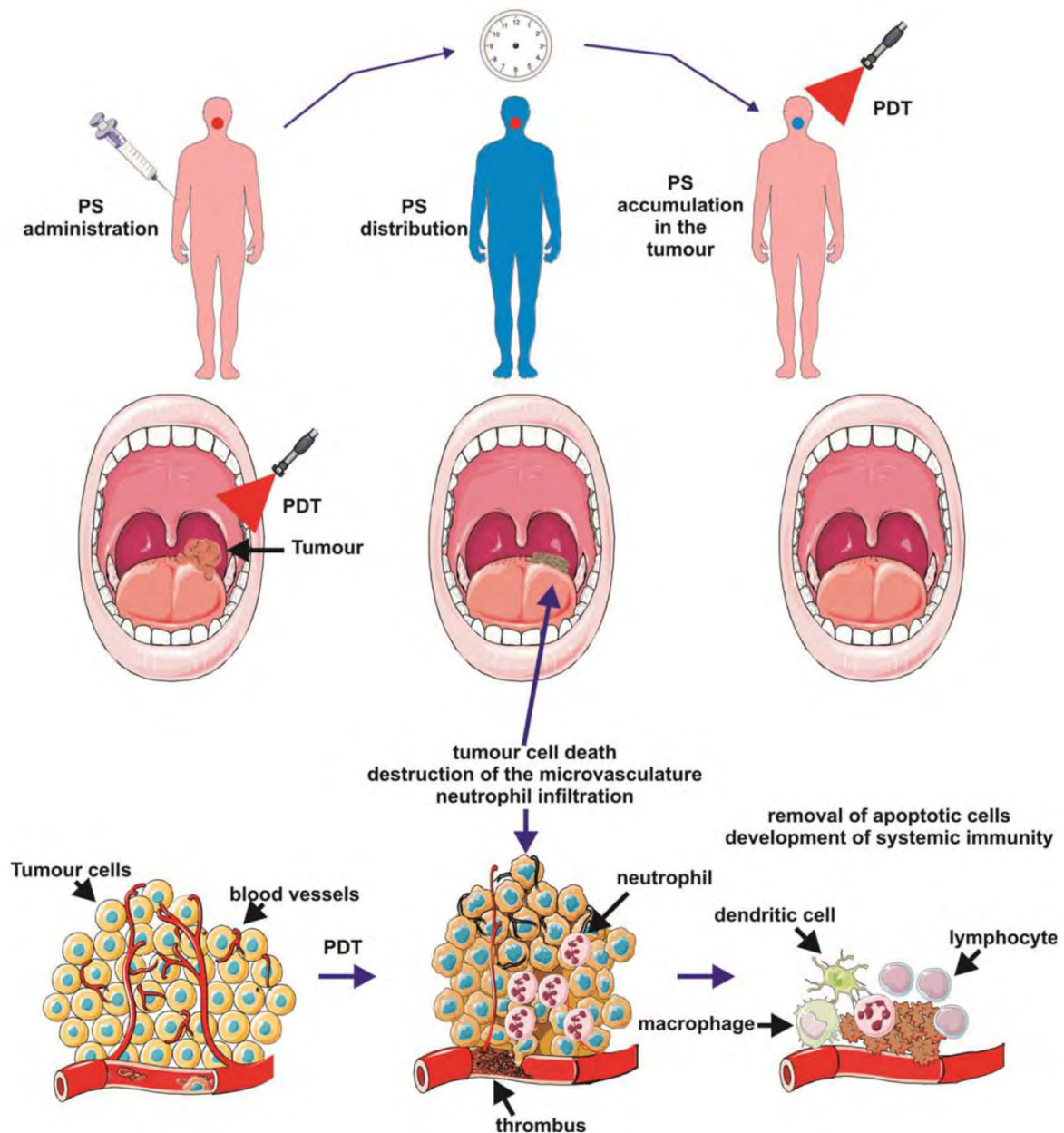


Figure 1.1: The Principles of Photodynamic Therapy (PDT). The photosensitizer is administered systemically or topically, followed by a period of systemic distribution and subsequent selective accumulation in the tumor. Upon light activation the photosensitizer produces cytotoxic reactive oxygen species (ROS) in the presence of molecular oxygen, resulting in tumor cell death via an apoptotic, necrotic, or autophagic mechanism. The concomitant induction of an acute local inflammatory reaction participates in the removal of dead cells, restoration of normal tissue homeostasis, and sometimes, in the development of systemic immunity (Taken from Agostinis et al. (2011)⁶).

1.2.2 MECHANISM OF ACTION

The “trinity” of photodynamic therapy (PDT) comprises a photosensitizer, light and molecular oxygen⁶. By themselves these components are non-toxic; together they initiate a potent photocytotoxic reaction. Photosensitizers used in cancer therapy usually have a tetrapyrrole structure and undergo a Type II photochemical reaction upon activation by light to produce ROS^{6,15} (Fig. 1.2). Upon absorption of light, the photosensitizer is raised from the ground state to an excited singlet state. In this unstable excited state, the photosensitizer emits the excess energy as fluorescence and/ or heat. Alternatively, the photosensitizer can form a more stable, triplet state by undergoing an intersystem crossing. In this triplet state, the photosensitizer can either decay back to its ground state or it can transfer its energy to molecular oxygen (O_2). Upon receiving energy from the photosensitizer, oxygen (O_2) forms singlet oxygen ($^1O_2$) in a Type II photochemical reaction. In a type I photochemical reaction, the photosensitizer reacts directly with an organic molecule in its environment and subsequent auto-oxidation of the reduced photosensitizer forms superoxide anion radical (O_2^-) via electron transfer, which can furthermore give rise to hydrogen peroxide (H_2O_2). Reduction of hydrogen peroxide can cause the formation of hydroxyl radical ($HO^\bullet$). Most photosensitizers are believed to predominantly produce singlet oxygen ($^1O_2$) via Type II reactions.

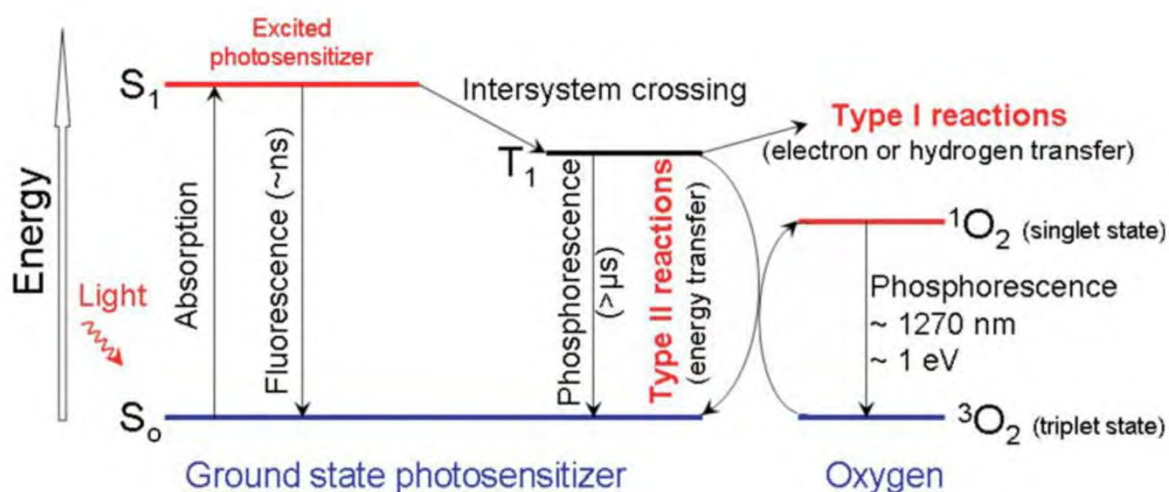


Figure 1.2: The “trinity” of photodynamic therapy (PDT). The photosensitizer moves from the ground singlet state (S_0) to an excited singlet state (S_1) upon absorption of light. The molecule in S_1 may undergo intersystem crossing to an excited triplet state (T_1) and then either form radicals via Type I reactions or transfer its energy to molecular oxygen, forming singlet oxygen ($^1O_2$), via Type II reactions. (ns: nanoseconds, μ s: microseconds, nm: nanometres, eV: electron volts, modified Jablonski diagram, taken from Agostinis et al. (2011)⁶).

1.2.3 PHOTSENSITIZERS AND LIGHT SOURCES

An ideal photosensitizer is taken up preferentially by tumour cells, has no dark toxicity and is relatively rapidly cleared from normal tissue, thereby minimizing phototoxic side effects⁶. It is a single pure compound for ease in quality control, has good stability for storage and low manufacturing costs. The ideal photosensitizer has a high quantum yield for singlet oxygen ($^1\text{O}_2$) production and a high absorbance peak between 600 and 800 nanometers (nm), in the red to deep red spectrum⁶. Light of longer wavelengths penetrates tissue more effectively (Fig. 1.3). Light between 600-800 nm is thus frequently called the optical window of tissue. However, light of wavelengths longer than 800 nm do not have sufficient energy to initiate a photochemical reaction⁶. The first photosensitizer used for cancer therapy was a water soluble mixture of porphyrins called hematoporphyrin derivative (HPD)^{4,5}. Its purified form is porfimer sodium with the commercial name Photofrin. Today this is still the most widely used photosensitizer despite its distinct disadvantages of low absorption at 630nm and a long-lasting skin photosensitivity⁶. Despite the wide use of Photofrin, a variety of second-generation photosensitizers with improved characteristics have been discovered and are currently applied in the clinic (approved or in clinical trials, Table 1.1)⁶. A commonly used second generation photosensitizer is 5-aminolevulinic acid (ALA) and its esters which are precursors of the photosensitizer protoporphyrin IX in heme biosynthesis⁶. Various types of cancers are currently being treated with a range of photosensitizers (approved or in clinical trials) including: skin, lung, esophagus, bile duct, bladder, ophthalmic, head and neck, pancreatic, breast, liver, colon, nasopharyngeal, sarcoma, brain, cutaneous T-cell lymphoma and prostate (Table 1.1, for a comprehensive review see Agostinis et al. (2011)⁶).

PHOTOSENSITIZER	STRUCTURE	WAVELENGTH, nm	APPROVED	TRIALS	CANCER TYPES
Porfimer sodium (Photofrin) (HPD)	Porphyrin	630	Worldwide		Lung, esophagus, bile duct, bladder, brain, ovarian
ALA	Porphyrin precursor	635	Worldwide		Skin, bladder, brain, esophagus
ALA esters	Porphyrin precursor	635	Europe		Skin, bladder
Temoporfin (Foscan) (mTHPC)	Chlorine	652	Europe	United States	Head and neck, lung, brain, skin, bile duct
Verteporfin	Chlorine	690	Worldwide (AMD)	United Kingdom	Ophthalmic, pancreatic, skin
HPPH	Chlorin	665		United States	Head and neck, esophagus, lung
SnEt2 (Purlytin)	Chlorin	660		United States	Skin, breast
Talaporfin (LS11, MACE, NPe6)	Chlorin	660		United States	Liver, colon, brain
Ce6-PVP (Fotolon), Ce6 derivatives (Radachlorin, Photodithazine)	Chlorin	660		Belarus, Russia	Nasopharyngeal, sarcoma, brain
Silicon phthalocyanine (Pc4)	Phthalocyanine	675		United States	Cutaneous T-cell lymphoma
Padoporfin (TOOKAD)	Bacteriochlorin	762		United States	Prostate
Motexafin lutetium (Lutex)	Texaphyrin	732		United States	Breast

Abbreviations: ALA, 5-aminolevulinic acid; AMD, age-related macular degeneration; Ce6-PVP, chlorin e6-polyvinylpyrrolidone; HPD, hematoporphyrin derivative; HPPH, 2-(1-hexyloxyethyl)-2-devinyl pyropheophorbide-a; MACE, mono-(L)-aspartylchlorin-e6; mTHPC, m-tetrahydroxyphenylchlorin; nm indicates nanometers; SnEt2, tin ethyl etiopurpurin.

Table 1.1: Clinically applied photosensitizers. Various photosensitizers (approved or in clinical trials) are listed including their structure, wavelength (nm) of light and type of cancer treated.

One of the most remarkable features of PDT is the tumor localizing property of the photosensitizer. This feature is thought to be attributed to various factors including leaky tumor blood vessels, absence of lymphatic drainage and the preferential binding of some of the most effective photosensitizers to low-density lipoprotein (LDL); suggesting that upregulated LDL receptors on tumor cells could be important^{16,17}.

Photosensitizers have also been covalently linked to various vehicle molecules in targeting studies so that the photosensitizer can be chosen based on its photochemical properties and not its uptake parameters¹⁸. These vehicle molecules have an affinity for neoplasia or receptors expressed on specific tumors including monoclonal antibodies and antibody fragments, peptides, proteins (such as insulin, transferrin and epidermal growth factor), carbohydrates, folic acid and somatostatin¹⁸.

Following uptake, the photosensitizer is activated by light. The penetration of light into tissue increases with its wavelength (Fig. 1.3), therefore photosensitizers with absorption peaks in the red to deep red spectrum (600- 800 nm) offer tumour control in deeper tissues⁶. Light of shorter wavelengths can however also be used, depending on the cancer treated. The choice of the light source is influenced by many variables including the photosensitizer absorption, disease (size, location, accessibility and tissue characteristics) and cost¹⁹. No light source is ideal for all applications even when using the same photosensitizer. The clinical effect of PDT relies on the light exposure

time and total light dose, light delivery mode (single, fractionated and metronomic) and fluence rate²⁰. Both incandescent light sources and lasers have been used in PDT and showed similar success²¹. Diode lasers are suitable for PDT as they are small, cost effective and easy to install and use. Light-emitting diodes (LEDs) are cost effective, alternative light sources with high fluence rates and relatively narrow spectral bandwidths²².

The use of different light sources enables the treatment of different types of cancers. Cutaneous cancers are easy to irradiate and lasers coupled to flexible fibres with diffusing tips allow for the treatment of hollow organs, such as the bladder, digestive tract and lung⁶. It is furthermore possible to implant a light source in a solid organ deep in the body under image guidance using inflatable balloons, covered on the inside with a strongly scattering material and formed to fit an organ²³. Similarly, interstitial PDT enables the delivery of light down fibres positioned directly in internal organs (such as prostate, pancreas, lung and liver) through needles, inserted through the skin using various forms of image guidance^{24,25}. No open surgery is necessary for these approaches.

An interesting approach to PDT is daylight-mediated PDT. It has been shown in 3 randomized controlled studies that daylight-mediated PDT was an effective treatment for thin actinic keratosis^{26–28}. Daylight-mediated PDT is nearly pain-free, more convenient for both patients and clinics and is especially suited for patients with large field cancerized areas, which can easily be exposed to daylight²⁹. It poses a particularly interesting avenue to explore for hospitals in developing countries, such as South Africa, where space is limited and budgets are inadequate.

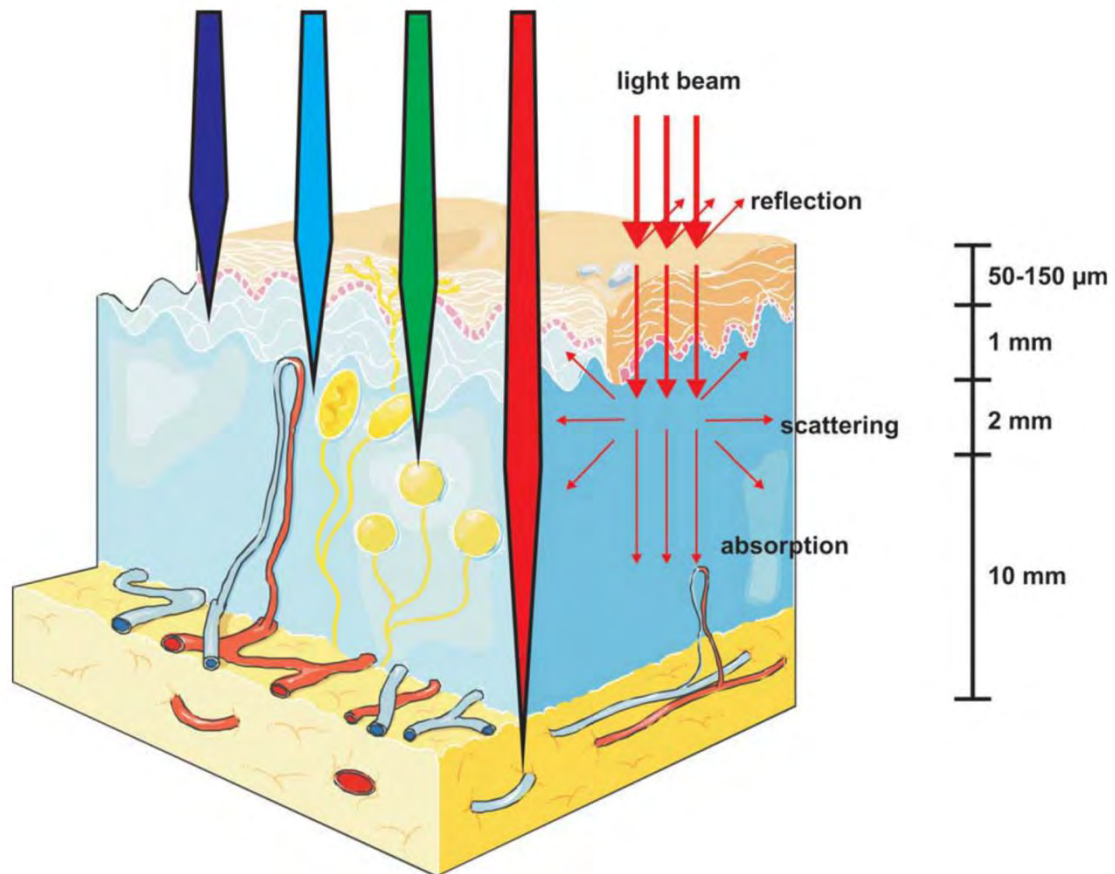


Figure 1.3 Light propagation through tissue. Light of longer wavelengths penetrates tissue more effectively (arrows indicate different wavelengths of light: violet 380-450 nm, blue 450-495 nm, green 495-570 nm, red 620-750nm, taken from Agostinis et al. (2011) ⁶).

1.2.4 THE LIGHT AND DARK OF PDT

The high selectivity of a useful photosensitizer for the tumor coupled with the precise delivery of light results in a high specificity of PDT. This specificity makes PDT unsuitable for metastatic cancers; however the discovery of the activation of systemic immunity by PDT gives hope for treatment of metastases in the future¹². PDT does not exhibit the same adverse side effects of chemo- and radiotherapy and can be used in conjunction with these and surgery. Photosensitizers approved for clinical practice do not accumulate in cell nuclei and are thus not carcinogenic and do not cause resistance via mutations³⁰. PDT has a unique cytotoxicity mechanism and mutations that result in chemo- and radioresistant tumours do not affect sensitivity to this treatment⁶. PDT can be repeated without compromising its efficacy. Excellent cosmetic outcomes and retention of mechanical integrity and functional anatomy of hollow organs are clear advantages of PDT^{31,32}. The treatment can be given in an ambulatory or outpatient setting which is patient friendly and reduces costs. PDT

can provide improvement of quality of life for selected patients that have exhausted other treatment options.

One of the limitations of PDT is pain experienced during the therapy which can be minimized through the use of local analgesics³⁰. Prolonged skin photosensitivity is another issue which is greatly reduced with the advent of second generation photosensitizers, approaching ideal characteristics³³. The penetration depth of light into tissue is limited. However, new approaches such as two-photon PDT which uses near-infrared light can achieve deeper tissue penetration¹⁹. Despite PDT's discovery at the beginning of the 20th century and its many advantages over other therapies its clinical use is still limited. This might be due to the scarcity of randomized phase 3 clinical trials⁶. The light activation and its associated technologies such as fiber optics and lasers can be complicated and frightening to the clinician that is not trained in this field. An option would be the assistance of a medical physicist or radiation oncologist that is trained in optical dosimetry and light delivery methods⁶.

PDT is a creation based on chemistry, physics, biology and medicine and its future development, application and success will thus be a product of the increased co-operation and exchange of ideas across these fields.

1.3 CELL DEATH

To elucidate the mechanisms of PDT-induced cytotoxicity an understanding of cell death is pertinent and is therefore outlined in the next section of this review. An immense body of work on cell death and its mechanisms has been produced in recent years. As one can imagine, such a rapidly evolving scientific field produced masses of novel data, with no systemic classification of cell death modalities in place. This led to the formation of the Nomenclature Committee on Cell Death in 2005 which intended “to provide a forum in which names describing distinct modalities of cell death are critically evaluated and recommendations on their definition and use are formulated, hoping that a non-rigid, yet uniform nomenclature will facilitate the communication among scientists and ultimately accelerate the pace of discovery”³⁴. This Committee has thus far published helpful recommendations on the classification and nomenclature of cell death modalities^{34–36} which have been incorporated into this review.

1.3.1 MORPHOLOGY

Programmed cell death is a ubiquitous process essential for cellular turnover in development, tissue homeostasis and health in multicellular organisms. The term “apoptosis” was introduced 40 years ago based on morphological observations in healthy tissues and used synonymously with programmed cell death³⁷. Morphological investigations of cell death quickly revealed more than one way to die^{36,38}. Besides apoptosis, and necrosis, autophagy has emerged within the cell death field, each exhibiting distinct morphological differences³⁶ (Fig. 1.4). Apoptosis is characterized by rounding of the cell, retraction of pseudopods, reduction in cellular and nuclear volume (pyknosis), nuclear fragmentation, minor modification of cytoplasmic organelles, plasma membrane blebbing and engulfment by resident phagocytes *in vivo*. Cell death associated with autophagy is morphologically defined as the lack of chromatin condensation, massive vacuolization of the cytoplasm, accumulation of double membrane autophagic vacuoles and little or no uptake by phagocytes *in vivo*. Necrosis was defined as cell death with the absence of apoptotic and cell death associated autophagic morphological traits; exhibiting cytoplasmic swelling (oncosis), rupture of the plasma membrane, swelling of cytoplasmic organelles and modification of chromatin condensation.

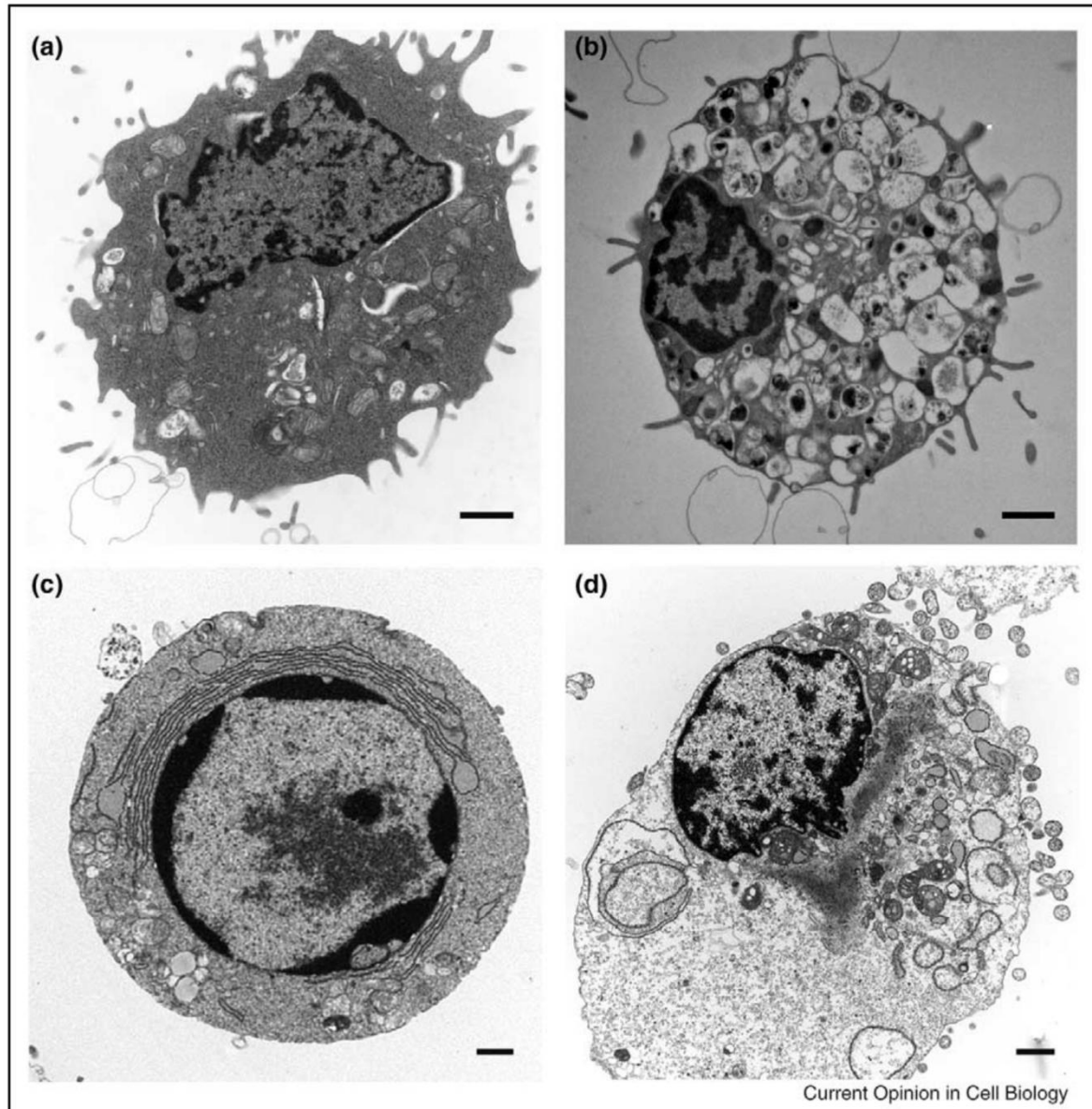


Figure 1.4: Morphological features of cell death. (a) Untreated, (b) autophagic, (c) apoptotic (d) and necrotic cells (transmission electron microscopy (TEM) images, scale bar: 1 μ m, taken from Edinger et al.³⁸)

1.3.2 APOPTOSIS

Looking at an apoptotic cell from the outside, several distinct cellular changes can be seen. The cell rounds up, retracts from its neighbors, undergoes dynamic plasma membrane blebbing, forms many small vesicles named apoptotic bodies and is engulfed by phagocytes for recycling of its components³⁷. Intracellularly, the cell undergoes numerous changes that lead to the shutting down of its life support systems³⁹. Caspases, a family of cysteine proteases, were first discovered to be responsible for the majority of these processes with over 400 mammalian caspase substrates identified⁴⁰. Active caspases have been shown to induce a proteolytic mayhem targeting various

essential cellular processes and structures such as transcription and translation, the multi-subunit proteasome complex, metabolic enzymes, DNA condensation and degradation, mitochondrial- ER- golgi- cytoskeleton- nuclear envelope- and nuclear fragmentation, detachment from the extracellular matrix and translocation of 'eat me' signals such as the membrane lipid phosphatidylserine to the outside of cell membranes for recognition and engulfment by phagocytes³⁹.

Apoptosis has been divided into two different pathways, intrinsic or extrinsic, depending on the initiating stimuli^{34,41} (Fig. 1.5).

1.3.2.a INTRINSIC APOPTOSIS

Intrinsic apoptosis results from diverse intracellular stress conditions such as oxidative stress, DNA damage, cytosolic calcium overload, mild excitotoxicity and accumulation of unfolded proteins in the endoplasmic reticulum (ER). These highly heterogeneous initiating stimuli trigger signaling cascades converging at the mitochondria^{42,43} (Fig. 1.5A). Pro- and anti-apoptotic signaling mechanisms compete at mitochondrial membranes in an attempt to allow cells to cope with stress and survive or die. When pro-apoptotic factors dominate, mitochondrial outer membrane permeabilization (MOMP) occurs via the pore forming activity of pro-apoptotic Bcl2 family members such as Bak/Bax and/or the opening of the multiprotein permeability transition pore complex (PTPC). Irreversible MOMP affecting most mitochondria within a single cell has several lethal effects: dissipation of the mitochondrial membrane potential which leads to cessation of mitochondrial ATP production and mitochondrial dependent transport activities; release of toxic proteins from the intermembrane space (IMS) to the cytosol (such as cytochrome c (CYTC), apoptosis inducing factor (AIF), endonuclease G (ENDOG), direct inhibitor of apoptosis binding protein (DIABLO), second mitochondrial derived activator of caspases (SMAC) and high temperature required protein A2 (HTRA2)); inhibition of the respiratory chain, favored by loss of CYTC which transfers electrons from complex III to complex IV in the electron transport chain³⁴. These lethal effects induce or intensify reactive oxygen species (ROS) overproduction in the cell, activating a feed forward circuit which amplifies the apoptotic signal.

Multiple toxic proteins released from the IMS to the cytosol have been identified with a variety of cellular targets⁴²⁻⁴⁴. The resulting apoptotic outcome can be grouped into caspase-dependent (such as CYTC) and independent (such as apoptosis inducing factor AIF). Cytosolic CYTC participates with APAF-1, dATP and procaspase 9 to form the apoptosome which activates caspase 9 (CASP9) which in turn cleaves and activates executioner procaspases 3, 6, 7. Active caspase 3/6/ 7 (CASP3/6/7)

execute a proteolytic cascade leading to cell death as outlined earlier^{39,45}. AIF relocates to the nucleus and mediates large scale DNA fragmentation, independent of caspases⁴⁶.

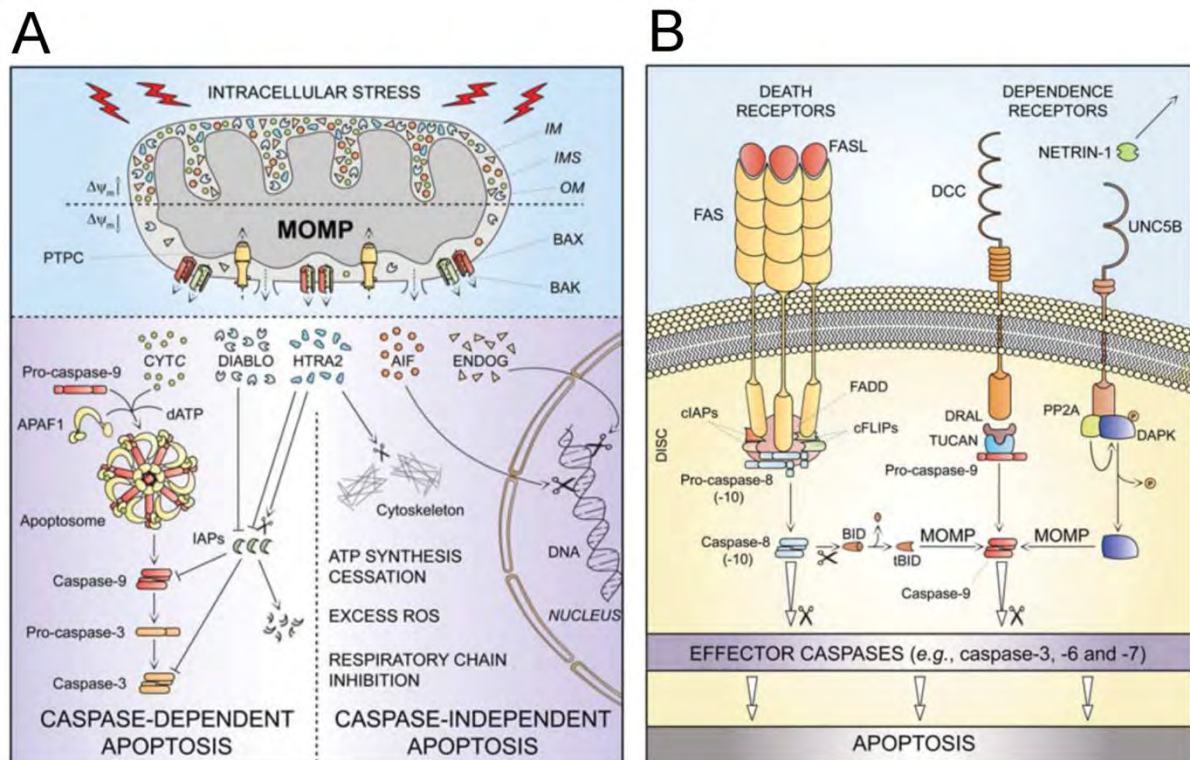


Figure 1.5: Apoptosis. (A) Intrinsic and (B) extrinsic apoptosis. (see text for details, taken from Galluzzi et al. (2012)³⁴).

1.3.2.b EXTRINSIC APOPTOSIS

Extrinsic apoptosis is induced by an extracellular stress signal and sensed and propagated through a specific transmembrane or death receptor (such as TNFAR, FAS/CD95, TRAILR1,2, Fig. 1.5B)⁴¹. Trimers of death receptors associate with each other via a death domain (DD). Upon death ligand (such as $Tnf\alpha$, FAS/CD95 ligand, TRAIL) binding to the receptor an adaptor protein binds with its DD to the DD of the receptor and its death effector domain (DED) to the DED of pro-caspase 8. Clustering of death receptors, adaptor proteins and pro-caspase 8 causes activation of caspase 8 (CASP8) by dimerization. Active CASP8 induces apoptosis independent of mitochondria (type I cells) or dependent of mitochondria (type II cells). In the former process, CASP8 cleaves and thereby activates executioner CASP3/6/7 directly. In the latter process, CASP8 mediates proteolytic cleavage of BH3 interacting domain death agonist (BID) generating the mitochondria permeabilizing fragment tBID; leading to MOMP, dissipation of the mitochondrial membrane potential, release of toxic proteins from the IMS including CYTC which causes activation of CASP9 and subsequently CASP3/6/7.

Several death ligands and their receptors have been associated with the extrinsic apoptotic pathway; two examples are highlighted here³⁴. Firstly, after FAS ligand binding, the death receptor FAS associates with FAS associated protein with a DD (FADD) and receptor-interacting kinase 1 (RIPK1, best known as RIP1), various isoforms of FLICE inhibitory protein (cFLIP), cellular inhibitor of apoptosis (cIAPs), E3 ubiquitin ligases and procaspase 8 to form a death inducing signaling complex (DISC), a platform which regulates the activation of CASP8/10. Secondly, the death receptor tumor necrosis factor receptor 1 (TNFR1), associates with the adaptor protein TNFR-associated DD (TRADD) needed for recruiting FADD and CASP8. It furthermore associates with several proteins not found in the FADD-assembled DISC, such as TNFR-associated factor 2 (TRAF2) and TRAF5 and RIP1. In this complex RIP1 can be polyubiquitinated by cIAPs leading to the recruitment of transforming growth factor β (TGF β)-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3 which together can initiate the canonical pathway of the survival factor NF- κ B. This illustrates that death receptor activation does not always lead to propagation of a lethal signal.

1.3.3 NECROPTOSIS

In the past, necrosis was viewed as predominantly accidental, a view which has dramatically changed in the last years. In a seminal paper, Laster et al. (1988) showed that tumor necrosis factor α (Tnf α) triggered cell death with either apoptotic or necrotic features⁴⁷. This meant that a cytokine, which is dependent on the molecular machinery of the cell to exert its effects, could trigger cell death with necrotic features; implying that necrosis could be regulated. Subsequent studies have established that indeed necrosis can occur in a regulated manner and has a prominent role in various physiological and pathological settings (for detailed review see Vandenabeele et al. (2010)⁴⁸). Regulated necrosis involving RIP1 and/or RIP3 has been coined necroptosis and occurs in response to Tnf α induced cell death^{48–50}. Ligation of TNFR recruits two ubiquitin ligases, TRAF2 and cIAP1, the adaptor molecule TRADD and RIP1. Ubiquitination of the resulting complex I leads to NF κ B activation which induces the expression of FLIP_L (hereafter FLIP). FLIP is a 'death twin' of CASP8 but lacks the catalytic cysteine. Thus when FLIP is present it forms a heterodimer with CASP8, preventing its activation and apoptosis is not induced. RIP1 is de-ubiquitinated by the enzymes CYLD and Cezanne, followed by the movement of RIP1 with associated TRADD to the cytoplasm. This complex, named complex II, is then joined by FADD, CASP8 and FLIP. In the absence of NF κ B activity or if protein synthesis is inhibited, FLIP is not present and the complex induces apoptosis by homodimer association of CASP8, inactivating RIP1 by proteolytic cleavage. Complex IIb or a ripoptosome can also be formed (in response to other signals such as extensive DNA damage) in which RIP1 directly

oligomerizes FADD (without TRADD) to recruit CASP8 and FLIP. This complex has attenuated CASP8 activity thus not inducing apoptosis but still inhibits RIP1, leading to cell survival. Association of RIP1 with RIP3 in a 'necrosome signaling complex' and inhibition of CASP8 leads to loss of RIP1 inhibition, RIP3 activation and subsequent necroptotic cell death. The formation of the necrosome is highly regulated by ubiquitination and mutual RIP1 and RIP3 phosphorylation. How RIP1 and RIP3 interact to promote necrotic cell death and the involvement of their "executioners" remains enigmatic. Several cellular events have however been placed downstream of RIP1 and RIP3 activation including increased ROS production, excessive energy consumption followed by a decline in ATP, increased glycolysis, activation of phospholipases and the induction of lysosomal membrane permeabilization⁴⁸⁻⁵⁰.

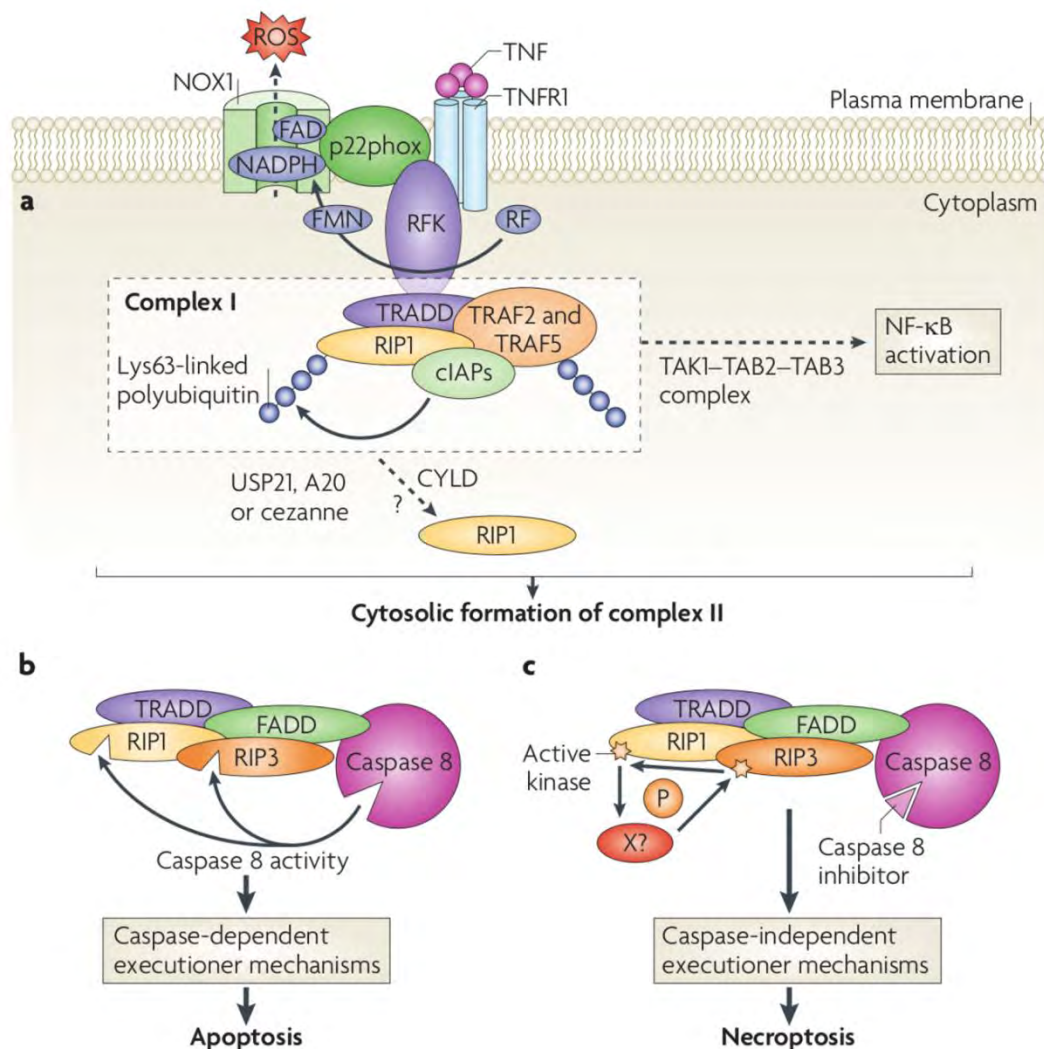


Figure 1.6: TNFR signaling pathways. (A) Formation of complex 1 upon TNFα ligation to TNFR. (B) Cytosolic formation of complex II resulting in apoptosis when caspase 8 is active, inactivating RIP1 and RIP3. Necroptosis occurs when caspase 8 is inhibited, resulting in the phosphorylation and activation of RIP1 and RIP3 (see text for details, taken from Vandenabeele et al. (2010)⁴⁸).

1.3.4 APOPTOSIS INDUCING FACTOR

Apoptosis inducing factor (AIF, Fig. 1.7) is an interesting protein in that it is involved in both life and death; in the latter it is linked to both caspase-independent apoptosis and regulated necrosis in the forms of necroptosis and parthanatos (Fig. 1.8)^{34,46,51–55}. AIF is synthesized from a nuclear gene into its precursor form of ~67 kDa and upon processing and targeting it is found in its mature form of ~62 kDa anchored to the inner mitochondrial membrane⁴⁶ (Fig. 1.7). Here it functions as a mitochondrial oxidoreductase in the respiratory chain, involved in energy metabolism and mitochondrial function and/or structure. It is vital for life as homozygous AIF knockout mice are shown to be embryonically lethal⁵¹. Various cellular insults and signaling cascades such as DNA damaging agents, kinase inhibitors, irradiation, staurosporine, atractyloside, ceramide, PAR polymers, Bax, tBID, ROS, Ca²⁺, VDAC, RIP1/TRAF2, JNK have been shown to cause AIF cleavage (mediated by two families of cysteine proteases, the calpains and cathepsins) into a truncated ~57kDa fragment (tAIF) which translocates through the cytoplasm into the nucleus^{46,51} (Fig. 1.7). In the nucleus, AIF forms a degradosome together with cyclophilin A and histone H2AX to induce chromatinolysis by chromatin condensation and large scale DNA fragmentation (~50 kb), leading to cell death⁵³. Conversely, heat shock protein 70 (Hsp70) associates with and retains AIF in the cytoplasm thus leading to survival⁵⁶. However, interaction of AIF with various other factors in the cytoplasm such as elf3 (protein synthesis inhibition) and cyclophilin A (nuclear translocation) favors apoptosis. In the worm *C.elegans*, the AIF ortholog has been shown to induce apoptosis with associated phosphatidylserine exposure on the cell membrane ('eat me'), an effect which has not been shown in mammals⁵⁷.

1.3.5 PARTHANATOS

Parthanatos, coined after poly(ADP-ribose) (PAR) polymer and the Greek Death God Thanatos, is a mode of cell death involving DNA damage response enzymes poly(ADP-ribose)polymerases (PARPs), in particular PARP-1³⁴. In response to DNA damage PARP-1 is activated to facilitate its repair, a process which generates PAR polymer via the utilization of nicotinamide adenine dinucleotide (NAD⁺)⁵². Overactivation of PARP-1 has toxic consequences to the cell due to NAD⁺ and ATP depletion and accumulation of the mitochondrotoxic polymer PAR which favors mitochondrial membrane potential dissipation and tAIF release with subsequent chromatinolysis⁵². In response to alkylating DNA damage (by MNNG), regulated necrosis is induced in mouse embryonic fibroblasts (MEFs) involving PARP-1, AIF, cyclophilin A, histone H2AX and RIP1 and has thus been coined caspase-independent necroptosis^{54,55,58}.

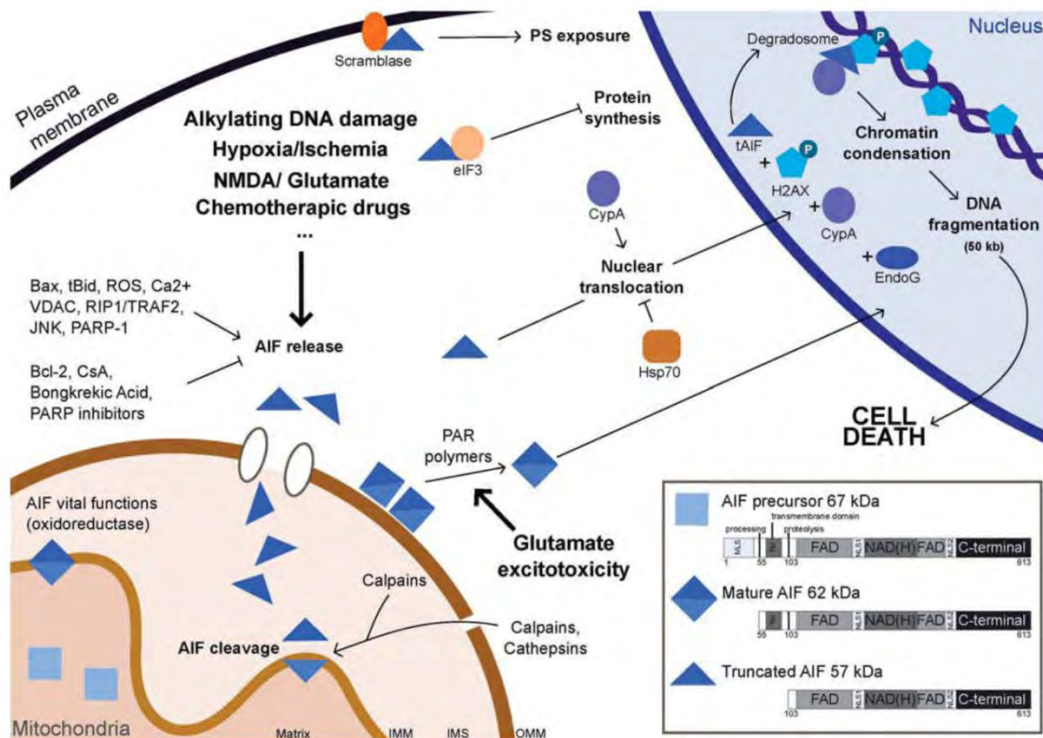


Figure 1.7: Apoptosis inducing factor (AIF) functions. Mature AIF resides in the mitochondria where it has vital functions. Upon cleavage by calpains and cathepsins truncated, lethal AIF translocates to the nucleus inducing cell death by DNA fragmentation. Various proteins either promote or inhibit AIF action (taken from Delavallée et al. (2011)⁵¹).

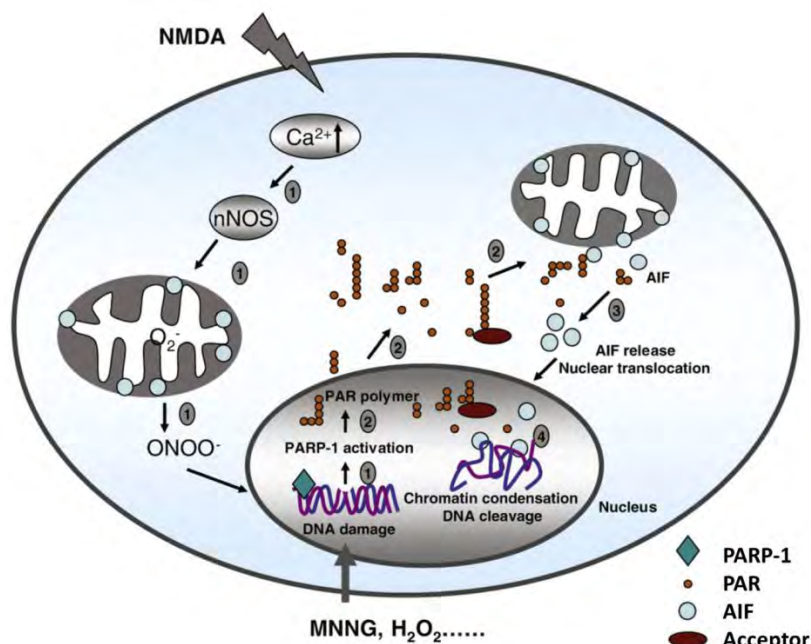


Figure 1.8: Parthanatos. In response to DNA damage poly(ADP-ribose)polymerase 1 (PARP1) is activated leading to the formation of PAR polymers at the expense of ATP. PAR polymers translocate from the nucleus to the mitochondria where they induce the release of apoptosis inducing factor (AIF). AIF then translocates to the nucleus initiating DNA fragmentation and cell death by parthanatos a form of regulated necrosis (taken from Wang et al. (2011)⁵⁹).

1.3.6 LYSOSOMAL MEDIATED CELL DEATH

Depending on the location and impact of a cellular insult, signaling cascades are elicited from different cellular compartments resulting in life or death of the cell^{60,61}. One such cellular compartment that has attracted considerable attention in recent years is the lysosome. Discovered in 1959 and subsequently dubbed “garbage disposal units” and “suicide bags” illustrates that these organelles have both vital and lethal functions⁶².

These acidic, single membrane cytoplasmic organelles form the terminal part of the endosomal pathway involved in endocytosis (plasma membrane receptors, extracellular matrix proteins), phagocytosis (pathogens, cellular debris) and autophagy (long-lived/damaged proteins and organelles)^{63,64}. This requires fusion of the lysosome with these vesicles, late endosomes, phagosomes and autophagosomes respectively; illustrating the dynamic nature of these organelles. Upon fusion, the contents are degraded resulting in the recycling of the hetero- and autophagic cargo. This degradation is mediated by over 50 different hydrolases such as proteases, amylases, lipases, nucleases, glycosidases, phospholipases, phosphatases and sulfatases; able to degrade a large variety of macromolecules, functioning optimally at low pH (3.8-5)⁶⁵.

This high content of degradative enzymes and the ubiquitous nature of lysosomes in a cell poses a potentially dangerous threat to its survival if lysosomal integrity is compromised. The rupture of lysosomes was observed in cells undergoing necrosis and was thought to be a part of the uncontrolled cellular ‘explosion’³⁸. Interestingly, lysosomal ultrastructure was found to be intact in apoptotic cells visualized by electron microscopy⁶⁶ and consequently, it has been shown that massive lysosomal membrane permeabilization (LMP) induces necrosis whereas partial/selective LMP induces caspase-dependent and independent apoptosis and regulated necrosis^{48,49,60,66} (Fig. 1.9). Various inducers of LMP have been identified such as reactive oxygen species, caspases (CASP3, 8, 9), Bax, RIP1, p53, calpains, cathepsin B, sphingosine, iron catalyzed Fenton reactions (inside lysosomes) and treatments/ insults with lysosomotropic agents, microtubule poisons, staurosporine, photodynamic therapy (PDT) and bacterial/ viral proteins⁶⁶⁻⁶⁹. Following LMP, a group of proteases, the cathepsins B, C and L (active at neutral pH) are released into the cytoplasm inducing changes in mitochondria (MOMP) and nuclei (DNA fragmentation)⁷⁰⁻⁷⁴ (Fig. 1.9). MOMP is induced directly or via BID/ Bax resulting in caspase-dependent apoptosis via CYTC and executioner caspases 3,6,7 activation or caspase-independent apoptosis via AIF. However, LMP has also been shown to induce apoptosis-like cell death without activation of the intrinsic apoptotic pathway (with cathepsins acting as initiator and effector proteases) and regulated necrosis such as necroptosis^{48,49}. Furthermore, moderate LMP does not always lead to cell death as the activity of cathepsins can be

negatively regulated by cytosolic inhibitors such as cystatins, serpins and Hsp70, resulting in cell survival⁷⁰. Lysosomes are thus intriguing aspects in the cell death response pathway.

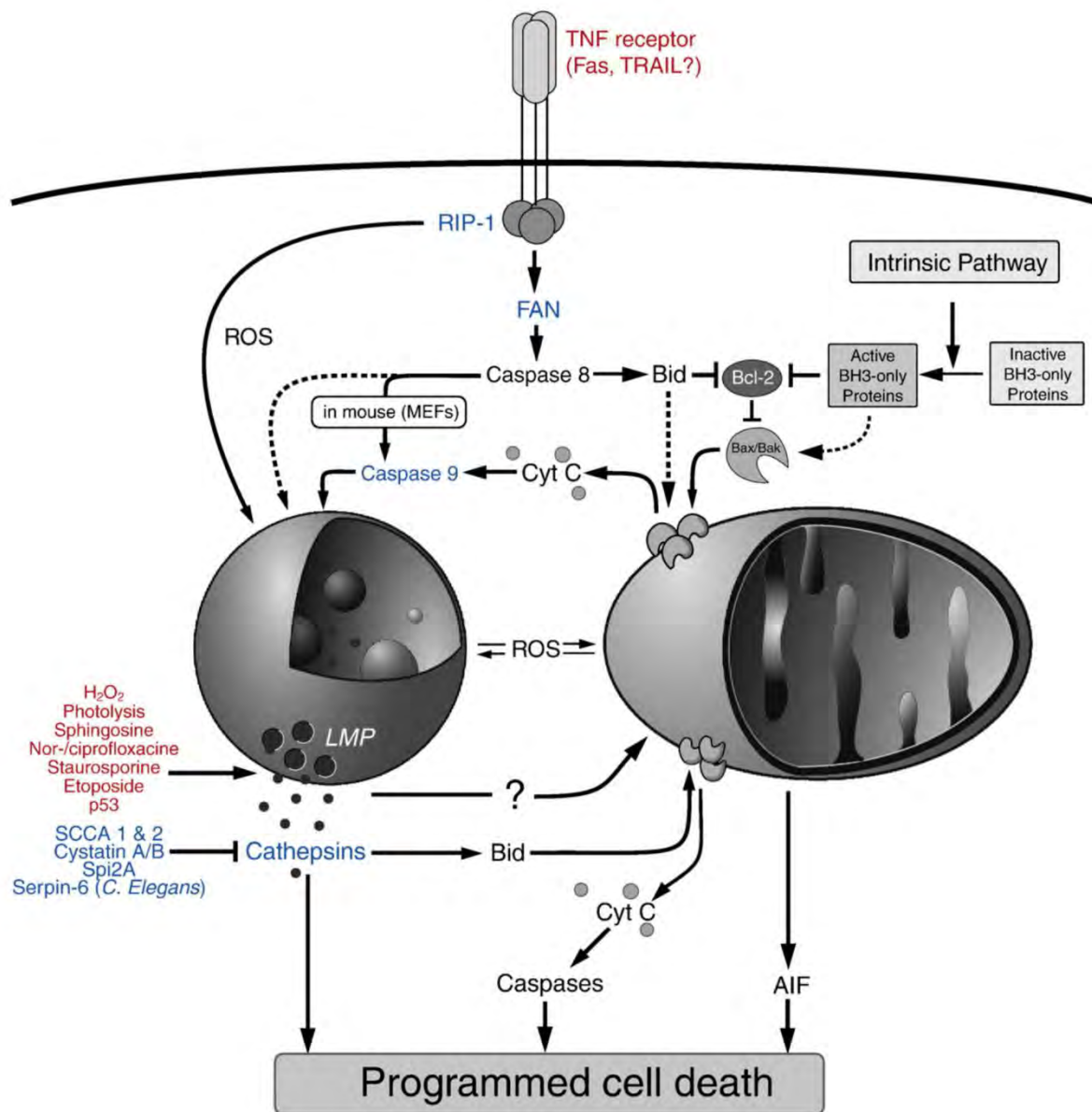


Figure 1.9: Lysosomal-mediated cell death. (see text for details, taken from Kirkegaard et al. (2009)⁶⁶).

Emergence of novel regulated cell death mechanisms has provoked debate whether these can be seen as standalone cell death pathways as many of them use components of the apoptotic pathway. However, from an oncological point of view the essential question is not whether regulated cell death pathways are truly independent but whether they can be utilized to achieve the ultimate goal of killing cancer cells. With this in mind, lysosomes play an important role in both the lysosomal and autophagic resistance mechanisms employed by cancer cells for survival.

Transformation of cells correlates with changes in the lysosomal compartment such as an increase in lysosomal volume, total protease activity and secretion of lysosomal proteases⁶⁶. Changes in cathepsin trafficking and subcellular localization have been associated with tumor growth, invasiveness, angiogenesis and cancer progression⁶⁷. Furthermore, multidrug resistant (MDR) cancers have been shown to harbor enlarged lysosomal compartments as well as enhanced endo/exocytosis for sequestering and exporting chemotherapeutic drugs⁶⁸.

Hsp70, the guardian of lysosomal integrity, is overexpressed in many tumor cells and found to be localized to the endosomal compartment and the cell membrane in these; the latter might sensitize cancer cells to death as Hsp70 serves as a recognition structure for natural killer cells⁶⁶. Cancer cells have also been shown to have a reduced half-life of protective lysosomal membrane LAMP1/2 proteins, thus sensitizing them to LMP⁶⁶. Furthermore, lysosomes of tumor cells can be more vulnerable to ROS due to the accumulation of free iron from enhanced protein turnover due to misfolding⁷⁵. This can result in iron catalyzed Fenton reactions inside lysosomes leading to LMP⁷⁶. It has also been shown that immortalization/ transformation sensitizes cells to lysosomal-mediated cell death⁷⁷. These changes in the lysosomal compartment of tumor cells present an exciting therapeutic avenue for cancer treatment with agents that target lysosomes^{49,68}. In line with this, it has been shown that lysosomotropic agents are able to reverse MDR in human cancer cells⁷⁸.

One of the hallmarks of cancer is the evasion of apoptosis⁷⁹. Dramatic changes in the lysosomal compartment of cancer cells might act as 'a killer from within' via sensitization to lysosomal-mediated cell death. This illuminates the path for anti-cancer therapy by circumventing apoptosis and multi-drug resistance.

1.3.7 CELL DEATH ASSOCIATED WITH AUTOPHAGY

Autophagy was first identified in yeast as a survival pathway⁸⁰ and has subsequently been shown to have a cytoprotective role in cells generating energy by capturing intracellular components for degradation and recycling⁸¹. It forms part of the protein and organelle 'quality control mechanism' in cellular homeostasis, via the formation of autophagosomes which subsequently fuse with lysosomes (autophagic flux) to recycle their cargo. In response to cellular stress, this system removes and recycles damaged proteins and organelles and generates energy for survival. The core molecular machinery of autophagy is a highly heterogeneous class of proteins named the autophagy-related genes (ATGs) which are tightly regulated⁸².

Accumulating evidence reveals the active contribution and connection of autophagy with several cell death pathways^{83,84}. The term 'cell death associated with autophagy' has been used for cells with massive cytoplasmic vacuolization, mediated by autophagy if autophagy inhibition results in reduced cell death³⁶. This could be a result of increased autophagic flux or inhibition of the degradation of autophagosomes, causing an accumulation of vacuoles. By using autophagy inhibitors, such as bafilomycin A, which prevents fusion of the autophagosomes and lysosomes, these processes can be characterized.

Autophagy is a double-edged sword in cancer cells with both tumor suppressive and tumor promoting effects⁸⁵. It improves the overall fitness of cells, preventing cellular damage and tumorigenesis by removal of oncogenic protein substrates, toxic unfolded proteins and damaged organelles (tumor suppressive) whereas it keeps tumor cells alive once they are established through autophagy-mediated intracellular recycling, providing substrates for metabolism and a functional pool of mitochondria (tumor promoting). Furthermore, cellular levels of ROS which promote LMP and necroptosis, can be lowered by the removal of damaged organelles through autophagy but the same process can also degrade ROS dissipating enzymes such as catalase which subsequently sensitizes cells to ROS⁴⁹. The completion of autophagy relies on the fusion of autophagosomes with lysosomes (autophagic flux). If this flux is inhibited, damaged proteins and organelles accumulate in the cell, posing a threat to its survival. This illustrates the potential of agents that target lysosomes in overcoming the tumor promoting effects of autophagy in cancers. Concomitantly, late-stage autophagy inhibitors which prevent autophagic flux, have been shown to induce LMP resulting in lysosomal-mediated cell death⁶⁸.

1.4 HYPERICIN

1.4.1 ORIGIN, CHEMICAL CHARACTERISTICS & SUBCELLULAR LOCALIZATION

Hypericin, a naturally occurring photosensitizer, belongs to the chemical class of naphthodianthrone and is part of the chromophoric system of phenanthroperylene quinones, occurring in plants of the genus *Hypericum*⁸⁶. This genus comprises approximately 450 species of which St John's Wort (*Hypericum perforatum*, Fig. 1.10) is the most medicinally and commercially recognized⁸⁷.

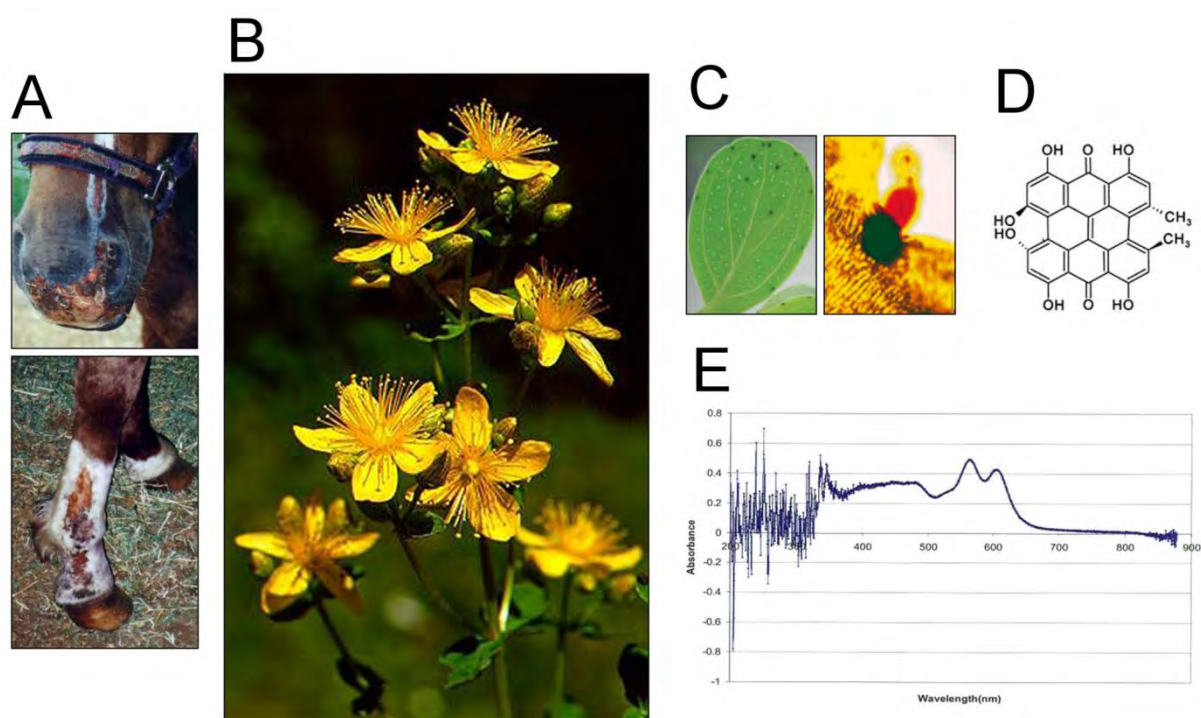


Figure 1.10: St John's Wort (*Hypericum perforatum*) and hypericin. Animals feeding on the plant (B) develop severe photosensitization effects of the skin upon exposure to sunlight (A, Hypericosis). Dark glands found in the leaves and petals (C), containing red hypericin (D), which absorbs light of ultraviolet and white light wavelengths (E).

St John's Wort is widely distributed around the world and has been used since antiquity for its myriad of medicinal properties (for a description of the cultural history see Appendix B)⁸⁸. It is a small, perennial aromatic herb growing up to one meter tall. Its leaves are characterized by the presence of multiple small translucent oil glands which give it a perforated appearance when held against the light. It has multiple bright yellow flowers and its calyx and corolla are marked with numerous dark glands which make the fingers red when rubbed between them. Animals feeding on this plant develop severe photosensitization effects of the skin when exposed to sunlight, a condition called hypericosis⁸⁹. This effect can mainly be attributed to the naphthodianthrone hypericin synthesized in the dark glands of this plant, one of the most powerful naturally occurring

photodynamic agents⁹⁰. The naphthodianthrone content of *H. perforatum* is quite low however (0.05-0.3%)⁹¹ and extraction processes are relatively costly as they require multiple cycles and fast handling of material⁹². Hypericin has also been found in endophytic fungi such as *Dermocybe* which grow in *Hypericum* species; possibly due to plant-microbe interactions leading to horizontal gene transfers and genetic recombination⁹². This could present an opportunity for the alternative production of hypericin which would be low-cost, enable use of renewable resources and produce reproducible results by fermentation technology⁸⁷. Hypericin was first isolated in 1939⁹³ and can be chemically synthesized by base catalyzed oxidative dimerization of emodium anthrone⁸⁶. The enzyme Hyp-1, catalyzing the conversion of emodin to hypericin with 84.6% efficiency, has been cloned and subsequently expressed in *Escherichia coli*⁹⁴.

Hypericin is labile, especially under light⁹². This highlights the necessity to work with hypericin under subdued light conditions. In organic solvents such as dimethylsulfoxide (DMSO), hypericin is present as a monoanion, tending to form monobasic salts⁸⁶. Hypericin salts form cherry-red solutions in organic solvents (absorbance λ_{max} 548 and 591nm in ethanol) with characteristic red fluorescence (λ_{max} 594 and 642nm in ethanol)⁸⁶. In aqueous solutions hypericin forms non-fluorescent high molecular weight aggregates⁹⁵. Notably, hypericin associates strongly with a large variety of proteins such as low- and high-density lipoproteins (LDL, HDL)^{96–99}, human serum albumin^{100–103} and amyloid fibrils¹⁰⁴ as well as macromolecules like PVP (poly vinyl pyrrolidone)^{105,106} causing the formation of red fluorescent monomers. Association of hypericin with these proteins and macromolecules as well as its encapsulation in guanidinylated liposomes¹⁰⁷, PEG-liposomes¹⁰⁸, PEG-cholesterol micelles¹⁰⁹ and nanoparticles^{110–112} facilitates its transport *in vivo* and transforms hypericin aggregates into the monomeric fluorescent state. Furthermore, the association of hypericin with proteins such as LDL enables the preferential uptake in tumor versus normal tissue *in vivo*, due to upregulated expression of LDL surface receptors on cancer cells related to their increased metabolism^{79,113}. *In vitro* however, hypericin is taken up by a variety of both normal (e.g. keratinocytes and melanocytes^{114,115}, fibroblasts¹¹⁶, mouse embryonic fibroblasts¹¹⁷, T lymphocytes¹¹⁸) and cancerous cells (e.g. bladder cancer¹¹⁹, squamous cell carcinoma^{120,121}, nasopharyngeal carcinoma¹²², Hela cervix carcinoma¹²³, glioma¹²⁴ and melanoma^{125–127}).

Hypericin is lipophilic and able to enter cells via passive diffusion or by receptor-mediated endocytosis when associated to LDL/ HDL^{96,113,128}. In addition, cholesterol has been shown to be a molecular determinant of hypericin uptake into cell membranes, as it is able to partition between cholesterol-rich lipid domains¹²⁹. The red fluorescent property of hypericin can be excellently combined with co-labeling of cells with fluorescent dyes specific for a subcellular organelle and

subsequent visualization with conventional or confocal fluorescence microscopy imaging and analyses.

Using these techniques, hypericin has been shown to exhibit a general association with lipid membranes and a perinuclear cytoplasmic localization. In a variety of cellular systems, hypericin co-localized with the cell membrane^{130,131} and membranes of various intra-cellular organelles, such as the endoplasmic reticulum (ER)^{107,117,120,128,132,133}, golgi apparatus^{107,117,132,133}, mitochondria^{107,124,134} and lysosomes^{113,117,124,128,135}. Hypericin has only been associated with the nucleus after prolonged incubation time¹³¹ and hardly after short incubation times¹⁰³. In addition, the hypericin-DNA interaction is very weak, thus the probability for genotoxicity is very low^{103,136}. It is evident from these studies that the intracellular localization of hypericin depends on a variety of factors such as the cell type, concentration of hypericin, incubation time and presence of serum proteins. For example, HeLa, T24 and MEF cells, incubated for 16 hours with 0.5 μ M hypericin, showed ER and lysosomal hypericin localization¹¹⁷. Glioblastoma cells (U373 MG), incubated with 1 μ M hypericin for 2 hours also revealed ER localization of hypericin¹³⁷. A higher concentration of hypericin (5 μ M) resulted in mitochondrial, ER and golgi hypericin localization in DU145 human prostate cancer cells after an incubation of 1 hour¹⁰⁷. However, the same hypericin concentration (5 μ M) showed a lysosomal hypericin localization after 3 hours in PAM212 murine keratinocytes¹³⁸.

Hypericin is characterised by a wide excitation range from 260-600 nm, containing several absorbance peaks (Fig. 1.9). Fluorescence emission occurs around 595nm⁸⁶. Light penetration into biological tissue of wavelengths below 630 nm is limited and attempts to shift the absorption spectrum of hypericin by chemical modification have been effective; however the resulting photosensitizers were not as potent as hypericin¹³⁹. Hypericin hydroquinone on the other hand, might be a compound with potential as a far-red photosensitizer as it produced a red light activated phototoxic response similar to that of hypericin¹⁴⁰. These new hypericin compounds thus provide hope for light activation at deeper tissue levels. The current potential of hypericin in therapy therefore lies in the identification and treatment of cutaneous malignancies and superficial lesions.

An *in vivo* study by Blank et al. (2002) on C26-derived tumors, revealed extensive vascular damage and tumor necrosis at 6 hours after intraperitoneal administration of hypericin (5 mg/kg)¹⁴¹. The depth of tumor necrosis was more pronounced at 590 than at 550 nm, with maximal depths of tumor necrosis (at 120 J/cm²) of 7.5+/-1.5 mm at 550 nm and 9.9+/-0.8 mm at 590 nm. These values were found to be rather high in view of the limited penetration of green-yellow light into the tissue. Moreover, these findings indicate that *in vivo* the depth at which hypericin-PDT elicits tumor

necrosis is substantially influenced by mechanisms such as vascular effect and not only determined by photophysical considerations (light penetration, number of absorbed photons)¹⁴¹.

Preferential accumulation of hypericin in cancerous tissue has attracted attention for its excellent use in the fluorescent photodynamic diagnosis (PDD) of cancer with various optical probes¹⁴². Ongoing clinical work has shown that hypericin has potential for the treatment of bladder cancer as it accumulates specifically in urothelial carcinoma lesions and is safe and efficacious as a diagnostic tool when intravesically administered^{106,143}. Hypericin has also been shown to selectively accumulate in malignant glioma *in vivo* after systemic administration in rats¹⁴⁴ and thus has potential for the intraoperative visualization and photodynamic therapy of glioma and possibly medulloblastoma. Interestingly, modification of hypericin has given rise to radioiodinated hypericin, a promising necrosis avid tracer for the non-invasive diagnosis of necrosis-related pathologies¹⁴⁵. Its prominent targetability to necrosis furthermore enables targeted radiotherapy following a necrosis-inducing treatment in the sequential dual-targeting theragnostic strategy (SMSDTTS)¹⁴⁶.

Upon activation by light, hypericin elicits a type II oxygen dependent photosensitization mechanism leading to the formation of singlet oxygen ($^1\text{O}_2$)¹⁴⁷. The high triplet quantum yield and efficient $^1\text{O}_2$ production from the lowest excited triplet state of hypericin was verified early on by laser spectroscopy^{148,149}. Hypericin has however also been shown to produce other reactive oxygen species (ROS) such as the photogenerated semiquinone radical anion^{150,151}, superoxide anion¹⁵⁰, reactive nitrogen species (RNS)¹⁵² and hydrogen peroxide (H_2O_2)¹³⁴. The extremely short half-lives and small radii of diffusion of these ROS implicate rapid interaction with biological targets in vicinity of their generation¹⁵³. Hydrogen peroxide is an exception to this and is able to diffuse through cell membranes acting as a messenger between cells¹⁵⁴. The subcellular localization of a photosensitizer is thus a critical determinant on the primary site of photodamage and activation of subsequent signaling pathways.

To date, hypericin is recognized as an anti-depressant¹⁵⁵, anti-bacterial^{156–159}, anti-fungal¹⁶⁰, anti-viral^{161–164}, anti-analgesic^{165,166} and anti-cancer agent^{92,153,167–169}. Hypericin is a potent phototoxin when activated by light, eliciting a multifaceted cytotoxicity response. A general trend in the literature is a concentration and light dose dependent cytotoxicity ranging from apoptosis and autophagy-related cell death to necrosis¹⁶⁹. Low doses of hypericin and/ or light have been shown to activate cell survival pathways¹⁶⁸. Hypericin has also been shown to have cellular effects in the dark, however these could be due to moderate exposure to ambient light as comments on the experimental light conditions are lacking in many publications. Nevertheless, these dark effects add to the powerful cytotoxic characteristics of this potent photosensitizer. Furthermore, hypericin is

able to perform an intra-molecular proton (or H atom) transfer, thus acidifying its surroundings resulting in a pH drop^{103,118,170}. A more detailed description of the above mentioned hypericin characteristics are outlined below.

1.4.2 THE DARK SIDE OF HYPERICIN

In the dark, i.e. unirradiated, hypericin has been shown to be tumor selective^{128,171,172}, cytostatic¹⁷¹, anti-metastatic¹⁷³, anti-angiogenic^{174,175}, anti-enzymatic¹⁷⁶, substrate of mitochondrial complex III¹⁷⁷ and an efficient pathway modulator^{120,168}. These responses were observed with high concentrations of hypericin (10µM and more) and may thus be negligible at lower concentrations.

Hypericin has been shown to selectively accumulate in lung metastases of two murine cancer models resulting in the reduction of tumor growth and increased survival, demonstrating the anti-metastatic activity of hypericin in the absence of irradiation¹⁷³. Hypericin content in lungs bearing metastases was approximately 2-fold higher than in the lungs of healthy animals, thus indicating minimal side effects on normal tissue.

In vitro, hypericin accumulation in bladder cancer cells correlated with loss of E-cadherin and thus a loss of intra-cellular adhesion¹⁷⁸. Hypericin furthermore accumulated specifically in undifferentiated compared to differentiated colon carcinoma cells, highlighting its potential to treat poorly differentiated tumors¹⁷⁹. Unactivated hypericin has been shown to inhibit various signaling modulators *in vitro* such as protein kinase C (PKC)^{180,181}, epidermal growth factor (EGF) binding¹⁸², EGF receptor tyrosine kinase activity^{183–185}, NF-κB and NF-κB proteosomal activity^{186,187}.

In addition, angiogenesis inhibition by high concentrations of hypericin (10-20 µM), including bovine aorta endothelial cell proliferation, tubular formation on matrigel, extracellular matrix degradation by urakinase, migration and invasion has also been demonstrated¹⁷⁴. These results indicate that hypericin potentially inhibits angiogenesis of non-cancerous tissue without light activation. However, these experiments employed bovine aorta endothelial cells and thus need to be verified for human endothelial cells.

Hypericin-induced loss of Hsp90 chaperone function furthermore resulted in destabilization and degradation of heat shock protein 90 (Hsp90) client proteins such as p53, cdk4, Raf1 and PIK and enhanced Hsp90 ubiquitination blocked the Ras/Raf pathway, resulting in proliferation arrest of murine DA3 mammary adenocarcinoma and SQ2 squamous cell carcinoma cells¹⁷². This could also

have implications for Hsp70 client proteins as it was shown that Hsp90-Hsp70 intermediate complex formation was prevented by hypericin¹⁸⁸.

In the same report the authors showed the enhanced degradation of hypoxia-inducible factor 1 α (HIF-1 α) in human glioblastoma and renal carcinoma cells and in the non-malignant ARPE19 retinal pigment epithelial cell line. HIF-1 α is the regulatory precursor of the HIF-1 transcription factor which promotes hypoxic stress and angiogenic responses¹⁸⁸. This process occurred independently of cellular oxygen levels and was mediated by lysosomal cathepsin-B enzymes with cathepsin-B activity, optimized in the cells through the hypericin-mediated reduction in intracellular pH. The hypericin-accelerated turnover of HIF-1 α overcomes the physiologic HIF-1 α protein stabilization which occurs in hypoxic cells.

Moreover, hypericin treatment without light activation downregulated cytochrome P450 IBI (CYP1B1) expression in squamous cell carcinoma cells, a natural tumour marker due to its overexpression in tumour cells supporting the cells resistance to many therapeutic drugs¹²⁰.

These unirradiated high-dose hypericin (≥ 10 μ M) responses including the tumour selective, cytostatic and survival pathway inhibitory characteristics, demonstrate its potential as a systemic antitumor agent concomitantly to PDT. Moreover, the *in vivo* association of hypericin with proteins such as LDL enables the preferential uptake in tumor versus normal tissue, due to upregulated expression of LDL surface receptors on cancer cells related to their increased metabolism^{79,113}. This indicates that the observed hypericin effects without light activation may be negligible on non-cancerous tissue *in vivo*.

1.4.3 THE LIGHT SIDE OF HYPERICIN

Photo-activated hypericin exhibits a multifaceted phototoxicity profile inducing cancer cell death^{92,167,168,189}. *In vivo* hypericin-PDT targeted both the vascular system of the tumour and its cells resulting in tumour elimination^{190–193}. Furthermore, hypericin-PDT has been implicated as a potent anticancer immunogenic modality as it has recently been shown to cause surface exposure of damage-associated molecular patterns (DAMPs) and induce immunogenic cell death *in vitro*^{194–196}. Targeting the vascular, endothelial and epithelial tumour cells, the tumour matrix and the immune system demonstrates the potency of hypericin-PDT. Inevitably a cell exposed to stress tries to defend and rescue itself from such stress in an attempt to survive. However if these attempts are futile, cell death can be initiated in regulated ways.

1.4.3.a CELLULAR DEFENCE AND RESCUE STRATEGIES IN RESPONSE TO HYPERICIN-PDT

A cell exposed to hypericin-PDT employs several defence and rescue strategies. These include engagement of the cellular antioxidant system and upregulation of survival pathways in an attempt to repair the cellular damage and survive the insult.

An increase in intracellular antioxidant systems, i.e. catalase, glutathione peroxidase and superoxide dismutase (SOD) and depletion of glutathione (GSH) was observed in human melanoma cells following hypericin-PDT¹²⁷. GSH depletion within an hour of hypericin-PDT has also been observed in nasopharyngeal carcinoma cells¹³⁴. In another study however, comparable SOD and GSH levels were observed between control and treated cells, immediately after irradiation¹⁹⁷. In addition, activation of CuZnSOD, Se-dependent glutathione peroxidase, glutathione S-transferase and glutathione reductase was documented in mouse mammary carcinoma cells after hypericin-PDT¹⁹⁸. Hypericin-PDT has also been shown to downregulate glutathione S-transferase activity in nasopharyngeal cancer cells¹⁹⁹. The difference in these results may be due to measuring antioxidant levels at different times after irradiation.

Activation of several survival pathways in response to hypericin-induced photostress has also been described. These include: c-Jun N-terminal kinases (JNK1) and p38 mitogen activated protein kinases (p38^{MAPK}) with irreversible inhibition of extracellular regulated kinase 2 (ERK2) in HeLa cervical carcinoma cells²⁰⁰, p38^{MAPK} and heme-oxygenase 1 (HO) in bladder cancer cells^{119,201} and p38^{MAPK}, ERK, JNK, Ras, and nuclear receptor (NOT or NR4A2) in squamous cell carcinoma cells¹²⁰.

Other important proteins such as matrix metalloproteinases (MMPs) involved in matrix remodelling, have been found to be either upregulated in bladder cancer and nasopharyngeal carcinoma cells (MMP1, 10, 13)^{119,202} or downregulated in well-differentiated nasopharyngeal carcinoma cells

(MMP9)¹²². Hypericin-PDT also stimulated proteins crucial for vascularisation and inflammation. These include stimulation of VEGF in a nasopharyngeal carcinoma cell line²⁰³, upregulation of VEGF, TNF α , IFN α , bFGF and downregulation of adhesion molecules in a human bladder carcinoma xenograft model²⁰⁴ and stimulation of pro-inflammatory cytokine expression such as interleukin-6 (IL-6)²⁰⁵. Pharmacokinetics of hypericin have been shown to be affected by the expression of the ABC transporters multidrug resistance associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) in HT29 colon cancer cells in response to hypericin-PDT²⁰⁶.

Photodynamic therapy results in photodamaged proteins and organelles and it is thus not surprising that in an attempt to clear these damaged toxic cellular components, cellular autophagy is induced. Hypericin-PDT resulted in the stress-specific recruitment of both macroautophagy and chaperone-mediated autophagy with the latter forming a dominant defence mechanisms against PDT, in normal and cancerous cells¹¹. These findings display the versatility of a cell in response to hypericin-induced photostress. Lethal damage however, overcomes these survival responses and elicits a very effective multifaceted death execution.

1.4.3.b CELL DEATH MECHANISMS

Several different cell death mechanisms have been observed in response to hypericin-PDT, including activation of intrinsic/extrinsic apoptosis, necrosis and cell death associated with autophagy^{92,167}. These cell death mechanisms are, at least in part, dependent on different uptake mechanisms, the subcellular localization of hypericin and the cell type.

1.4.3.b.i HYPERICIN AND THE ENDOPLASMIC RETICULUM

Hypericin induced photodamage to the ER has been shown to involve oxidative damage to the sarco(endo)plasmic Ca²⁺ ATPase (SERCA)2 pump¹¹⁷. SERCA2 couples ATP hydrolysis to calcium (Ca²⁺) transport from the cytosol into the ER, maintaining the physiological levels of Ca²⁺ in the ER 3-4 orders of magnitude above that of the cytosol. Destruction of SERCA and subsequent depletion of the ER Ca²⁺ pool furthermore induced the unfolded protein response sensor (UPR) PKR-like endoplasmic reticulum kinase (PERK)^{117,119,207}. The unfolded protein response is initiated to reduce the accumulation and aggregation of unfolded or misfolded proteins in order to restore normal ER function; in this case however, cells died via a PERK-dependent cell death mechanism involving downregulation of the miR-106b-25 cluster and upregulation of the Bcl-2 homology 3-only family member Bim in ER-photostress induced apoptosis²⁰⁸. In addition, photogenerated ER-ROS induced initial reticulophagy followed by propagation to mitochondria (via contact sites between mitochondria and the ER), resulting in mitophagy and apoptosis²⁰⁹. In line with this it was shown that

PERK was required as a component of mitochondria-associated ER membranes to maintain the ER-mitochondria juxtapositions and advance ROS-mediated mitochondrial apoptosis in response to ROS-mediated ER stress²⁰⁷. Similarly, cells deficient of Bax/Bak inhibited apoptosis after ER-stress induced hypericin-PDT photodestruction of SERCA2, triggering a caspase-independent mode of cell death utilizing aberrant macroautophagy for self-killing^{117,189}.

1.4.3.b.ii HYPERICIN AND MITOCHONDRIA

Mitochondria are the main source of ROS and ROS signalling in non-inflammatory cells as well as producing ATP via oxidative phosphorylation²¹⁰. This involves the shuttling of electrons along inner mitochondrial membrane complexes via multiple transfer sites to provide and sustain a proton (H^+) gradient between the mitochondrial intermembrane space (IMS) and the matrix. This proton gradient generates and sustains the mitochondrial membrane potential ($\Delta\Psi_m$) which is dissipated by F_1F_0 -ATP synthase as it ferries one proton back into the mitochondrial matrix per ADP molecule which it phosphorylates to ATP.

Light-activated hypericin has been shown to induce direct mitochondrial impairment by targeting complex III of the electron transport chain, resulting in damage to the quinone reducing center, making several enzymes of the TCA cycle (e.g. aconitase, succinoxidase) also located on the matrix side possible targets^{177,197}. Indeed, both aconitase¹⁹⁷ and succinoxidase^{211,212} are depleted and/or inhibited by light-activated hypericin. Furthermore, activation of reverse pumping of F_1F_0 -ATP synthase in an attempt to maintain the $\Delta\Psi_m$ at the expense of ATP, results in ATP depletion after hypericin-PDT^{170,177}.

Phototoxic hypericin has also been shown to affect an outer mitochondrial target, hexokinase (HK), resulting in its dissociation from mitochondria¹⁷⁰. This was observed downstream of an intracellular pH decrease (0.6-0.8 pH unit) followed by an increase in cytoplasmic HK activity and a decrease in ATP content, reflecting its severe impact on energy metabolism¹⁷⁰. HK associates with the porin voltage-dependent anion channel (VDAC) in mitochondria and catalyses the phosphorylation of glucose to glucose-6-phosphate in the glycolytic pathway. Association of HK with VDAC furthermore suppresses the release of intermembrane space proteins and thus inhibits apoptosis²¹³. Support of the glycolytic phenotype (Warburg effect) and apoptosis inhibition are both hallmarks of cancer cells²¹⁴; the interaction of HK with VDAC is thus the target for many anticancer drugs in an attempt to reverse the 2 hallmarks mentioned²¹⁵. The hypericin-PDT induced dissociation of HK from mitochondria is therefore very favourable for anticancer treatment.

The exact correlation between hypericin-induced HK-VDAC dissociation and onset of apoptosis has not been investigated; however it is possible that VDAC becomes accessible after HK dissociation for pro-apoptotic proteins to bind to, such as Bax, promoting mitochondrial outer membrane permeabilization (MOMP), followed by release of the intermembrane space proteins and mitochondrial apoptosis.

Bax/Bak has been shown to be a mediator in hypericin-PDT induced cell death as Bax^{-/-}/Bak^{-/-} double knockout (DKO) cells die in a caspase-independent fashion¹¹⁷. Re-expression of Bax in DKO cells abrogates this and switches the caspase-independent mode back to mitochondrial-mediated apoptosis¹¹⁷. The latter is mainly, but not entirely, dependent on intrinsic caspase-mediated apoptosis as it is only partially inhibited by caspase antagonists¹¹⁷. This suggests the release of other toxic mitochondrial intermembrane space proteins (such as AIF) along with cytochrome c (CYTC) from mitochondria after MOMP, orchestrating caspase-independent apoptosis.

Involvement of the intrinsic apoptotic cascade including dissipation of the mitochondrial membrane potential followed by release of CYTC from mitochondria and activation of initiator CASP9 and executioner CASP3 after hypericin-PDT, has been documented in various cancer cells *in vitro*^{92,167–169}.

Photoactivated hypericin has also been shown to induce the extrinsic pathway of mitochondrial apoptosis. In Jurkat T-lymphocytes, TRAIL receptor-mediated apoptosis takes place with the activation of CASP8²¹⁶. In nasopharyngeal carcinoma cells, Fas, Fas-FADD and CASP8 activation followed by Bid cleavage and engagement of mitochondrial apoptosis was observed²¹⁷. Fas and Fas ligand were furthermore upregulated in the RIF1 mouse tumour model¹⁹¹. In contrast to these findings, with respect to CASP8, its inhibition had no effect on HeLa cervical carcinoma cells apoptosis²⁰⁰. Moreover, CASP8 activation was not observed in a variety of both murine and human cancer cell lines^{200,218–220}. Differences in these results could be related to the subcellular localisation of hypericin. Conditions favouring the plasma membrane retention could cause production of photogenerated ROS in the vicinity leading to receptor-mediated apoptosis.

1.4.3.b.iii HYPERICIN AND LYSOSOMES

Lysosomal membrane permeabilization (LMP) results in the release of toxic factors, has been implicated in triggering regulated cell death mechanisms such as apoptosis and necrosis^{49,63}. Lysosomal integrity was assessed after hypericin-PDT in human prostate carcinoma cells by monitoring the activity of hexosaminidase, usually found in lysosomes and released into the

cytoplasm following their rupture. Hexosaminidase activity was found to be constant in the cytoplasm over 24 hours and accounted for 25 % of the total activity which did not correlate with the very rapid and complete loss of mitochondrial function¹⁹⁷. Similarly, hypericin-PDT did not affect lysosomal stability¹¹⁷. The same study showed that inhibition of lysosomal hydrolases did not affect mitochondrial CYTC release and overall apoptosis in MEF and HeLa cells, suggesting a lysosomal independent cell death pathway¹¹⁷. In another study however, hypericin associated to LDL was found in its active fluorescent monomer form in the lysosomes of glioma cells and the observed mitochondrial membrane permeabilization was suggested to be a consecutive event of the primary photo-induced lysosomal damages¹²⁴.

1.4.3.b.iv HYPERICIN AND NECROSIS

Cell death by necrosis has been described after hypericin-PDT in various cell types, usually at higher treatment doses^{92,167–169}. Direct photosensitization of mitochondria can result in rapid imbalance of mitochondrial bioenergetics, ROS overproduction and possibly direct compromise of mitochondrial membranes, conditions that have been linked with primary necrosis⁹. However, for photosensitizers localizing to lysosomes, resulting LMP induction could be linked to apoptosis or regulated necrosis. An increase in Hsp70 mRNA and protein levels has been observed in bladder cancer cells after hypericin-PDT treatment¹¹⁹; whether this is a cytoprotective mechanism to stabilize lysosomal membranes to survive the insult still needs to be verified experimentally.

1.4.3.b.v HYPERICIN AND IMMUNOGENICITY

Recent reports investigated the potential of hypericin-PDT as an immunogenic anticancer modality. In these studies, the hypericin-induced ROS-mediated ER stress resulted in surface exposure of damage-associated molecular patterns (DAMPs), such as HSP70 and calreticulin (ecto-CALR), followed by phagocytosis of the cancer cells by dendritic cells in the absence of ERp57¹⁹⁶. Hypericin-PDT furthermore induced immunogenic cell death (ICD) in cancer cells, characterized by phenotypic maturation of dendritic cells as well as induction of a protective antitumor immune response¹⁹⁵. These processes involved a novel PERK-dependent subroutine for the early and simultaneous emission of two critical DAMPs (surface-exposed CRT and secreted ATP), following hypericin-PDT induced, ROS-mediated ER stress¹⁹⁵. Moreover, a role of ROS-induced autophagy assisting in the evasion from determinants of ICD by weakening the functional interaction between dying cancer cells and the immune system, was identified²²¹.

To reiterate, the multifaceted phototoxicity profile of hypericin is vast and may depend on various factors such as cell type, hypericin concentration, subcellular localisation and activating light source

(including type, wavelength, dose, irradiation time, fluence rate). The versatility of inducing different modes of cell death highlights the potential of this powerful photosensitizer for the treatment of cancer. A summary of hypericin-induced cellular responses is shown below (Fig. 1.20).

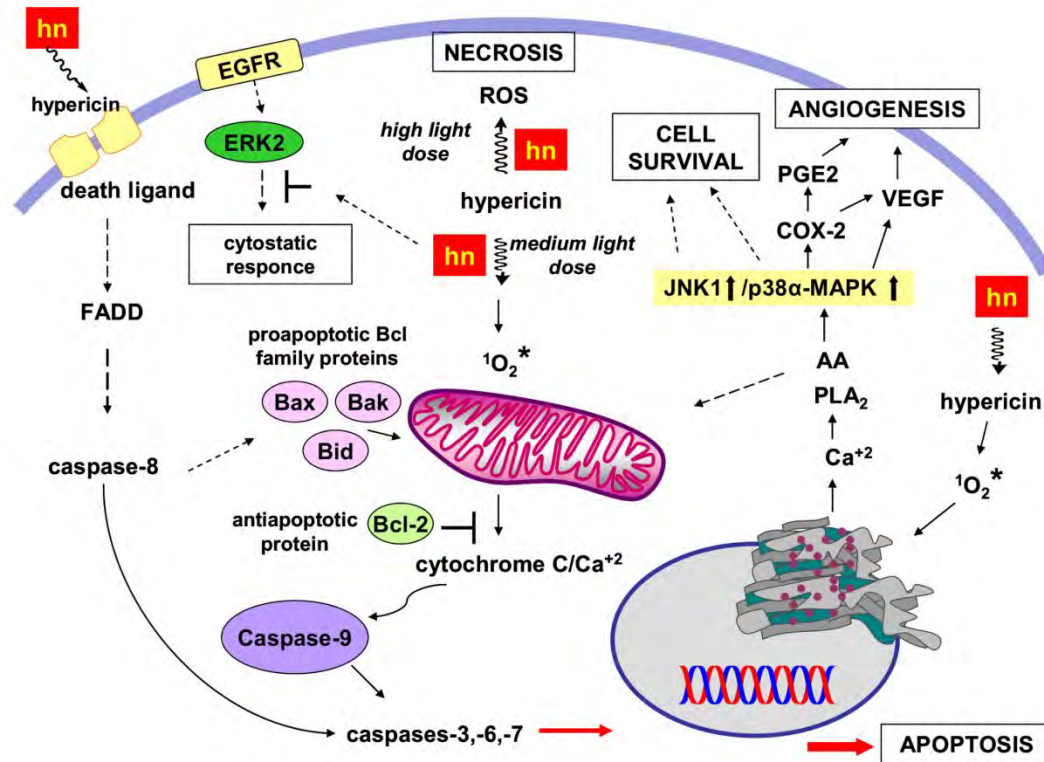


Figure 1.11: Hypericin induced cell death and survival pathways. (taken from Karioti et al. (2010)⁹²)

1.5 MELANOMA

1.5.1 SKIN AND MELANOCYTES

The skin is the body's largest organ and provides us with a physical barrier to the external environment. It forms a protective shield against ultraviolet (UV) radiation via a UV-absorbing pigmentary system and prevents desiccation by storing water and fat. Furthermore, it is involved in the protection against mechanical, chemical and microbial insults and forms part of the body's thermal regulation and vital immuno-detection system²²². The skin is generally divided into three distinguishable layers, the external epidermis, underlying dermis and deep sub-cutis or hypodermis²²² (Fig. 1.12A). The epidermis is characterised as an external, avascular, stratified epithelium disrupted by pores of hair follicles and glands. The epidermis is separated from the dermis by a basement membrane and consists of layers of keratinocytes and melanocytes. The dermis is a connective tissue layer which consists of dense fibrous and elastic components produced by fibroblasts with associated ground substance. It contains the vascular, neural and lymphatic systems of the skin with hair follicles, sensory nerve receptors, excretory and secretory glands. The deepest layer, the hypodermis/ sub-cutis, forms the connection to the underlying skeletal components and consists of fatty connective tissue.

The neural crest derived melanocytes are found in the basal layer of the epidermis and in the hair follicles of the dermis²²³ (Fig. 1.12). They have important functions such as photoprotection, combating photooxidative stress and sequestering excess metal ions²²⁴. Melanocytes have multiple, long dendrites which associate with keratinocytes in a series of networks. In the hair follicle and the basal layer of the epidermis (i.e. on the basement membrane), the melanocyte to associated keratinocyte ratio is 1:5, whereas in the top of the epidermis it is 1:36²²² (Fig. 1.12B). This association results in the photoprotection from UV radiation of keratinocytes and underlying tissue, brought about by the pigment melanin. Melanin is produced by melanocytes via an ancient phylogenetically conserved pathway called melanogenesis²²⁵ (Fig. 1.12B/C). This occurs by the conversion of tyrosine to melanin in a series of oxidation reactions, involving the rate limiting enzyme tyrosinase and tyrosinase related proteins 1 and 2. Toxic intermediates such as 5, 6-dihydroxyindole and 5, 6-dihydroxyindole-2-carboxylic acid, quinones, indole-quinones and hydrogen peroxide are produced during melanin synthesis²²⁶. The melanocyte protects itself from these toxic intermediates by structurally confining melanogenesis to an organelle, the melanosome²²⁷ (Fig. 1.12C). Endogenous melanogenic cytotoxicity can however occur if these toxic intermediates leak out of the melanosome into the cytoplasm. To circumvent this, Chen et al. (2009) have hypothesised that melanocytes use

the ATP-binding cassette (ABC) transporter system (e.g., ABCB5 and ABCC2) to regulate homeostasis by trapping toxic intermediates into subcellular organelles such as small vesicles, endosomes, lysosomes and melanosomes²²⁸. These are then released from the cell by various mechanisms including exocytosis and melanosome export.

Melanosomes are produced in melanocytes in four distinct stages (I-IV) and melanin is formed in the last two stages of melanosome biogenesis^{223,226,229} (Fig. 1.12C). Once mature and filled with the black, electron-dense melanin pigment on a fibrillar matrix, melanosomes are transferred to associated keratinocytes via the long dendritic processes of melanocytes. Melanosomes cluster predominantly above keratinocytes nuclei for protection against UV-induced DNA photodamage.

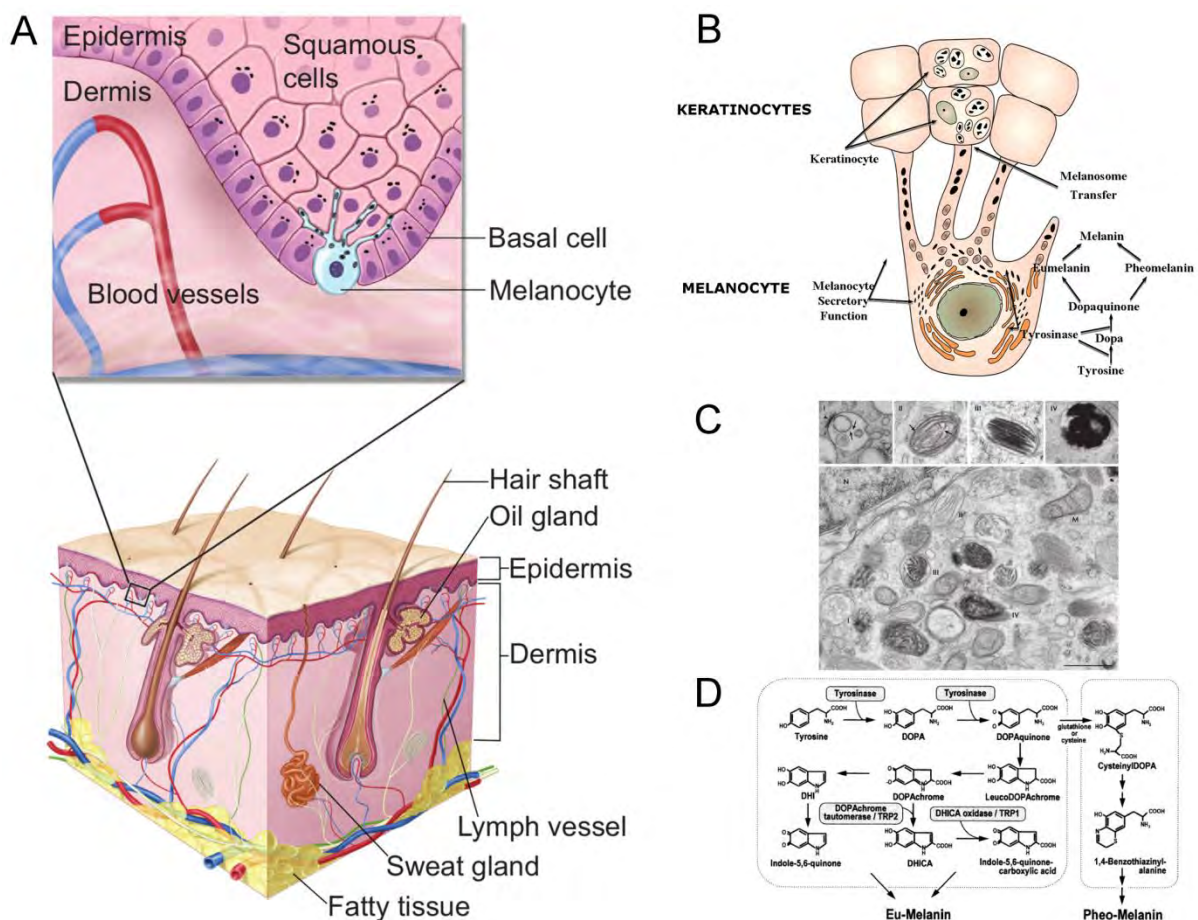


Figure 1.12: Human skin, melanocytes and pigmentation. (A) Human skin consists of 3 layers, the epidermis, dermis and hypodermis. In the epidermis melanocytes are found on the basement membrane (basal layer) associating with keratinocytes through a series of networks of multiple, long dendrites. (B) Melanocytes associate with keratinocytes to transfer the pigment melanin for photoprotection from ultraviolet radiation. (C) The pigment melanin is confined to the melanosome, an intracellular organelle made in 4 stages: I-IV (transmission electron microscopy (TEM) images, note the electron dense pigment melanin, scale bar: 0.5 μ m). (D) Melanogenesis produces the pigment melanin in melanocytes with tyrosinase as the rate-limiting enzyme. Melanin synthesis is structurally restricted to melanosomes due to toxic intermediates formed in the process. (Amended from^{227,230}).

Sunlight is made of several types of ultraviolet (UV) radiation, penetrating the skin in a wavelength dependant fashion²²⁹. The very small percentage of UVC (100-280 nm) not filtered by atmospheric ozone is absorbed by the stratum corneum. UVB (280-320 nm) is able to penetrate up to the stratum basale. UVA (320-400 nm) and white light (400-760 nm) penetrate into the dermis. Melanin effectively absorbs UV radiation resulting in photoprotection of the skin.

1.5.2 MELANOMAGENESIS

The etymology of melanoma originates from the Greek *melas* (male gender) and *melan* (neutral gender) meaning black and dark. The verb is *melanono* (μελανώνω), to make dark and the noun is *melanoma* (μελάνωμα), describing the process of making black, dark or black dark formation. The earliest writing about melanoma dates back to Hippocrates of Cos in the 5th century BC. The earliest physical evidence (4th century BC) of diffuse melanotic melanoma metastases were discovered in pre-Colombian mummy skeletons from Peru, carbon dated to ~2400 years old. Centuries later the first record of an excised melanoma tumor appeared in Western medical literature in 1787 by the Scottish surgeon John Hunter working at the St George's Hospital Medical School in London (for further key events in melanoma history the subject is reviewed by Rebecca et al. (2012)²³¹).

Both nature and nurture effects interplay in creating melanoma, manifested as a heterogeneous disease with an array of etiologies²³². Epidemiologic data suggests that chronic UV radiation exposure induces DNA damage and subsequent genetic alterations^{233,234}. Recent advances in molecular oncology have furthermore elucidated several genetic patterns responsible for different melanoma subtypes^{231,235-238}. Genome instability generates genetic diversity that forms the basis for the hallmarks of cancer: constitutive proliferative signaling, growth suppressor inhibition, cell death evasion, replicative immortality activation, angiogenic- invasive- and metastatic induction, energy metabolism reprogramming and evasion of immune destruction⁷⁹.

Melanoma arises from the melanocytes of the skin via a series of transformations²³⁹⁻²⁴¹. These include progression from a benign to a dysplastic nevus to radial- and vertical-growth phases and finally metastatic melanoma (Fig. 1.13). Each of these stages is characterized by molecular lesions manifesting in various biological events²⁴¹ (Fig. 1.13). Originating from a benign nevus, activation of the mitogen-activated protein kinase (MAPK) signaling pathway such as v-Raf murine sarcoma viral oncogene homolog B1/ proto-oncogene B-Raf (BRAF) and N-Ras may occur, associated with 15-50% of melanomas respectively²⁴¹. In the premalignant dysplastic nevus, melanocytes display abnormal growth with irregular borders, a change in color and often an associated allergic response. These biological changes are harbored by underlying molecular changes in the cyclin-dependent kinase

inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways²³⁵. Further progression of melanoma is associated with decreased differentiation and clonal proliferation leading to the radial growth phase (RGP)²⁴¹. The RGP melanoma is considered an intra-epidermal lesion that can involve some local microinvasion of the dermis. The RGP cells can furthermore progress to vertical growth phase (VGP) cells which breach the basement membrane and invade the dermis as nodules or nests of cells and are considered to have metastatic potential. Striking changes in the control of cell adhesion occur in the vertical-growth phase and metastatic melanoma²⁴¹. Interestingly not all melanomas develop through these phases - RGP and VGP can both develop directly from melanocytes or naevi and both can progress directly to metastatic malignant melanoma²⁴².

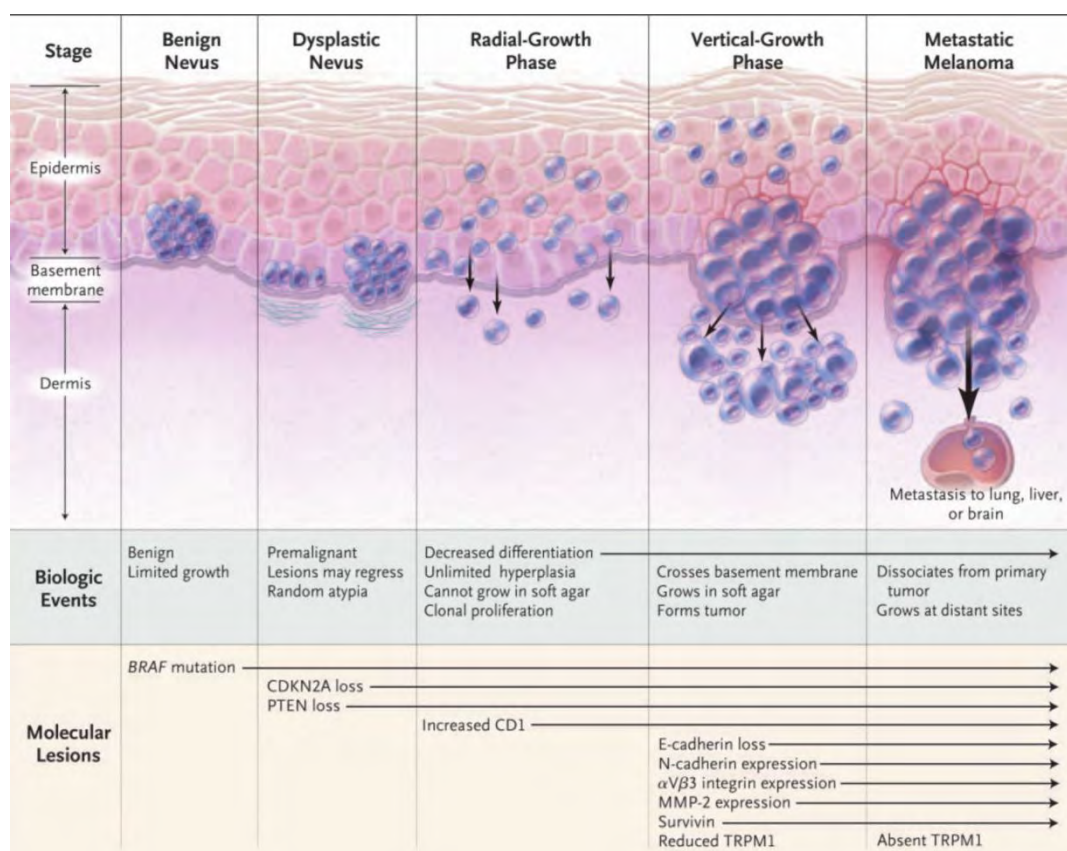


Figure 1.13: Biologic Events and Molecular Changes in the Progression of Melanoma. In the benign nevus, activation of the mitogen-activated protein kinase (MAPK) signaling pathway such as v-Raf murine sarcoma viral oncogene homolog B1/ proto-oncogene B-Raf (BRAF) may occur. In the premalignant dysplastic nevus, cytologic atypia reflects lesions within the cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways. Further progression of melanoma to the radial-, vertical-growth phase and metastatic melanoma is associated with decreased differentiation and the decreased expression of melanoma markers regulated by microphthalmia-associated transcription factor (MITF). Striking changes in the control of cell adhesion occur in the vertical-growth phase and metastatic melanoma. These changes include the loss of expression of the melanocyte-specific gene melastatin 1 (TRPM1), E-cadherin and increased expression of N-cadherin, α V β 3 integrin, and matrix metalloproteinase 2 (MMP-2). (Taken from Miller et al. (2006)²⁴¹)

1.5.3 MELANOMA STATISTICS

Melanoma accounts for 4% of all dermatologic cancers but remains responsible for 80% of deaths from skin cancer with the average patient diagnosed with disseminated metastases surviving for an average of 5 years (Cancer facts and figures, 2003, Atlanta, American Cancer Society, 2003). Each year, more than 68,000 Americans are diagnosed with melanoma, and another 48,000 are diagnosed with an early form of the disease²³⁰. Incidences of melanoma skin cancer have increased over the past decades, with 132,000 melanoma skin cancers occurring globally each year (World Health Organization <http://www.who.int/uv/faq/skincancer/en/index1.html>). South Africa and Australia have the highest incidences of melanoma in the world. To date South Africa does not have reliable statistics but 2009 estimates for the Cape region showed 69 new cases per year per population of 100,000 Caucasians (Australia: 65 per 100,000). This incidence has increased immensely, from 1990-1995 (22.2 per 100 000 for females and 27.5 per 100 000 for males) to 2000-2003 (33.5 per 100 000 for females and 36.9 per 100 000 for males) to 2009 estimates (Cancer Association of South Africa http://www.melanoma.co.za/D_doccnr_MFS.asp).

1.5.4 MELANOMA TREATMENT

In its early stages malignant melanoma may be cured by surgical resection. However, once progressed to the metastatic stage current therapies offer no cure²⁴³. In addition, recurrence rates of resected melanoma remain high and traditional chemo- and radiation therapy evoke resistance^{244,245}. Recent molecular discoveries have enabled a greater understanding in the biology of melanoma which has led to the development of new therapeutics and targeted drugs, which include immunotherapy (e.g. interleukin-2 (IL-2)), radiotherapy, biochemotherapy and gene therapy^{231,238,245,246}. A limited number of these therapies have progressed to human clinical trials but their outcomes remain negligible. One promising therapy is high-dose interferon (IFN) alpha-2b therapy, approved as the only adjuvant therapy for melanoma by the US Food and Drug Administration. However overall survival remains negligible or very small²³⁶. Various molecular pathways altered in melanoma have been identified and are currently under clinical investigation, such as drugs targeting BRAF (sorafenib, vemurafenib, GSK2118436) and N-Ras (tipifarnib)²³⁸. In addition, a number of melanoma-specific and melanoma-associated tumor antigens have been cloned with the hope that these potential antigens may be developed to stimulate tumor-specific T cells to eliminate melanoma cells²⁴⁷. Despite these advances, there remains the need for the development of novel and effective approaches to treat melanoma, one such approach is photodynamic therapy (PDT) as an adjuvant therapy or in combination with current therapeutics to combat melanoma.

1.5.5 MELANOMA RESISTANCE

The one aspect that sets melanoma apart from all other cancers is its ability to produce the pigment melanin. Melanosomes have been associated with chemotherapeutic drug trapping, followed by increased melanosomal biogenesis and export from the cell^{228,248,249}. These studies revealed that drug-resistant melanoma cells treated with a sub-lethal dose of cisplatin showed the presence of predominantly stage II–III and III melanosomes. Melanosomes at these stages are typically cigar-shaped with some nascent melanin deposition on proteinaceous fibrils, whereas stage IV melanosomes are typically round and completely filled with melanin²²⁷. Stage II–III and III melanosomes possess a greater capacity to trap cytotoxic drugs in the nascent melanin and are thus likely to be involved in drug resistance²²⁸. Moreover, treatment with the tyrosinase inhibitor phenylthiourea (PTU) induced the pharmacological modulation of melanosomes to stage II or II–III and significantly increased resistance to cisplatin by 12-fold²⁴⁹. Chen et al. (2009) have furthermore hypothesized that melanoma cells express several ABC transporters (e.g. ABCB5, ABCC2 and ABCC1) in melanosomes and diverse subcellular organelles of the endosomal pathway, constituting a buffer system that prevents nuclear or mitochondrial import of cytotoxic drugs²²⁸. Furthermore, the same transporters, especially ABCB5, might function as heterodimers on lysosomal and/or melanosomal membranes to trap cytotoxic melanin metabolites in those organelles, thereby protecting the cells from endogenous melanosomal cytotoxicity generated by late-stage melanosomes²²⁸.

These studies demonstrate the potential of melanoma to use different melanosomal stages in therapeutic resistance. By shifting production of melanogenesis to early stage melanosomes, melanoma cells are able to trap chemotherapeutic drugs in the nascent melanin, followed by export of melanosomes from the cell. Unraveling the mechanisms of melanoma resistance to current therapies is crucial for the design of novel targets- one such target could be the pigmentary system of these cancer cells, the one aspect that sets melanoma apart from other cancers.

1.5.6 PHOTODYNAMIC THERAPY FOR MELANOMA

Using photodynamic therapy for the treatment of melanoma has been investigated in various cancer cell lines, animals and humans with a range of different photosensitizers and treatment regimens (for comprehensive reviews see refs.^{250–252}). In summary, various *in vitro* and *in vivo* responses to PDT are observed in different melanoma models. These include: apoptosis^{114,253–259}, necrosis^{260–262}, tumor regression^{260–264} and prolonged survival of animals^{261,264,265}. In a mouse model, PDT with the benzoporphyrin derivative verteporfin induced destruction of the melanoma-associated intralymphatic tumor cells and lymphatic vessels, preventing metastasis and relapse²⁶⁶. However, *in*

vivo PDT with different systemically administered photosensitizer (chlorins, phthalocyanine and verteporfin) has seldom resulted in complete tumor remission²⁶⁴, followed by recurrence²⁶² and toxic side effects to normal tissue²⁶⁷ and liver and kidney toxicity^{260,261}. Clinically, PDT with the photosensitizer verteporfin has been used to treat ocular melanoma and was found to be partially effective as a second line of therapy²⁶⁸ and effective as a first line of therapy^{269,270}. It was furthermore shown that PDT with systemically administered chlorin e6 was well tolerated and effective on skin metastases of melanoma²⁷¹. These limited reports illustrate the necessity for further research, especially with photosensitizers with enhanced tumor targeting characteristics, thereby minimizing cytotoxic side effects to normal tissue.

1.5.6.a HYPERICIN AND MELANOMA

Hypericin, an extract from St John's Wort, is a promising photosensitizer in the context of clinical photodynamic therapy (PDT) due to its excellent photosensitizing properties and tumorigenic characteristics^{92,167,168}. However, limited *in vitro* reports are found in the literature describing melanoma treatment with hypericin-PDT and no *in vivo* investigations have, to the best of our knowledge, been published.

An early study of hypericin in the dark by Blank et al. (2003) revealed the formation of polykaryons in B16.F10 mouse melanoma cells after treating the cells with 10 μ M hypericin for 72 hours²⁷². The authors suggest that formation of these multinucleated cells forms part of a specific cell death mechanism (mitotic catastrophe) in response to the therapy; this view was strengthened by other biochemical characteristics in different cell types used in the study, however these were not carried out for the B16.F10 melanoma cells. Further investigations need to be undertaken to investigate the involvement of hypericin without light activation in melanoma cells.

Another study employed a dose of 1- 20 μ g/mL hypericin with a one minute exposure at 4°C to an argon laser (514 nm, 5 W), a KTP-532 laser (532 nm, 5 W), or a 20-A xenon lamp²⁷³. Viability was reduced over 90 % in human melanoma cells by this combined treatment and trace toxicity was observed after separate exposures to hypericin or light alone.

Hadjur et al. (1996) investigated the effect of visible light ($\lambda > 590$ nm) activated hypericin on one pigmented (G361) and two unpigmented human melanoma cell lines (M18 and M6)¹²⁷. The uptake and maximum level of hypericin incorporation was not significantly different between the cells. No dark cytotoxicity was observed for 0- 10⁻⁶ M hypericin. Upon activation by light, a drug- and light-dose dependent cytotoxicity was found; with unpigmented cells more sensitive to the treatment than pigmented cells. Thiobarbituric acid-reacting substances (TBARs), indicating lipid peroxidation,

were significantly increased in unpigmented cells after treatment but unchanged in pigmented cells. The treatment furthermore induced changes in antioxidant activities of the cells. An increase in total SOD activity, a decrease in glutathione peroxidase (GSHPx) and catalase (Cat) activities and decreased GSH levels were found in unpigmented cells. In contrast, GSHPx was unaffected but an increase in Cat activity was found in pigmented cells. These results illustrate the role the pigment melanin may play in the cytotoxic response to hypericin-PDT in melanoma cells.

Recent findings from our laboratory have confirmed these earlier results. We found a pigmentation dependent susceptibility of human melanoma cells to 3 μ M hypericin-PDT, with pigmented cells being less susceptible than unpigmented cells¹¹⁴. Upon depigmentation with tyrosinase inhibitors, kojic acid and phenylthiourea, pigmented melanoma cells become more susceptible to the treatment^{126,274}. Moreover, 72 hours after hypericin-PDT the cell viability of the depigmented melanoma cells remained significantly less than the control cells. Over the same time period the cells not treated with kojic acid approached a cell viability similar to the control. These findings illustrate the involvement of the pigment melanin in melanoma cell resistance to hypericin-PDT and thus highlight new therapeutic possibilities of phototoxic cancer therapies in conjunction with depigmenting agents.

A very recent report identified a role of hypericin-PDT ROS-induced autophagy in assisting in the evasion from determinants of immunogenic cell death (ICD) by weakening the functional interaction between dying cancer cells and the immune system²²¹. In this study, 150 nM hypericin-PDT induced ER-mediated ROS stress in A375m melanoma cells. Reduction of autophagy (by ATG5 knockdown) did not alter ATP secretion and enhanced ecto-CALR induction which correlated strongly with the inability to clear oxidatively damaged proteins. Autophagy attenuated A375m cells furthermore displayed an increased ability to induce DC maturation, IL6 production and proliferation of CD4⁺ or CD8⁺ T cells, accompanied by IFN γ production. These findings on the evasion ICD by the induction of autophagy could point to possible resistance mechanisms of melanoma cells to the therapy.

These studies indicate the potential of hypericin-PDT for melanoma, however further elucidation of the cellular response mechanisms are crucial in testing whether this therapy can one day be extrapolated to the clinic. Identifying possible cell death resistance mechanisms furthermore allows for the design of targeted therapies circumventing such resistance, possibly enabling effective melanoma eradication.

AIM OF THE STUDY

Hypericin, an extract from St John's Wort, is a promising photosensitizer in the context of clinical photodynamic therapy (PDT) due to its excellent photosensitizing properties and tumoritropic characteristics^{92,167,168}. However, limited reports on the efficacy of this photomedicine for the treatment of melanoma have been published^{114,126,127,221,272,273,275,276}.

The aim of this study was to investigate the response mechanisms of melanoma cells to hypericin-PDT in an *in vitro* tissue culture model.

This investigation was three-fold:

1. Are melanoma cells susceptible to hypericin-PDT?
2. Is hypericin taken up by melanoma cells and does it specifically associate with intracellular organelles?
3. What melanoma cell death mechanisms, if any, are elicited in response to hypericin-PDT?

CHAPTER 2: MATERIALS & METHODS

2.1 IN VITRO MELANOMA TISSUE CULTURE MODEL

2.1.1 MELANOMA CELL LINES

Unpigmented A375 melanoma cells were purchased from the American Type Culture Collection (ATCC CRL-1619). These cells originated from a 56 year old female with malignant melanoma of the skin and the resulting cell line is described as unpigmented with epithelial morphology, adherent growth and able to grow in immunosuppressed mice. The cells are furthermore described as hypotriploid with a modal number of 62 chromosomes. There are 9 marker chromosomes that are commonly found in each cell and normal N2, N6, and N22 are present at one copy per cell.

Moderately pigmented 501mel melanoma cells were a gift from Prof Sharon Prince from the Department of Human Biology, University of Cape Town. These cells are described as metastatic; however the laboratory of Prof S Prince has shown that they did not form tumours in nude mice (personal communication).

Pigmented UCT Mel-1 melanoma cells were obtained from liquid nitrogen stores in the Department of Human Biology, University of Cape Town. These melanoma cells were originally excised from a 67 year old female patient at Groote Schuur Hospital in Cape Town, South Africa²⁷⁷. The primary tumour was found on the right ankle and the secondary tumour resulting in the cell line was found in the right inguinal lymph node. The biopsy was pigmented and the resulting cells were described as epithelioid with triangular dendritic morphology, a modal chromosome number of 74 and able to grow in soft agar and nude mice.

2.1.2 TISSUE CULTURE CONDITIONS

All cell lines were cultured in complete culture medium consisting of Dulbecco's modified Eagle's medium (Highveld Biological, Appendix) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Highveld Biological, Appendix) and 100 U/ml penicillin/ 100µg/ml streptomycin (P3032/S91370 Sigma, Appendix) in a tissue culture incubator (MCO-175M, Sanyo) at 37°C with a humidified atmosphere of 5 % CO₂. The cells were examined regularly using a brightfield microscope (Olympus Microscope Model CK2, Olympus Microscopes) and images of cells were captured with a digital camera (Powershot S50, Canon). Media was changed every 3 days and cells were passaged by trypsinization with trypsin/ EDTA (Appendix) when confluent. Cells were counted manually for experiments using a haemocytometer (Neubauer improved, Marienfeld) and routinely checked for mycoplasma contamination with the nuclear stain Hoechst (Appendix A) or with a MycoAlert™

mycoplasma detection kit (LT07-318, Lonza), according to the manufacturer's instructions. All cells were cultured to 90% confluence for all experiments to obtain similar pigmentation levels.

2.1.3 GROWTH DYNAMICS

Melanoma cells were seeded into 35mm tissue culture dishes (A375 and 501mel: 2×10^4 , UCT Mel-1: 5×10^4) and allowed to adhere and grow overnight. Cells were counted manually using a haemocytometer (Neubauer improved, Marienfeld) on day 1, 3, 5 and 7 after seeding. Resulting data was expressed as a ratio of the number of cells seeded (day 0). These experiments were repeated twice with 3 technical repeats in each.

2.1.4 TYROSINASE ASSAY

The two initial steps of melanogenesis involves the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and subsequent oxidation to DOPAquinone, both catalysed by the same enzyme, tyrosinase. Utilization of radiolabelled ^{14}C tyrosine with the addition of DOPA enables the assessment of the enzymatic activity of tyrosinase from total cell extracts by detection of the radiolabelled reaction products^{278,279}. This assay was modified from the original protocol^{278,279}.

Equal cell numbers were cultured to confluency in 6 cm tissue culture dishes for 3 days followed by trypsinisation with TE (Appendix). After centrifugation the cell pellet was washed with 1xPBS (Appendix) and transferred to a 1.5 ml microfuge tube on ice. The PBS wash procedure was repeated to remove all traces of medium, after which the pellet was re-suspended by vortexing in 1ml of 0.1M sodium phosphate buffer (Appendix). The samples were repeatedly freeze-thawed in liquid nitrogen and then centrifuged at 12000rpm at 4 °C for 20 min. The supernatant was transferred to clean 1.5 ml microfuge tubes and stored at -80 °C till use.

The sample protein concentration was determined using the BCA Protein Assay Kit according to the manufacturer's instructions (23225, Thermo Scientific Pierce) and 120 µg/ml was used for the assay. The cell extract was combined with a solution of L-[U- ^{14}C]Tyrosine (20 µCi/ml, 1.85 MBq/ml, CFB74, Amersham) and 0.25 mM DOPA (Appendix A, D9628, Sigma- Aldrich) at 37 °C for 1 hour. Samples were then transferred to glass microfiber filters with a diameter of 25 mm (1822-025, Whatman) and left to air-dry. Once dry, the discs were washed 3 times in 0.1N HCl (Appendix A, Merck) at room temperature for 15 min per wash, followed by 2 further 5 min washes in 95 % ethanol (Merck) and a final 5 min wash in acetone (Merck). The discs were then placed in scintillation vials (Sigma-Aldrich) and left to air-dry overnight. The following day, 5 ml scintillation fluid (Quicksafe A scintillation cocktail, Zinsser Analytic) was added per vial and the samples were read on a Tri-Carb 2100 TR liquid

scintillation analyser (Packard). Results were expressed as incorporated radioactive tyrosine counted per minute (cpm). Controls included a negative control (no cell extract), a washing control (no cell extract, no radioactive tyrosine or DOPA) and a scintillation fluid control (no cell extract, no radioactive tyrosine or DOPA and no washing steps).

2.2 PHOTODYNAMIC THERAPY

2.2.1 HYPERICIN

Hypericin from *Hypericum perforatum*, 95 % pure by high-performance liquid chromatography, was obtained from Sigma (56690). A 2mM stock solution (Appendix) was made in dimethyl sulfoxide (DMSO, Merck) and stored as aliquots at -80 °C. Working solutions were prepared fresh at a concentration of 50µM in phosphate-buffered saline (1x PBS, Appendix) and then diluted further directly in the complete cell culture medium containing fetal bovine serum (Appendix). All experiments were carried out under subdued light conditions.

2.2.2 LIGHT ACTIVATION

Hypericin was activated with 1 J/cm² UVA. A light box with two F15T8 15W/UVA PUVA lamps (320-410 nm, peak 351 nm, Waldmann) was used to administer the dose. The power output was checked using a portable UV meter (Waldmann Type 585100). The following equation was used to obtain the time of exposure.

$$\text{Time} = \frac{\text{Light dose (J/cm}^2\text{)}}{\text{Irradiance (W/cm}^2\text{)}}$$

Equation 2.1: Equation used to determine the time using the light dose (J/cm²) and irradiance (W/cm²).

The time obtained was 6 minutes and 10 seconds. The power output was routinely checked to ensure that the same dose was given in all experiments; if the power output varied the time was adjusted to give the right dose. The cells were irradiated from the top in 1x PBS. No difference in cell viability was found when cells were irradiated with the tissue culture dish lid on or off. For the cell viability experiments cells were irradiated with lids off whereas for all other experiments lids were kept on the dish. Controls were sham-irradiated by covering the plate/ dish with foil.

2.2.3 HYPERICIN-PDT

Melanoma cells were exposed to 4 hours of hypericin in complete media followed by a wash in 1xPBS and light activation with 1 J/cm² UVA in 1x PBS for all experiments (Fig. 2.1). Following light

activation, complete media was gently added to the cells. At different times after treatment the cells were subjected to various analyses.

For each experiment cells were exposed to 4 different treatments:

- vehicle with sham-irradiation (control -light)
- vehicle with light-activation (control +light)
- hypericin with sham-irradiation (hypericin -light)
- hypericin with light-activation (hypericin +light)

The concentration of the vehicle (DMSO) was kept constant at a maximum of 0.5 % for the cell viability and 0.15% for all cell death experiments. A vehicle concentration of 0.5 % did not affect melanoma cell viability. All experiments were carried out under subdued light conditions.

The same tissue culture consumables were used for all experiments (Greiner bio-one), to ensure no differences in light activation. A recent report describes lipophilic photosensitizers adhering to tissue culture plates, using the same consumables is thus of advantage in PDT¹⁵⁶.

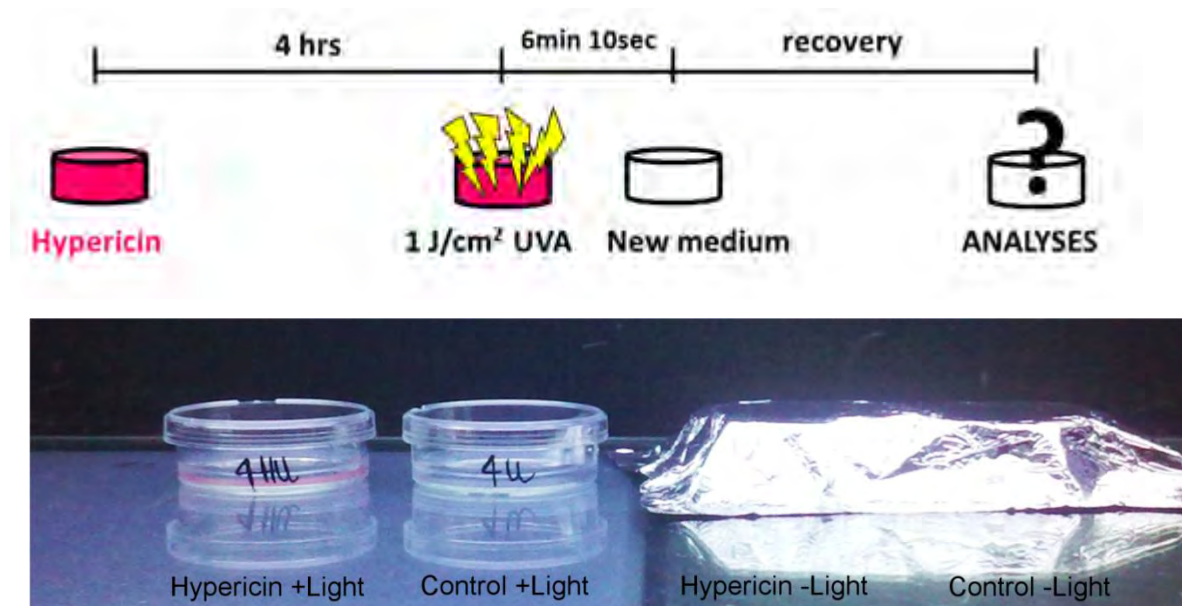


Figure 2.1: The experimental setup for hypericin-PDT. Cells were exposed to hypericin for 4 hours followed by light activation with 1 J/cm^2 (6 min 10 sec), administered in a light box with 2 UVA lamps. Each experiment included 4 different treatments: light-activated hypericin (hypericin +light), light-activated vehicle (control +light), sham-irradiated hypericin (hypericin -light) and sham-irradiated vehicle (control -light). Upon light activation hypericin fluoresced red (hypericin +light). Cells were given new media after light activation and analyses were conducted at different times after treatment.

2.3 CELL VIABILITY

Melanoma cell viability at 24 hours after hypericin-PDT was determined using the XTT assay (Cell proliferation Kit II (XTT), 11465015001, Roche). Dehydrogenases and reductases of metabolically active cells are able to reduce the yellow XTT tetrazolium salt to form orange formazan, monitored by absorbance at 450nm. Therefore absorbance is directly proportional to cell viability. Cells were seeded into a 96 well plate at a density of 3×10^4 cells per well in 200 μ l complete medium. The next day, adherent cells were exposed to various hypericin concentrations for 4 hours. Cells were then washed once with 100 μ l 1x PBS (Appendix) followed by irradiation with 1 J/cm² UVA in 50 μ l of 1x PBS. After irradiation, PBS was removed and cells were incubated in 200 μ l complete media for 24 hours. Four hours before the end of the 24 hour period, 51 μ l of combined XTT solution (50 μ l XTT labelling reagent plus 1 μ l electron coupling reagent) was added per well, according to the manufacturer's instructions. At the end of the 24 hour period the absorbance was measured at 450nm using an ELISA plate reader (VERSAmax™ tuneable microplate reader, Molecular Devices).

Two sets of hypericin concentration ranges were tested, namely 0.2- 10 μ M and 1-2.5 μ M. For these sets, the final vehicle (DMSO) concentration was 0.5 % or 0.125% respectively, for all wells. The percentage of viable cells was established by normalization to the vehicle-treated, sham-irradiated control (control –light). All treatments were carried out with 3 or more biological repeats, each with 3 technical repeats.

2.4 ELECTRON MICROSCOPY

Ultrastructural investigation was carried out at 4 hours post hypericin-PDT. All solutions used in this protocol can be found in Appendix C. After treatment, suspended and adherent cells were pooled, fixed with 2.5 % Sorenson's phosphate buffered glutaraldehyde (pH 7.2-7.4) and processed for transmission electron microscopy. Briefly, the glutaraldehyde fixed cells were centrifuged at 1000 RPM to form a pellet, the supernatant discarded, and rinsed in 0.1 M Sorenson's phosphate buffer (pH 7.2). The cell pellets were post-fixed in 1 % osmium tetroxide buffered in Palade's veronal acetate buffer, dehydrated through a graded series of acetone (70-100 %), impregnated with Spurr's epoxy resin and polymerized at 70 °C. Ultrathin sections (60-80 nm) were cut, stained with uranyl acetate and lead citrate by standard procedures and viewed on a Philips transmission electron microscope. For estimations of vacuolisation and glycogen accumulation, semi-thin toluidine blue-stained sections were analysed. Approximately 50 cells were scored from resin sections for each sample and the scoring was determined by 2 independent viewers.

2.5 HYPERICIN UPTAKE ASSAY

Similar to previous protocols²⁸⁰, cells were seeded at a density of 3×10^4 in a 35 mm tissue culture dish. The following day, cells were exposed to 4 hours of hypericin. At the end of this time-point cells were placed on ice, the hypericin containing media was removed and cells were washed with ice-cold 1x PBS (Appendix). Complete extraction buffer (40 μ l, Appendix) was added to the dish and cell lysates were collected with a rubber policeman. Cell lysates were then centrifuged at 4°C for 20min at 12000 rpm. The supernatants were divided for the determination of protein content and for the quantification of hypericin fluorescence on a fluorimeter (Cary Eclipse Fluorimeter, Varian). Hypericin was excited with 488 nm and 563 nm with an emission of 608 nm for both. Resulting data was normalized to total protein and presented as arbitrary fluorescent units (AFU) per microgram of protein.

Protein concentrations were determined using the BCA assay according to the manufacturer's instructions (BCA protein assay kit, 23225, Thermo Scientific Pierce). Briefly, protein standards were prepared using bovine serum albumin (BSA) which was used to produce a standard curve. Samples were diluted 1:5 using the complete extraction buffer. The working reagent was added to the sample in a 96 well plate in triplicate and the absorbance was measured using a spectrophotometer (Microplate Reader RT-2100C, Rayto) at 595 nm. The amount of protein was calculated using the optical density (OD) reading and the equation generated by the slope of the standard curve.

2.6 INTRACELLULAR LOCALISATION OF HYPERICIN

2.6.1 ORGANELLE SPECIFIC GFP-FUSION PROTEINS/DYES

Several organelle-specific GFP-fusion proteins or dyes were used to label intracellular organelles in melanoma cells, summarised in the table below. All constructs used in this study were prepared according to standard techniques²⁸¹.

Cells were seeded on microscope cover glasses in 35 mm tissue culture dishes at a density of 2×10^4 cells and allowed to grow over 2 nights. Cells were then transiently transfected with plasmids (for concentrations refer to Table 2.1). TransFectin (1 μ l, 170-3351, Biorad) was used for transient transfection of A375 and 501mel and GeneCellin (0.5 μ l, GC500, BioCellChallenge) was used for UCT Mel-1; according to the manufacturer's instructions with minor modifications. For TransFectin, the transfection reagent containing medium was taken off the cells after 4 hours and replaced with fresh complete media. For GeneCellin, the transfection reagent was left on the cells for 20 hours. Both

transfection reagents were added to the cells in a total volume of 1ml. Controls of the parental GFP/YFP plasmid were included.

At 20 hours after transfection the cells were exposed to 3µM hypericin for 4 hours. To stain the nuclei, 10 µl of 100 µg/ml Hoechst (1 µg/ml, Appendix) was added to the hypericin containing medium, 20 minutes before the end of the 4 hour time-point. Similarly, to stain lysosomes, 7.5 µl of 10000nM lysotracker yellow (75 nM, Appendix) was added at 30 minutes before the end of the hypericin incubation. At the end of the staining period the cells were not washed and viewed immediately.

Intracellular organelle	Plasmid/ Dye	Experimental conditions	Function	Source
Endoplasmic reticulum	pGFP-Bcl2CB5	200 ng of plasmid	Anti-apoptotic ER targeted BCL2 family member	Addgene plasmid 18000 ²⁸²
Endoplasmic reticulum	pEYFP-ER	200 ng of plasmid	Calreticulin, multifunctional quality control of protein folding	Dr G. Schafer, Dr C. Kaschula, ICGEB, University of Cape Town, Clontech 6906
Mitochondria	pEGFP-pOTC	200 ng of plasmid	Ornithine transcarbamylase (OTC), mitochondrial matrix protein	Dr L.M. Davids ²⁸³ , University of Cape Town
Early and late endosomes Late endosomes and lysosomes Early and intermediate stage melanosomes	pEGFP-rab7WT	100 ng of plasmid	Microtubule-based transport, facilitates transport of tyrosinase and TYRP1 from trans-golgi to melanosomes	Addgene plasmid 12605 ²⁸⁴
Mature melanosomes	pGFP-Rab27a	200 ng of plasmid	Localises to the mature melanosomal membrane, acts as a receptor for myosinVa in the actin-dependent transport of mature melanosomes	Prof J. Lambert, University of Ghent ²⁸⁵
Mature melanosomes	pGFP-MyosinVa	200 ng of plasmid	Actin-dependent motor protein, links mature melanosomes to the actin network	Prof J. Lambert, University of Ghent ²⁸⁶
Lysosomes Melanosomes	LysoTracker® Yellow-HCK-123	75 nM for 30 min in medium, live	Acidotropic probe labelling acidic organelles	Invitrogen Molecular Probes, L12491
Nuclei	Hoechst 33342	1 µg/ml for 20 min in medium, live	Binds to DNA	Invitrogen Molecular Probes H1399

Table 2.1: Organelle-specific GFP-fusion plasmids and dyes used to visualise intracellular organelles.

Microscope cover glasses were taken out of the 35mm tissue culture dishes for viewing and placed onto a drop of tissue culture medium (DMEM only, no FBS) on a microscope slide and viewed immediately on the confocal fluorescent microscope. This enabled the “live” viewing of cells. This procedure was done in the vicinity of the confocal microscope to ensure immediate viewing of cells.

2.6.2 CONFOCAL FLUORESCENT MICROSCOPY

Fluorophores were excited at the following excitation wavelengths: GFP 488 nm, YFP 514 nm, LysoTracker 458 nm, Hoechst 405 nm, Hypericin 561 nm and emission was collected at the following wavelengths, through appropriate filters: GFP 490-543 nm, YFP 516-586 nm, LysoTracker 464-604 nm, Hoechst 410-516 nm, Hypericin 585-734 nm. The Plan-Apochromat 63x/1.4 oil immersion DIC M27 lens was used. Both the Carl Zeiss 510 LSM meta with NLO confocal microscope (LSM and ZEN 2009 software) and the Carl Zeiss LSM 780 confocal microscope (ZEN 2009, 2011 software) were used in this study. Profiles taken through different locations of the cell displaying the fluorophores, were created using the free Zen 2011 (blue edition) software.

2.7 FLUORESCENT ACTIVATED CELL SORTING (FACS)

FACS was employed to analyse phosphatidylserine exposure using Annexin V-FITC and loss of cell membrane integrity using VIVID^{287,288}. Cell size and granularity and intracellular hypericin were also recorded. Cells were seeded at a density of 5×10^4 in 35 mm tissue culture dishes and allowed to grow overnight. The following day cells were treated with hypericin-PDT and analysed at 30', 1, 4, 7 and 24 hours after treatment. For the 24 hour time-point, cells were seeded and treated a day earlier. The experiment was timed in such a way that the time-points ended together at which the cells were placed on ice. The processing time was 4 hours as a total of 24 dishes were processed. Controls included unstained cells, untreated cells (i.e. not sham-irradiated or vehicle-treated) and cells treated with 5µM doxorubicin for 24 hours. All processing was done on ice.

At the end of the time-points the culture medium was collected and kept on ice (to include suspended cells in the analysis). Adherent cells were lifted by trypsinization and pooled with the suspended cells and centrifuged for 5 min at 1200 rpm. Resulting cell pellets were re-suspended in ice-cold 1xPBS and centrifuged for 5 min at 1200 rpm. The pellet was re-suspended in 500µl ice-cold 1x PBS, to which 0.5 µl VIVID (L34955, Invitrogen Molecular Probes) was added. Cells were stained on ice for 30 minutes, centrifuged for 5min at 1200rpm, re-suspended in 1 ml of 1 % BSA in 1x PBS and centrifuged again for 5min at 1200rpm. Thereafter cells were re-suspended in 200 µl 1x Annexin V binding buffer (Appendix C) to which 1µl Annexin V-FITC (556420, BD Biosciences) was added. Cells were stained with Annexin V for 15 minutes at room temperature after which samples

were transferred to 5ml FACS tubes (REF352052, BD Biosciences) and 10000 events were acquired immediately on a LSRII flow cytometer (BD Biosciences).

The following LSRII parameters were used: blue 488 nm laser: FITC 530/30 505LP, PE 576/26 550LP; violet 405 nm laser: Pac Blue 440/40; voltages: FITC 520, PE 530, Pac Blue 370, FSC 250 and SSC 320. Annexin V was detected with FITC, hypericin with PE and VIVID with Pac Blue. Flow cytometry data analysis was performed with FlowJo version 7.6.5 (TreeStar).

Unstained cells and single-stained mouse κ beads (552844, BD Biosciences) were used as controls and to calculate compensation for every run. The beads were stained in 200 μ l PBS with 5 μ l of the following antibodies: FITC Anti-Human IL-2 (340448, BD Biosciences), Pacific Blue Mouse Anti-human CD3 (558117, BD Biosciences), PE Mouse Anti-Human Ki-67 Set (556027, BD Biosciences). A three-point gating strategy was applied (Appendix E).

2.8 WESTERN BLOTTING

The expression of specific cell death proteins was analysed after hypericin-PDT. All solutions used in this protocol can be found in Appendix C.

For preparation of whole cell lysates, cells were grown in 6 cm tissue culture dishes to 90% confluency and treated with 3 μ M hypericin-PDT. At 1, 4, 7 and 24 hours after treatment, suspended cells were collected and adherent cells were harvested by scraping in 70 μ l complete extraction buffer with a rubber policeman. Resulting cell lysates were added to 1.5ml tubes, vortexed briefly and incubated overnight at 4°C. The next day, the cell lysates were centrifuged at 4 °C for 20 min at 12000 rpm. The supernatant was frozen in aliquots at -80 °C until use. Protein concentrations were determined using the BCA assay according to the manufacturer's instructions (BCA protein assay kit, 23225, Thermo Scientific Pierce), as described in section 2.4.

To separate the various proteins in the cell extracts, equal amounts of proteins (25 μ g) were diluted in 5x loading dye, loaded into the wells of a stacking SDS-polyacrylamide gel and resolving SDS-polyacrylamide gel (7.5/ 15 %). Electrophoresis was applied to the gel at 80 V for 30 minutes followed by 120 V for approximately 2.5 hours in 1x running buffer. The separated proteins were then transferred onto a nitrocellulose membrane (Hybond ECL Amersham, RPN203D, GE Healthcare) in 1x transfer buffer at 50V for 2 hours. The blotted membrane was then immersed in sufficient Ponceau S and stained for total protein for 5 minutes. After staining the membrane was immersed in

an aqueous solution containing 5 % acetic acid for 5 minutes, the solution was then changed and the membrane immersed for another 5 minutes. The membrane was then washed in water (2x 5min). The membrane was scanned digitally (CanoScan LiDE210, Canon) and washed twice in TBS-Tween (TBS-T) for 5 minutes each thereafter. The membrane was then blocked in 5 % milk TBS-T for 1 hour at room temperature. After blocking the membrane was exposed to the primary antibody (see Table 2.2) overnight at 4 °C. The following day the membrane was washed in TBS-T (3x 5min) and the secondary antibody was added (see Table 2.2) for 1 hour at room temperature. Thereafter the membrane was washed with TBS-T (3x 5min) and exposed to SuperSignal West Pico chemiluminescent ECL detection reagent (Thermo Scientific) for 1 minute. For immunodetection, the x-ray film (LEEFV, AGFA) was manually exposed to the membrane, developed (G150, AGFA) and fixed (G354, AGFA) for viewing. Resulting films were scanned and analysed densitometrically.

The total protein Ponceau S stained membrane was used as the loading control^{289,290}. The optical densities (OD) of the exposed film bands of the protein of interest were quantified using ImageJ and normalized to the OD of the total protein loading control. The resulting ratio was furthermore normalized to the vehicle-treated, sham-irradiated control of the respective experiment.

Cells treated with 5 µM Doxorubicin were included as a positive control and subjected to the same protocols.

Protein	Source	Function	Primary antibody dilution	Secondary antibody	Secondary antibody dilution
Apoptosis inducing factor (AIF)	Apoptosis inducing factor (AIF) A7549 Sigma	Caspase-independent apoptosis and parthanatos	1:8000	GAM-HRP 170-6516 Bio-Rad	1:3000
Caspase 3 (CASP3)	Caspase-3 Cleaved (Asp175) Antibody 9661 Cell Signaling Technology	Executioner caspase of intrinsic and extrinsic apoptosis	1:1000	GAR-HRP 170-6515 Bio-Rad	1:1500
Caspase 8 (CASP8)	Caspase-8 (1C12) Mouse mAb 9746 Cell Signaling Technology	Initiator caspase of extrinsic apoptosis	1:1000	GAM-HRP 170-6516 Bio-Rad	1:1500
Poly(ADP-ribose) polymerase 1 (PARP1)	PARP-1 (H-250): sc-7150 Santa Cruz Biotechnology	DNA damage repair enzyme, cleaved during apoptosis	1:2000	GAR-HRP 170-6515 Bio-Rad	1:1500

Table 2.2: Primary and secondary antibodies used for Western Blot analyses of cell death proteins.

2.9 DATA ANALYSES

All data was presented and analysed using Graphpad Prism (Version 5, Graphpad Software Inc.) Statistical analyses were performed by One-Way Anova with Bonferroni post-test (comparing all groups) or Dunnett post-test (comparing all groups to a control group). Differences in values were stated as significant if the p-value was less than 0.05. All experiments were carried out at least 3 times and presented as mean±SEM, unless otherwise indicated.

2.10 METHODS SUMMARY

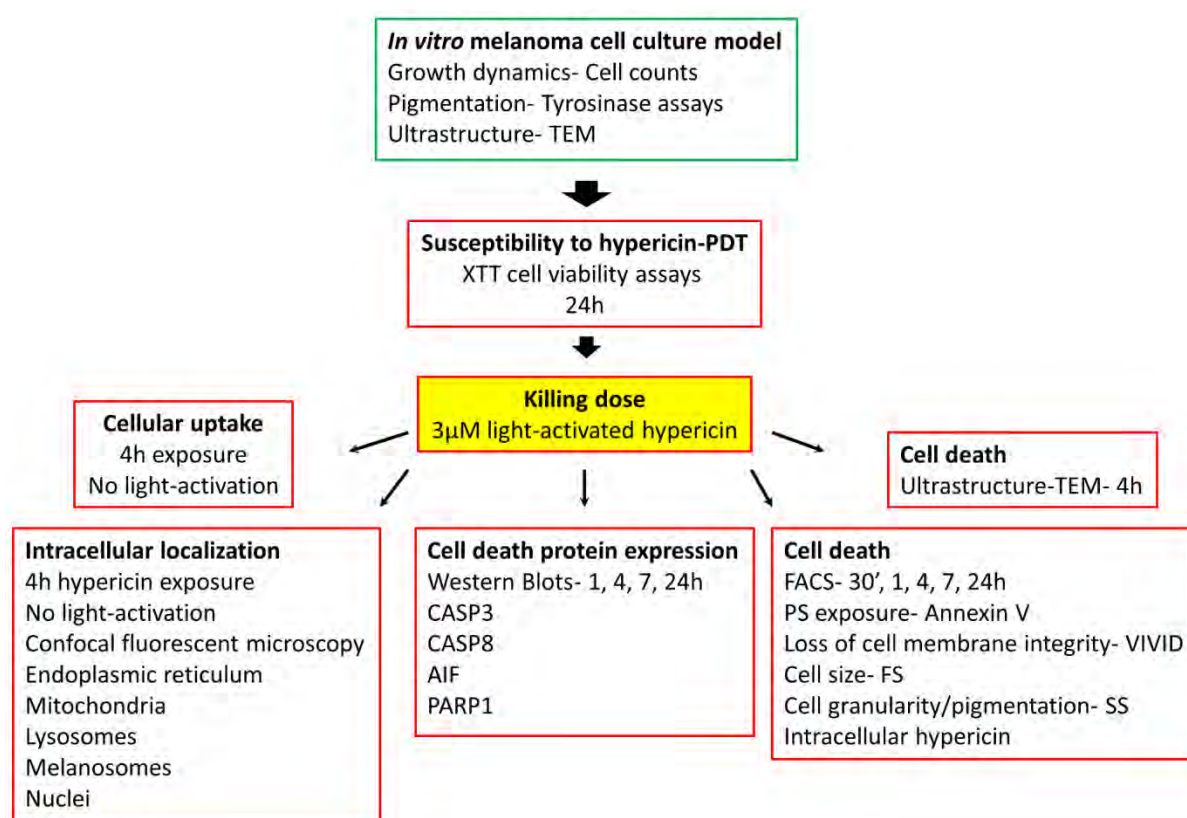


Figure 2.2: Summary of methods employed in this study.

CHAPTER 3: RESULTS

3.1 IN VITRO MELANOMA CELL CULTURE MODEL

The *in vitro* tissue culture model system used for this study consisted of several melanoma cell lines with varying degrees of pigmentation. These melanoma cells exhibited different morphological characteristics (Fig. 3.1A). The A375 melanoma cells displayed an epithelial-like morphology with no dendrites and grew in clusters. The 501mel and UCT Mel-1 cells displayed triangular and bipolar dendrites respectively. Pelleting the cells revealed a pigmented pellet for UCT Mel-1 cells whereas the other cell lines displayed white pellets (Fig. 3.1B). The pigmentation levels of UCT Mel-1 cells correlated with overall confluency in the tissue culture dish²⁷⁷, cells were thus cultured to 90 % confluency for all experiments. Assessing growth dynamics over seven days revealed significantly faster growth rates for A375 (105 ± 20 fold) compared to both 501mel (51 ± 9 fold) and UCT Mel-1 cells (15 ± 3 fold normalized to day 0, Fig. 3.1C).

To verify the visually observed pigmentation differences of the melanoma cells (Fig. 3.2A) a tyrosinase activity assay was employed (Fig. 3.2B). The two initial steps of melanogenesis involve the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and subsequent oxidation to DOPAquinone. Both these steps are catalysed by the same enzyme, tyrosinase (Fig. 1.12). Utilization of radiolabelled ^{14}C tyrosine with the addition of DOPA enables the assessment of the enzymatic activity of tyrosinase from total cell extracts by detection of the incorporated radiolabelled reaction products^{278,279}. The UCT Mel-1 cells exhibited the highest tyrosinase activity (25887 ± 744 cpm/120 μg protein) which was significantly different from A375 (5191 ± 811 cpm/120 μg protein), 501mel (6286 ± 964 cpm/120 μg protein) and the control cells (MCF7 breast cancer cells: 1519 ± 624 cpm/120 μg protein). The A375 and 501mel tyrosinase activity was not significantly different from each other but significantly different to the control cells. To confirm these results ultrastructurally we chose one unpigmented (A375) and one pigmented (UCT Mel-1) cell line. Transmission electron microscopy (TEM) images revealed the presence of melanosomes filled with the electron-dense pigment melanin in both A375 and UCT Mel-1 cells. These were few in the unpigmented cells and numerous in the pigmented cells, thus confirming the tyrosinase activity assay results.

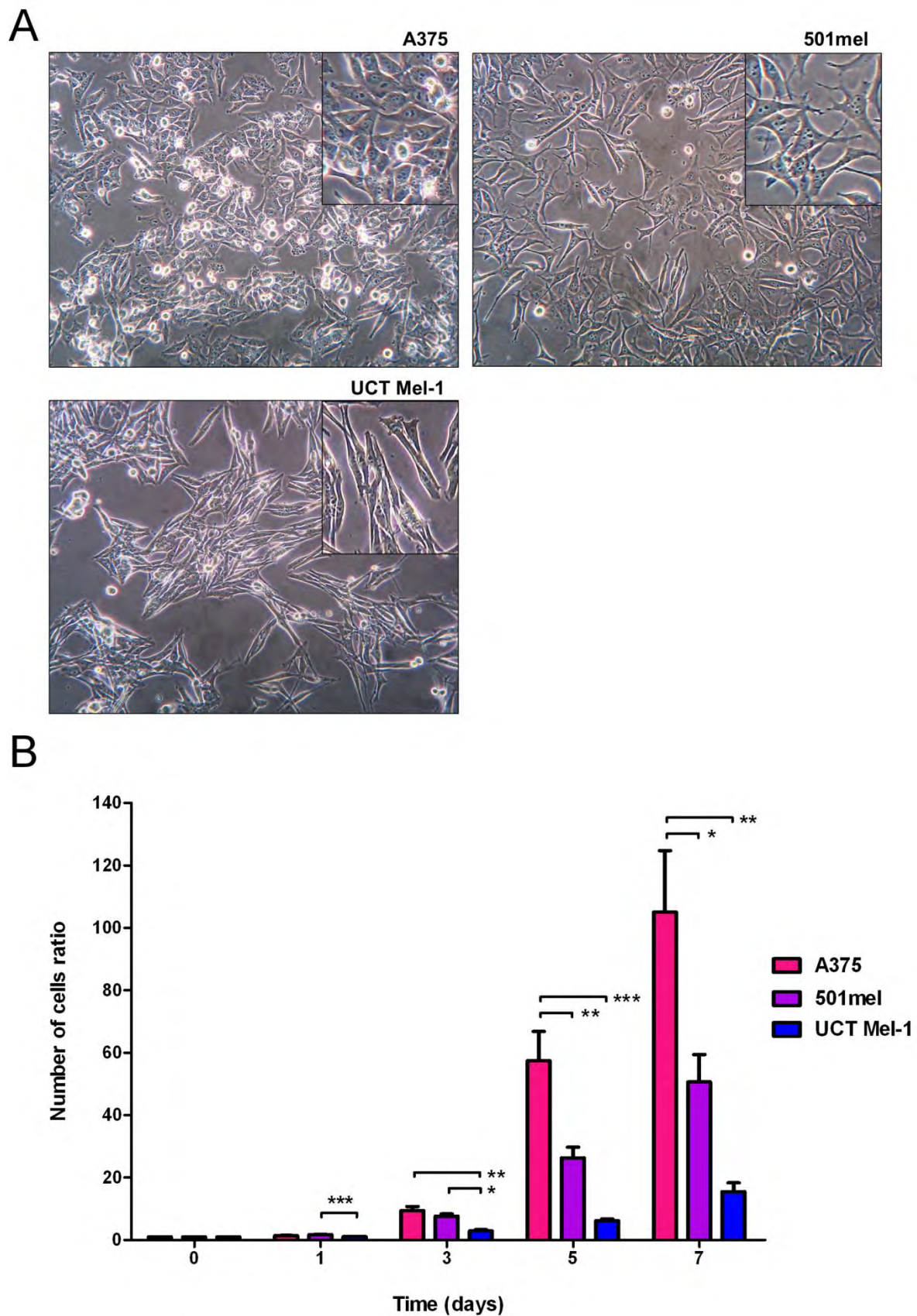


Figure 3.1: *In vitro* melanoma cell culture. (A) Phase contrast images (200x magnification). A representative result is shown (n≥3, inset: higher magnification). (B) Growth dynamics over a period of 7 days. Data is shown as mean ± SEM fold increase vs day 0 (n=2, ***p<0.0001, **p<0.05, *p<0.05).

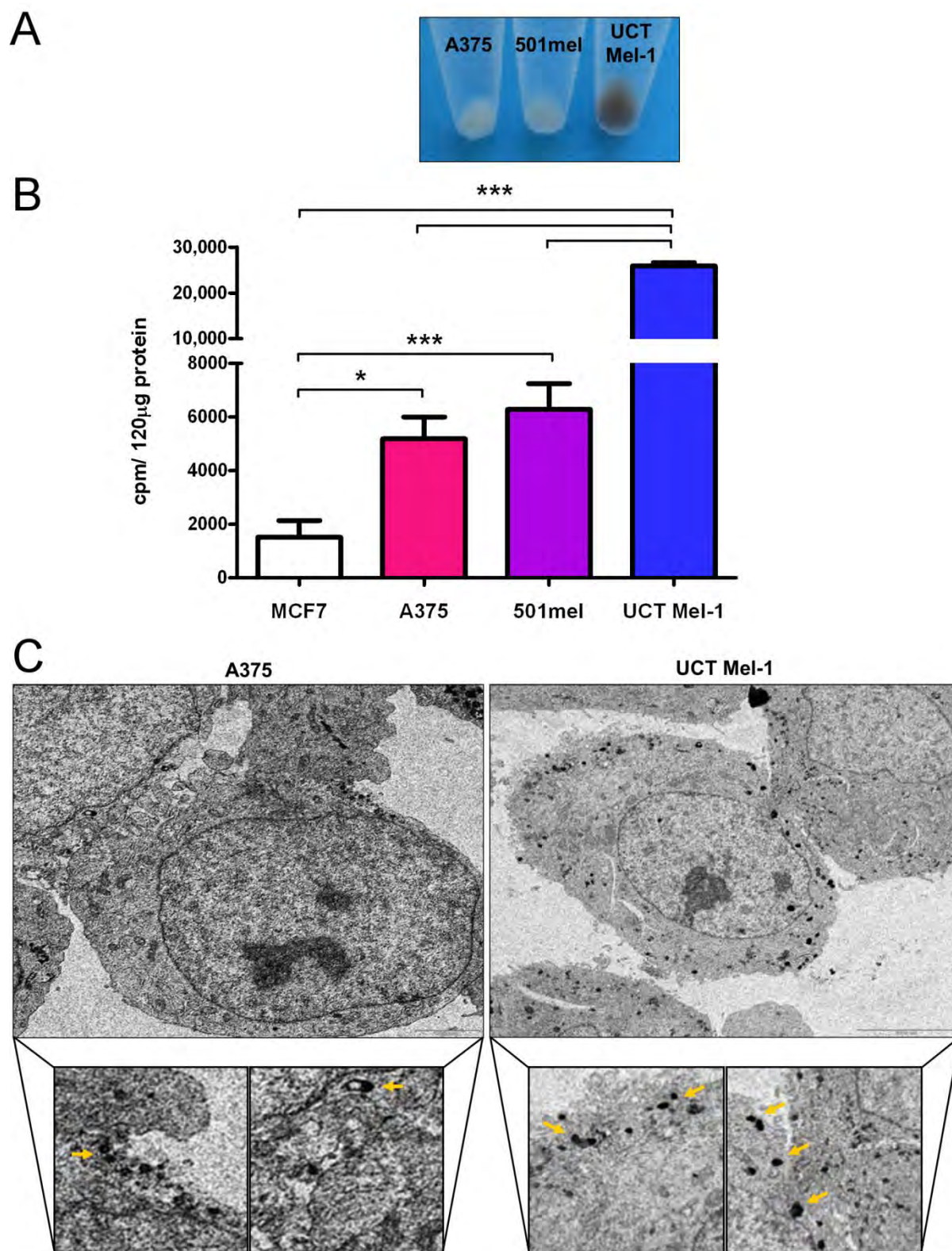


Figure 3.2: Melanoma pigmentation characterization. (A) Cell pellets. (B) Mean $\pm$ SEM tyrosinase activity in counts per minute (cpm)/120 μ g protein ($n=3$, *** $p<0.0001$, * $p<0.05$). MCF7 breast cancer cells were included as a negative control. (C) Transmission electron microscope images (TEM). Yellow arrows indicate melanosomes. A representative result is shown ($n=3$, scale bar: 2 μ m).

3.2 PART I: MELANOMA SUSCEPTIBILITY TO HYPERICIN-PDT

To investigate the response of melanoma cells to light-activated hypericin we chose a range of hypericin concentrations from 0.2- 10 μM (Fig. 3.3). We used the XTT cell viability assay to assess melanoma cell viability 24 hours after hypericin-PDT. Dehydrogenases and reductases of metabolically active cells are able to reduce the yellow XTT tetrazolium salt to form orange formazan, monitored by absorbance at 450 nm. This absorbance is therefore directly proportional to cell viability. Cells were treated with different hypericin concentrations for 4 hours followed by light activation (hypericin +light) or sham-irradiation (hypericin –light) and subsequent assessment of cell viability.

Hypericin absorbs light of both ultraviolet A (UVA) and visible wavelengths (Fig. 1.1). We chose UVA to activate hypericin in our experimental system, to investigate whether hypericin could be activated in a cost-effective clinical regime potentially exploiting sunlight as a light source.

Treatments of vehicle (0.5 % DMSO) with sham-irradiation had no effect on the cell viability of melanoma cells. This vehicle-treated, sham-irradiated control was thus used for normalization of data (black bar, Fig. 3.3). Similarly, treatments of vehicle with light activation did not significantly affect cell viability (yellow bar, Fig. 3.3).

Hypericin treatment without light activation did not affect melanoma cell viability adversely; in fact it exhibited a stimulatory effect in some cases (Fig. 3.3). A significant increase in cell viability was found after sham-irradiated 0.4 μM hypericin treatment of A375 (122 ± 6 %) and 5 μM hypericin treatment of UCT Mel-1 (129 ± 9 %) melanoma cells (Fig. 3.3A/C).

Hypericin concentrations below 3 μM with light activation had no effect on melanoma cell viability (Fig. 3.3). After 1 μM light-activated hypericin treatment all melanoma cells remained viable (Fig. 3.3). Interestingly, a significant increase in cell viability was observed in A375 melanoma cells after treatment with 0.4 μM light-activated hypericin (117 ± 6 %, Fig. 3.3A). This stimulatory effect on cell viability with light-activated hypericin was not observed in 501mel or UCT Mel-1 cells (Fig. 3.3C/D).

In contrast, light-activated 3 μM hypericin significantly reduced melanoma cell viability to 50 % or less than the control (A375: 32 ± 4 %, 501mel: 39 ± 5 % and UCT Mel-1: 50 ± 8 %, Fig. 3.3). Treatments up to 10 μM light-activated hypericin revealed a further significant reduction in cell viability to 17 ± 0.8 % for A375, 14 ± 0.6 % for 501mel and 17 ± 1.2 % for UCT Mel-1 (Fig. 3.3).

All the cell lines exhibited a similar response when compared to each other (Fig. 3.4). It was clear from these investigations that treatment with light-activated 1 μM hypericin had no effect on cell

viability of melanoma cells, whereas treatment with light-activated 3 μ M hypericin caused a 50 % or less reduction in viability. We further investigated hypericin concentrations between 1 and 3 μ M to more accurately pinpoint the cytotoxic threshold (Fig. 3.5).

These results showed that light-activated 2.5 μ M hypericin treatment of A375 resulted in a cell viability of 29 ± 4 % comparable to that of 3 μ M treatment 32 ± 4 % (Fig. 3.5A). For the other melanoma cells this was not the case as cells were more viable after 2.5 μ M hypericin treatment compared to 3 μ M (501mel: 82 ± 3 % and UCT Mel-1: 68 ± 5 %, Fig. 3.5B/C) thus re-emphasizing that A375 melanoma cells are the most susceptible to light-activated hypericin (Fig. 3.6).

Strangely, slight discrepancies were noted in this set of experiments (Fig. 3.5) compared to that of the first set (0.2- 10 μ M hypericin, Fig. 3.3). In this set of experiments A375 cell viability was significantly reduced at 1 μ M light-activated hypericin treatment (69 ± 6 %) and 2.5 μ M sham-irradiated hypericin treatment (84 ± 4 %, Fig. 3.5A). This contradicts the results of the first set of experiments in which 1 μ M light-activated hypericin treatment resulted in an A375 cell viability of 103 ± 6 % (Fig. 3.3A). Similarly, A375 cell viability was 113 ± 7 % after 3 μ M sham-irradiated hypericin treatment (Fig. 3.4A). We suggest that this could possibly be related to different batches of XTT reagent used for the assay as these experiments were performed at different times. Overall however, a reduction of cell viability was obtained - the desired outcome of any cancer therapy.

We thus decided to use the light-activated 3 μ M hypericin killing-dose in subsequent investigations.

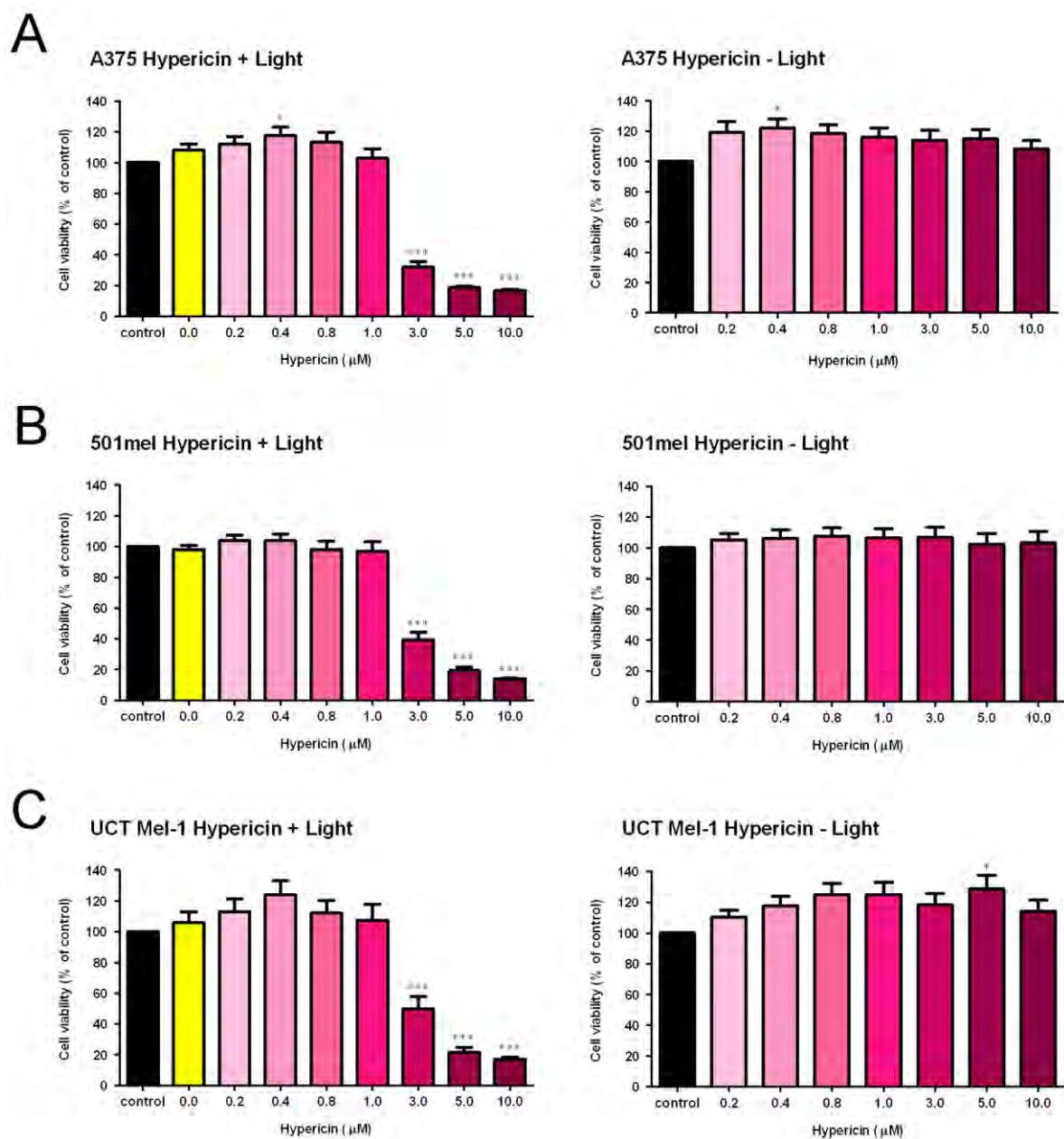
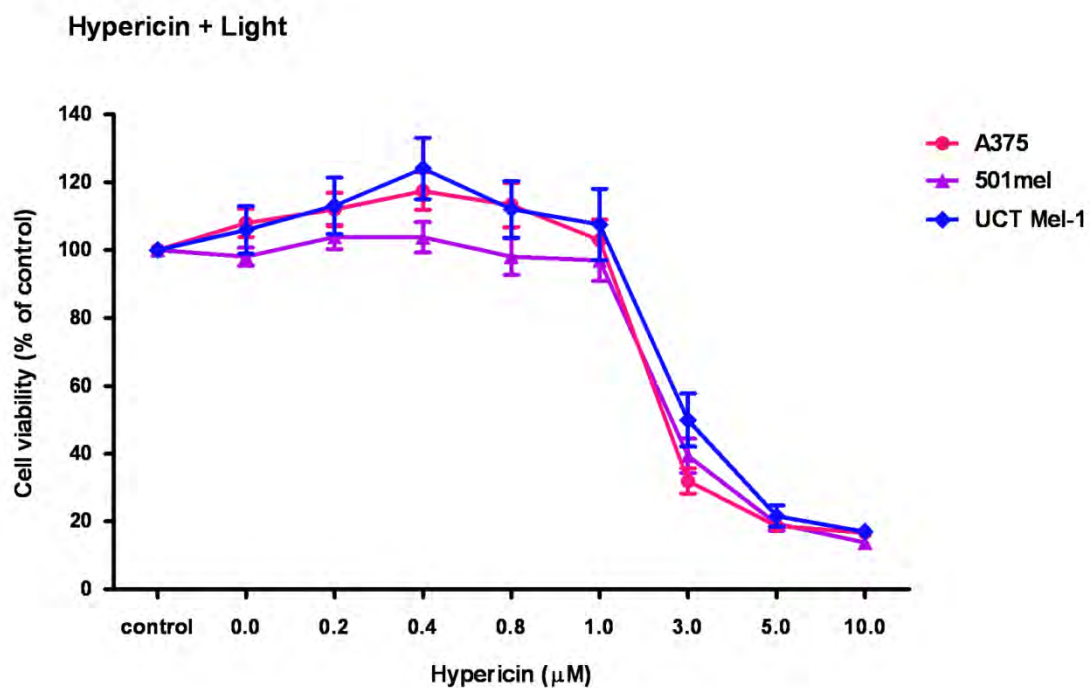


Figure 3.3: Cell viability at 24 h post 0.2-10 μM hypericin-PDT. (A) A375, (B) 501mel and (C) UCT Mel-1 melanoma cells were treated with hypericin and light-activation (left panel) or sham-irradiation (right panel). Data is shown as mean±SEM (n≥3) of percentage of viable cells normalized and compared to the vehicle-treated, sham-irradiated control for both light-activated and sham-irradiated hypericin (black bar, ***p<0.0001, **p<0.01, *p<0.05).

A



B

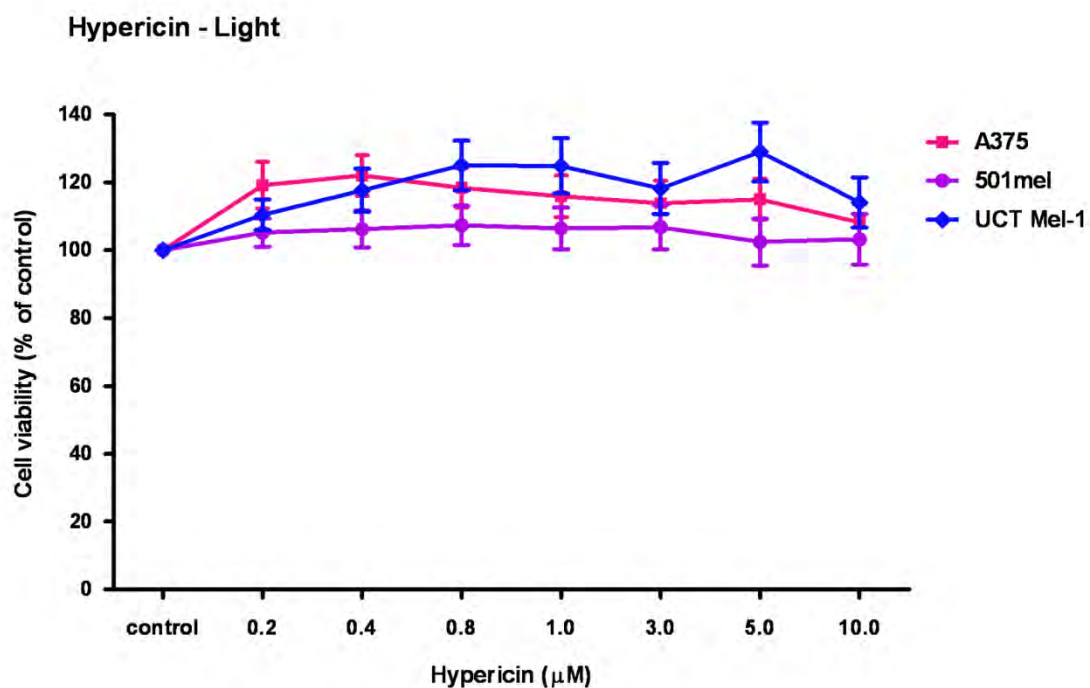


Figure 3.4: Cell viability similarities at 24 h post 0.2-10 μM hypericin-PDT. Cells were treated with hypericin and (A) light or (B) sham irradiation. Data is shown as mean $\pm$ SEM ($n\geq 3$) of percentage of viable cells normalized to the vehicle-treated, sham-irradiated control.

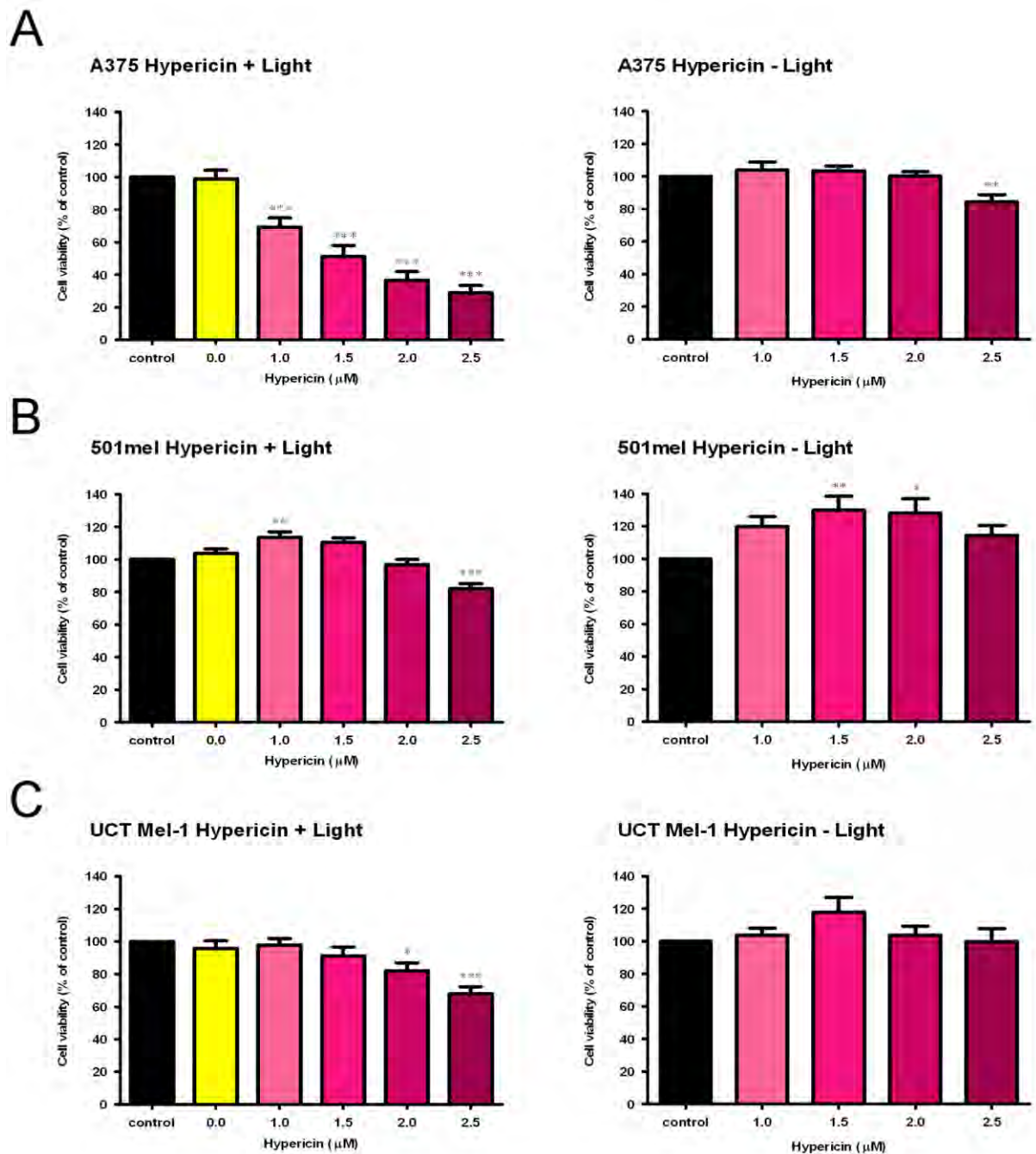
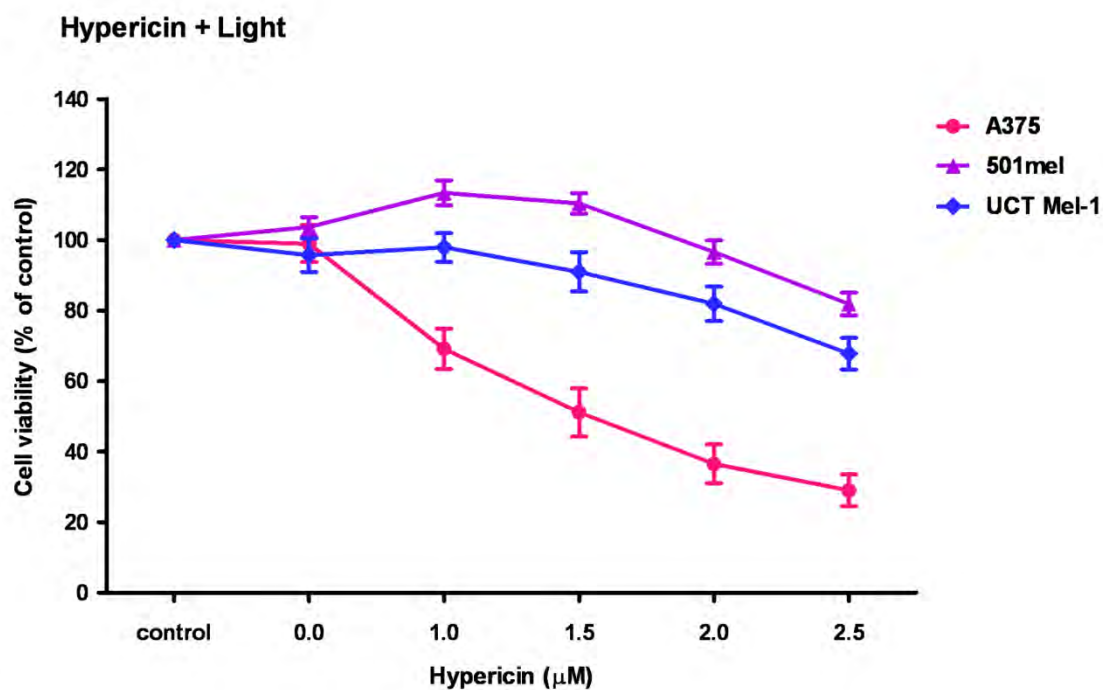


Figure 3.5: Cell viability at 24 h post 1- 2.5 μM hypericin-PDT. (A) A375, (B) 501mel and (C) UCT Mel-1 melanoma cells were treated with hypericin and light-activation (left panel) or sham-irradiation (right panel). Data is shown as mean±SEM (n≥3) of percentage of viable cells normalized and compared to the vehicle-treated, sham-irradiated control (black bar, ***p<0.0001, **p<0.01, *p<0.05).

A



B

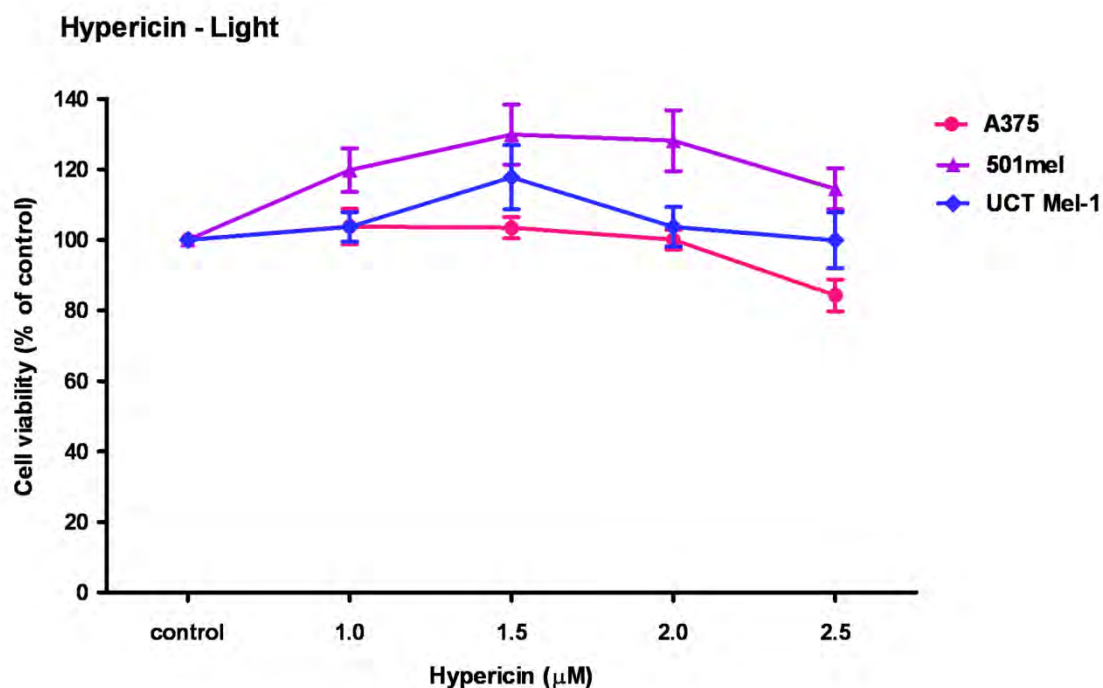


Figure 3.6: Cell viability differences at 24 h post 1- 2.5 μM hypericin-PDT. Cells were treated with hypericin and (A) light-activation or (B) sham-irradiation. Data is shown as mean $\pm$ SEM ($n\geq 3$) of percentage of viable cells normalized to the vehicle-treated, sham-irradiated control.

3.3 PART II: HYPERICIN AND MELANOMA CELLS

The photosensitizer, oxygen and light constitute the “trinity” of photodynamic therapy (PDT). Effective cellular uptake and intracellular localisation of the photosensitizer is crucial for PDT. We thus chose to investigate these parameters for hypericin in melanoma cells.

3.3.1 CELLULAR UPTAKE

Hypericin is a naphthodianthrone with a wide absorbance spectrum (Fig. 1.10) and characteristic red fluorescence (Ex: 548/593, Em: 594/642)²⁹¹. This fluorescence characteristic of hypericin was used to quantify its relative quantity inside the cell. Cells were exposed to 3 μ M hypericin for 4 hours without light activation. Cell lysates were collected and assayed for hypericin fluorescence with an excitation of 488 nm or 563 nm and an emission of 608 nm for both, normalized to the protein concentration (Fig. 3.7). The different excitation wavelengths were used to investigate whether hypericin can be excited with another wavelength apart from the optimal one, 563 nm, in our experimental system. Control cells not treated with hypericin showed no fluorescence (Fig. 3.7). Both excitation wavelengths revealed the same pattern of fluorescence, however more pronounced at 563 nm. All melanoma cells displayed intracellular hypericin fluorescence with 0.28 ± 0.01 relative fluorescent units (RFU)/total protein for the A375 cells, significantly higher than 501mel (0.22 ± 0.01 RFU/total protein) and UCT Mel-1 (0.22 ± 0.01 RFU/total protein) at 488nm excitation (Fig. 3.7A). The same trend was observed at 563 nm excitation (A375: 0.37 ± 0.01 , 501mel: 0.30 ± 0.01 and UCT Mel-1: 0.30 ± 0.021 RFU/ total protein, Fig. 3.7B). Hypericin fluorescence in 501mel and UCT Mel-1 was not significantly different from each other. These results showed that hypericin was effectively taken up by melanoma cells after 4 hours of exposure.

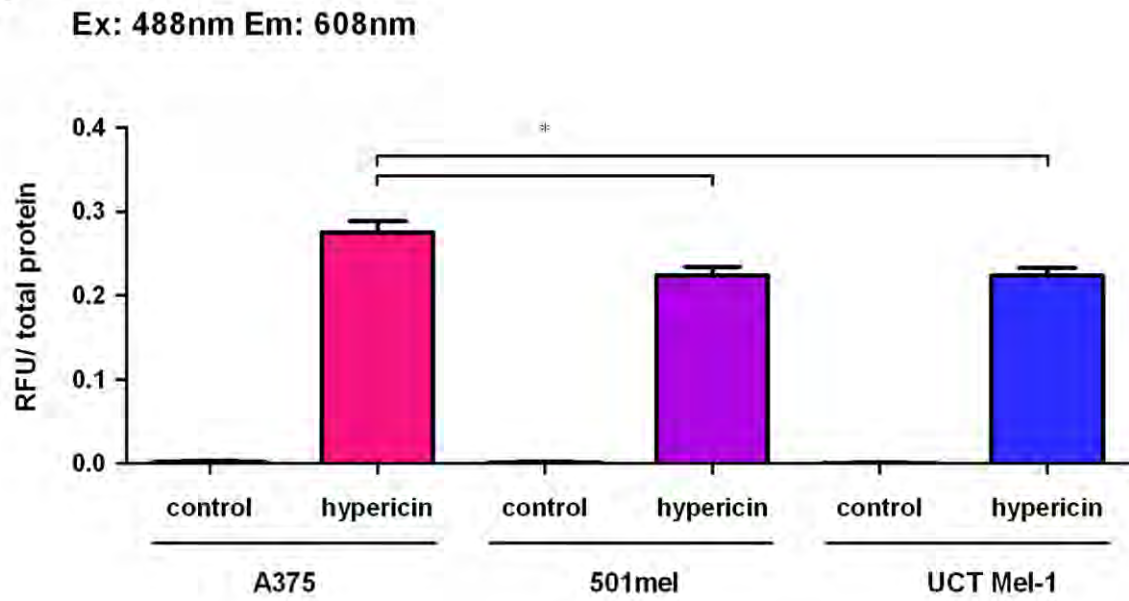
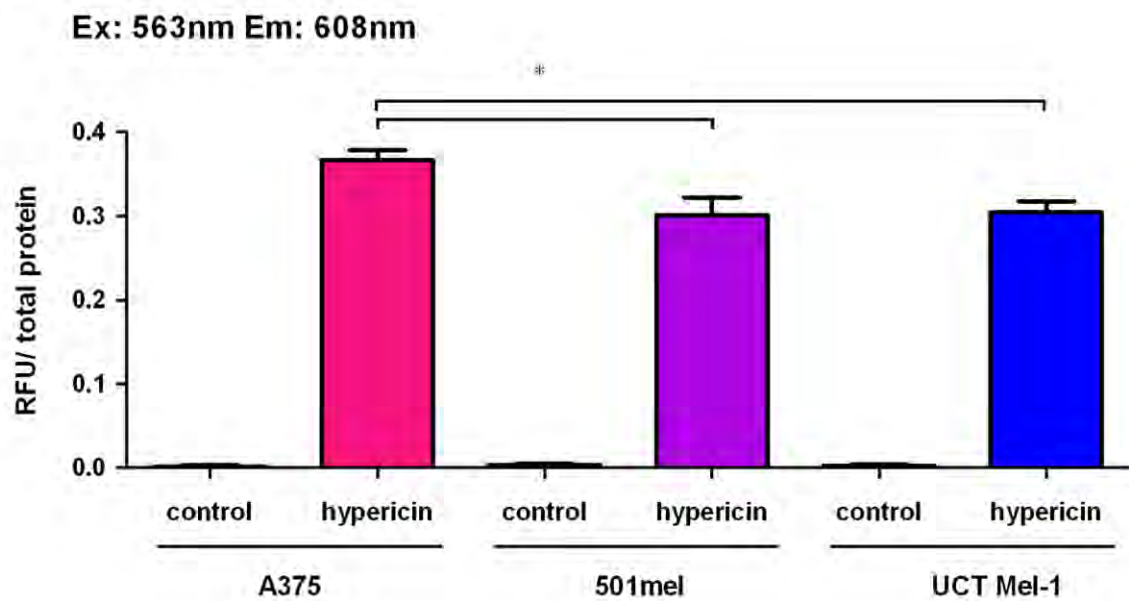
A**B**

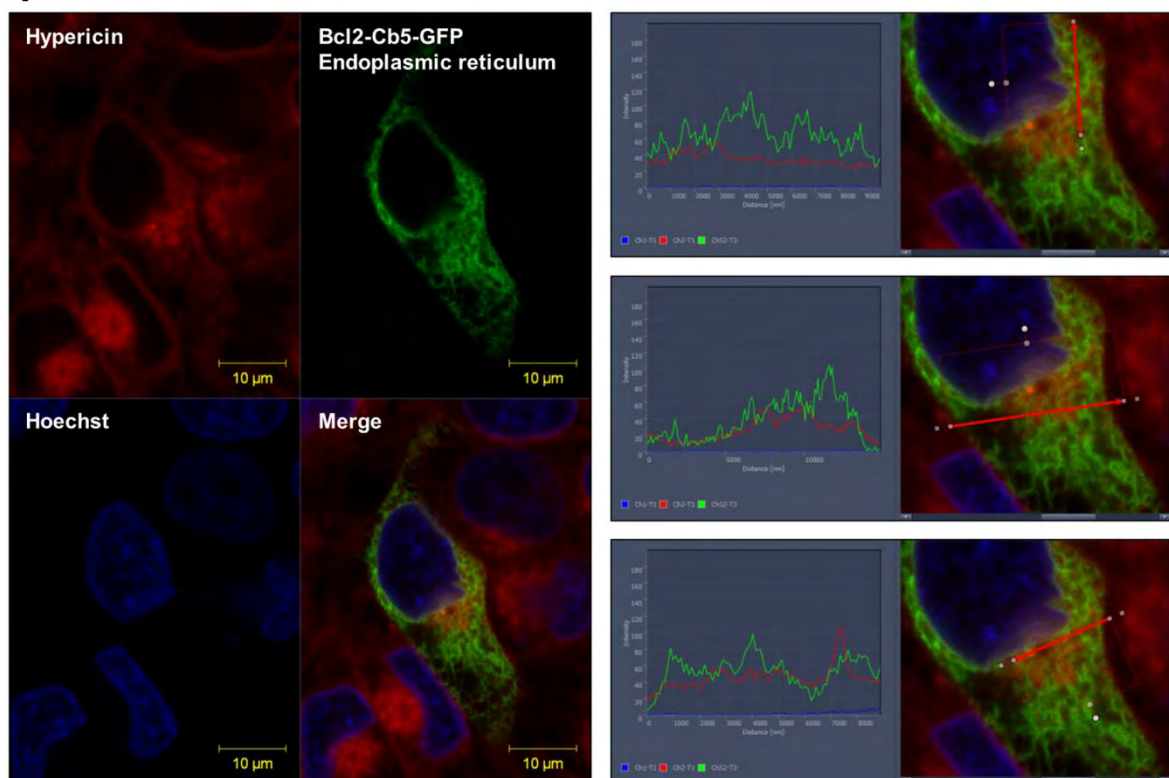
Figure 3.7: Hypericin uptake assay. Cells were exposed to 3 μ M hypericin for 4h without light activation. Hypericin was excited with (A) 488 nm or (B) 563 nm, with emission of 608 nm for both. Data is shown as mean $\pm$ SEM relative fluorescent units per microgram of protein (RFU/ μ g of protein) of (n=3, *p<0.05).

3.3.2 INTRACELLULAR LOCALISATION

The principle of PDT is the primary production of reactive oxygen species (ROS). These ROS have short half-lives, small radii of diffusion and thus elicit their action in the vicinity of their production. This calls for the investigation of the intracellular localisation of a photosensitizer as it inevitably affects its mechanism of action. We transiently expressed organelle-specific green- or yellow-fluorescent fusion proteins and stained the cells with organelle-specific dyes to fluorescently label different intracellular organelles in order to visualise hypericin localisation. Cells were exposed to 3 μ M hypericin for 4 hours without light activation and fluorophores were visualised with live confocal fluorescent microscopy. Initially we investigated this in fixed cells; however we found hypericin presents a diffuse pattern upon fixation and thus employed live visualisation of cells. Using profiles taken at different locations through the cell indicated co-localization of the fluorophores and accordingly intracellular localisation of hypericin in melanoma cells. Controls including GFP/YFP parental plasmid transfection or dyes by themselves were included to test the experimental system employed and revealed no cytotoxicity or aberrant targeting.

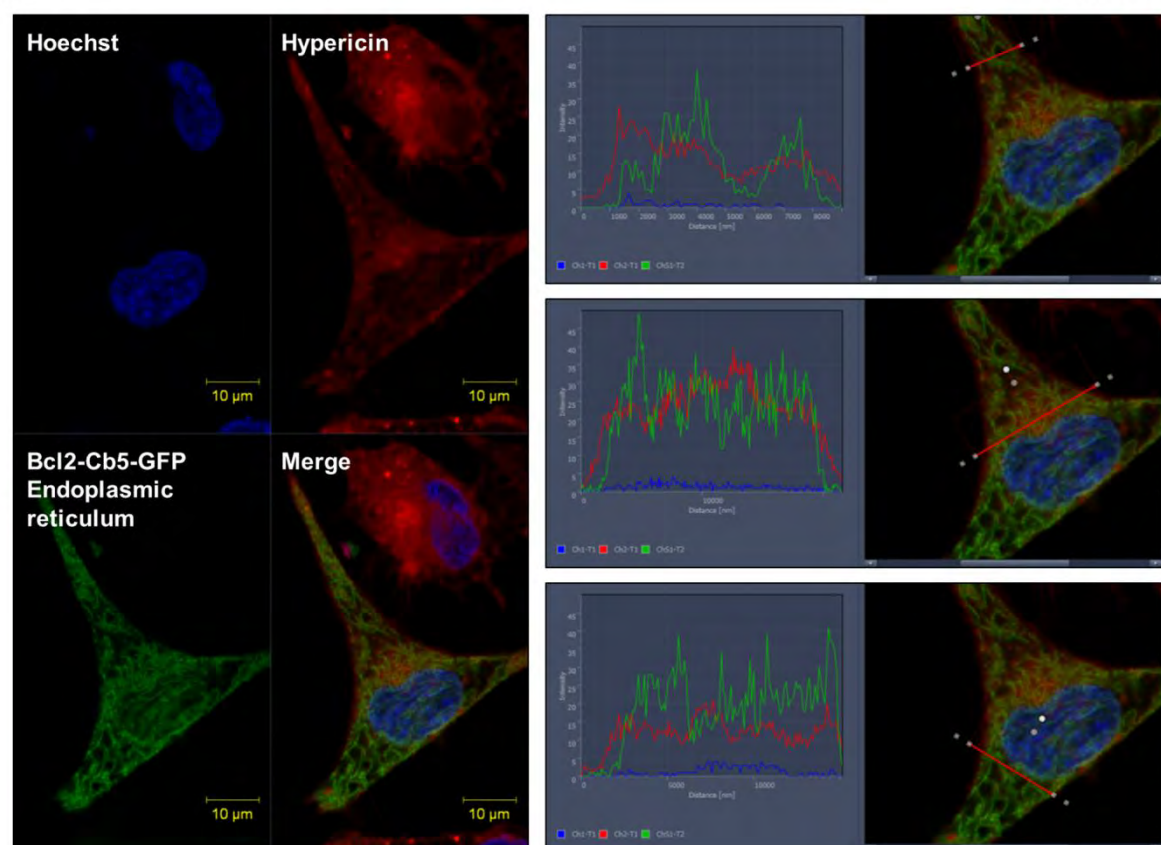
A

A375



B

501mel



C

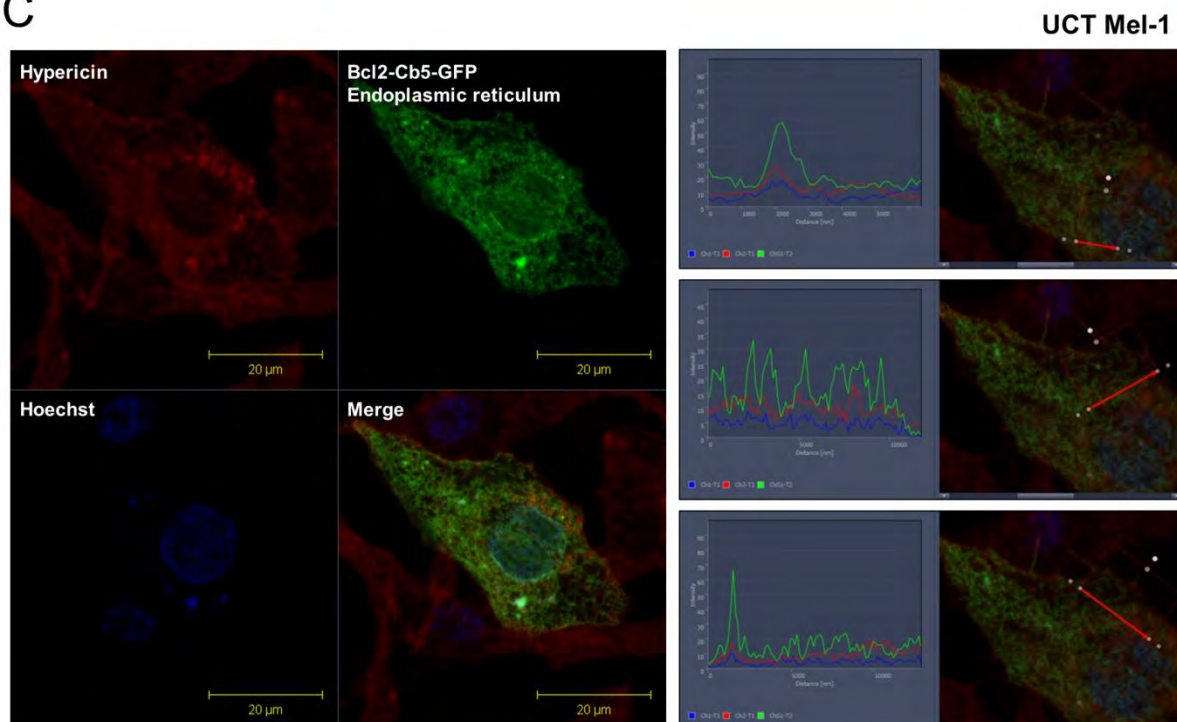
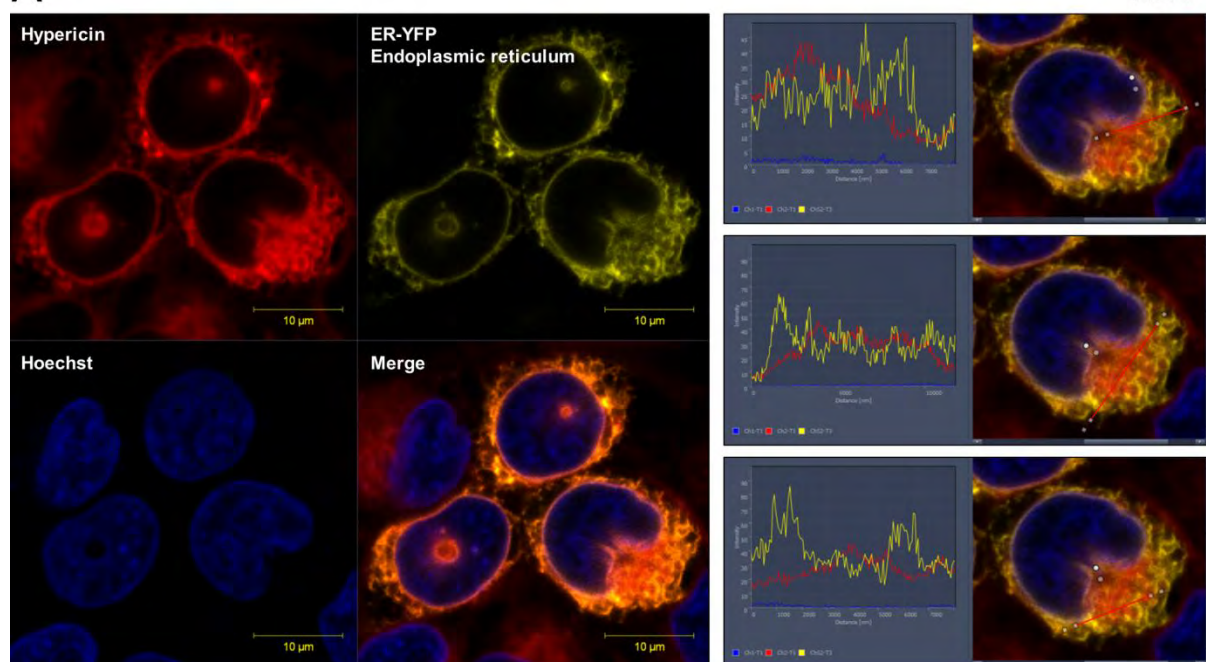


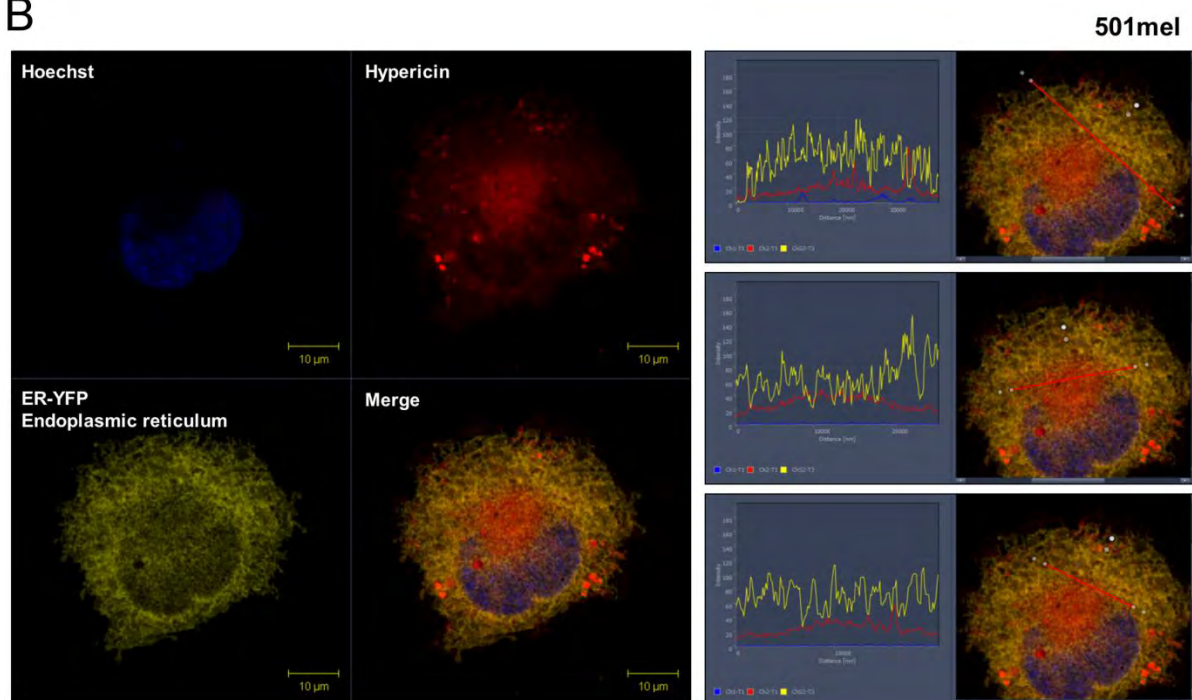
Figure 3.8: Intracellular localization of hypericin in relation to the endoplasmic reticulum (Bcl2-Cb5-GFP). Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells transiently expressing Bcl2-Cb5-GFP (green). Cells were exposed to 3 µM hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 µm).

To investigate co-localization of hypericin with the endoplasmic reticulum (ER), melanoma cells were transiently transfected with Bcl2-Cb5-GFP (Fig. 3.8) and ER-YFP (Fig. 3.9). The Bcl2-Cb5-GFP plasmid visualises the anti-apoptotic Bcl2 family member targeted to the ER²⁸². The ER-YFP plasmid visualises calreticulin, an ER-located, multifunctional protein involved in the quality control of protein folding. Confocal fluorescent microscopy images revealed a vast ER network in all melanoma cells, extending through most parts of the cell. Although profiles taken through the cells at different locations didn't show exact matching of the fluorophores, a large overlap between ER and hypericin signal was found as visualized by the overlap of the two channels (Fig. 3.8/3.9). Hypericin thus partially co-localised with the ER.

A



B



C

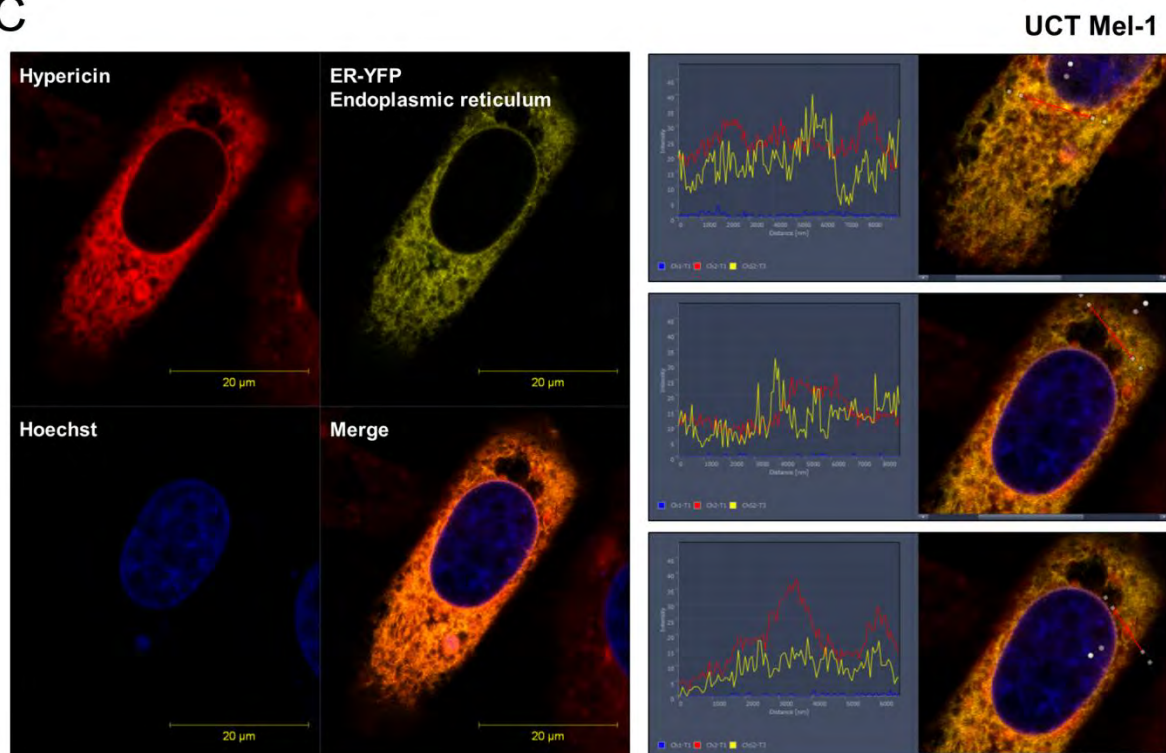
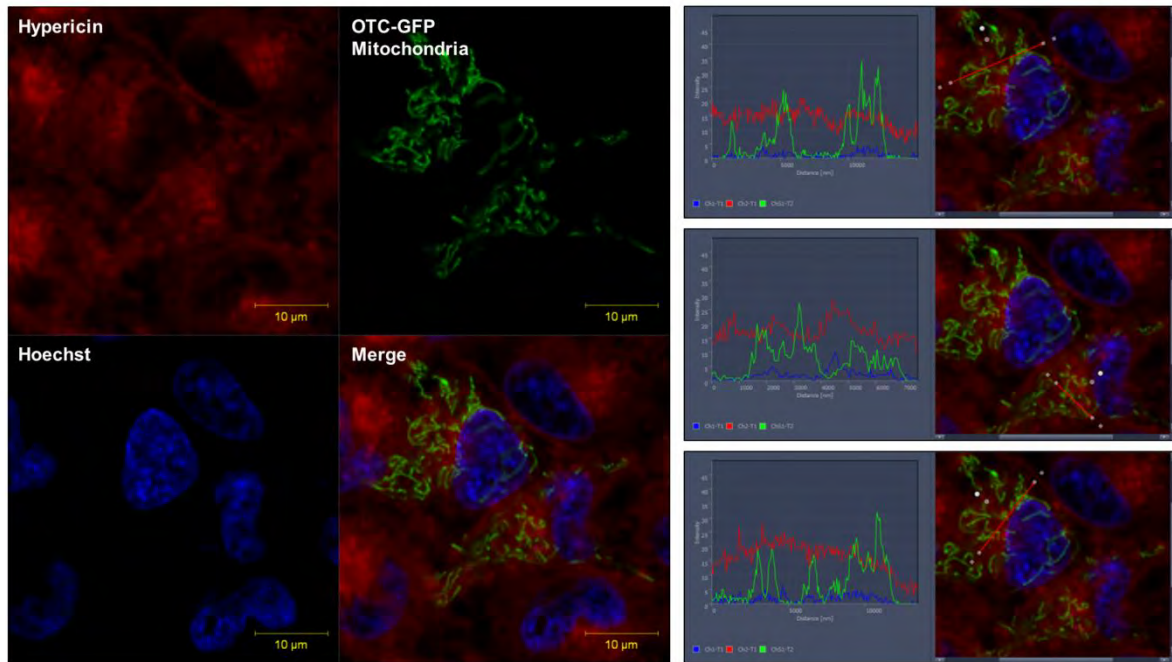


Figure 3.9: Intracellular localization of hypericin in relation to the endoplasmic reticulum (ER-YFP). Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells transiently expressing ER-YFP (yellow). Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

Mitochondria were visualised by transient expression of ornithine transcarbamylase (OTC)-GFP, a mitochondrial matrix protein^{283,292} (Fig. 3.10). Hypericin partially co-localized with the extensive tubular mitochondrial network in A375, 501mel or UCT Mel-1 melanoma cells (Fig. 3.10). Upon visualisation of the fluorophores in 501mel cells, the mitochondria unexpectedly changed shape from a tubular to a beaded appearance (Fig. 3.10B2). Mitochondria in controls not treated with hypericin did not undergo these changes. Activation of hypericin with the laser of the confocal microscope and subsequent ROS production in the vicinity of mitochondria possibly explains such extensive morphological alteration.

A

A375



B1

501mel

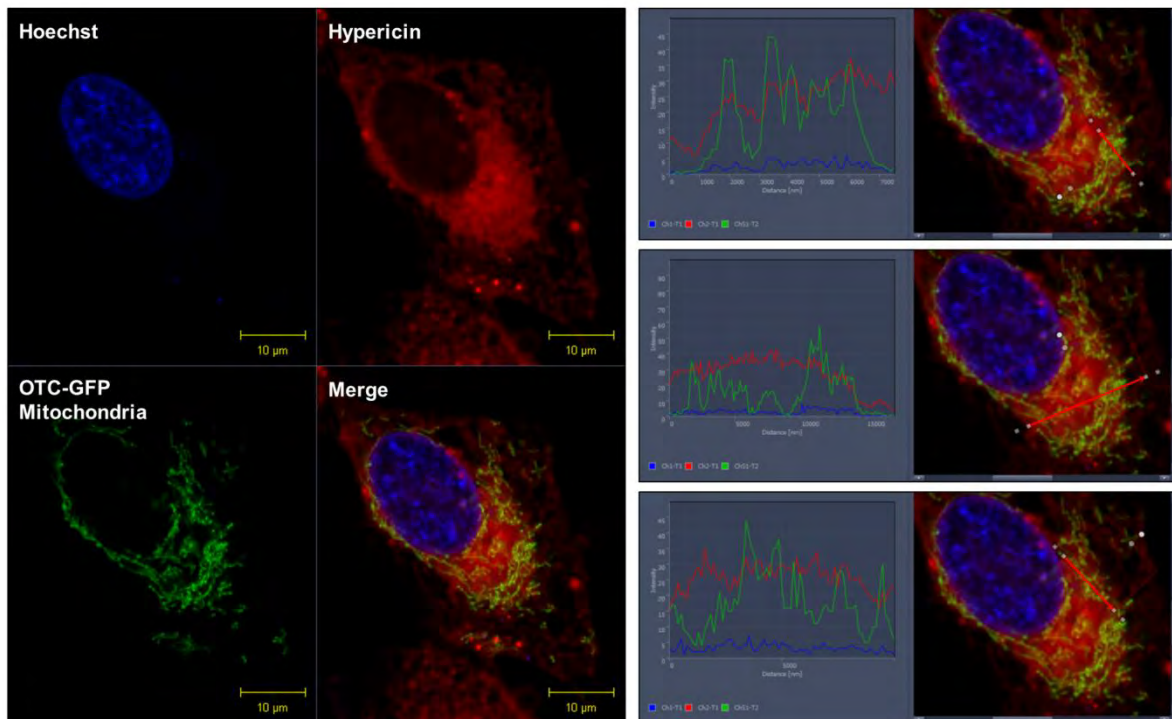
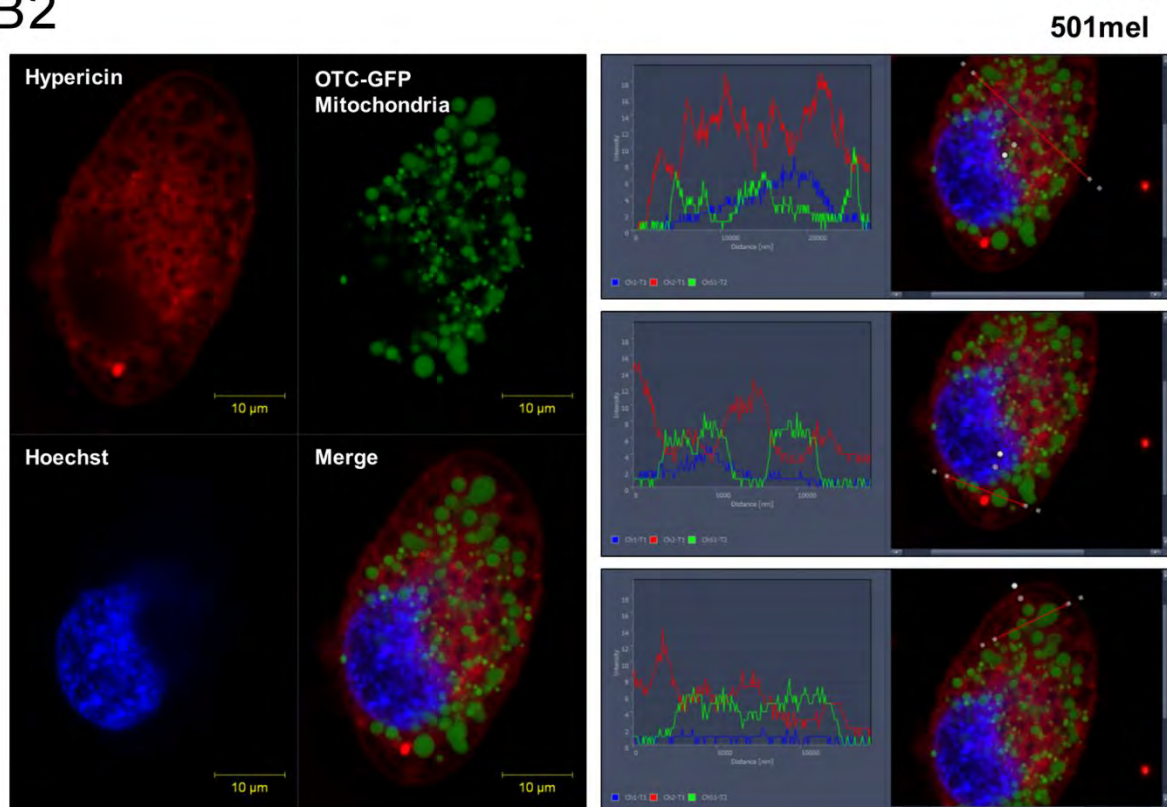
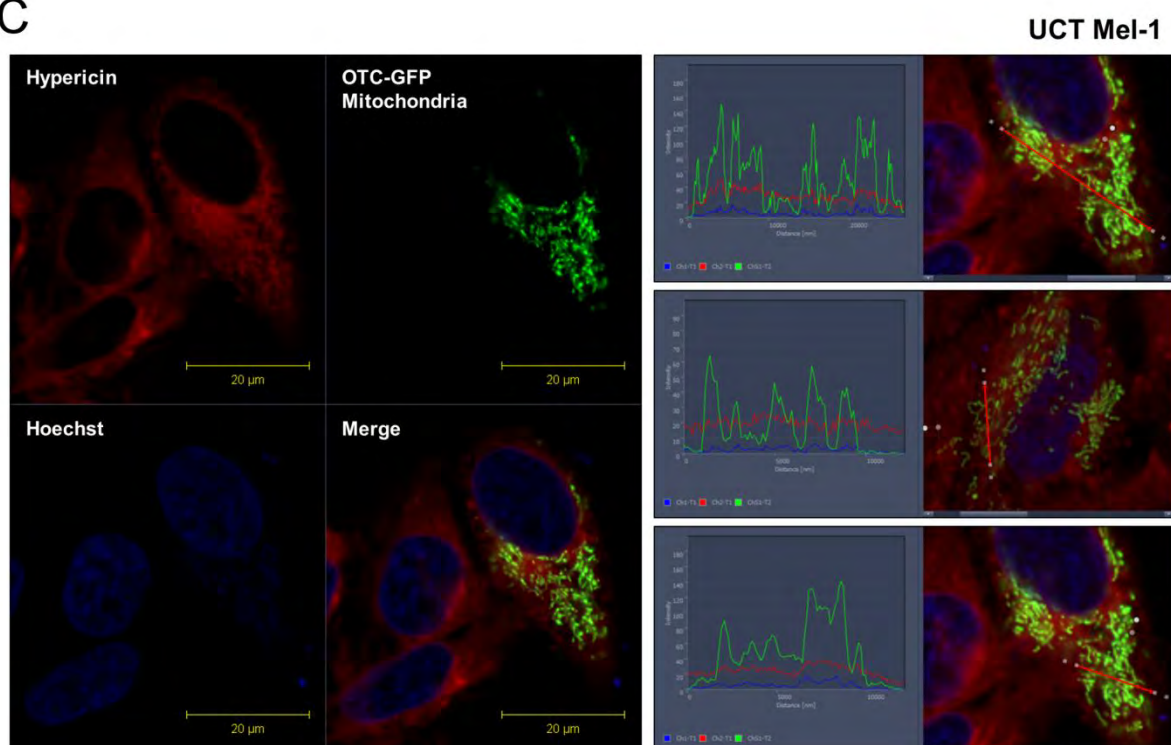


Figure 3.10: Intracellular localization of hypericin in relation to mitochondria. Live fluorescent confocal microscopy images of (A) A375, (B1/2) 501mel and (C) UCT Mel-1 cells transiently expressing OTC-GFP (green). Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

B2

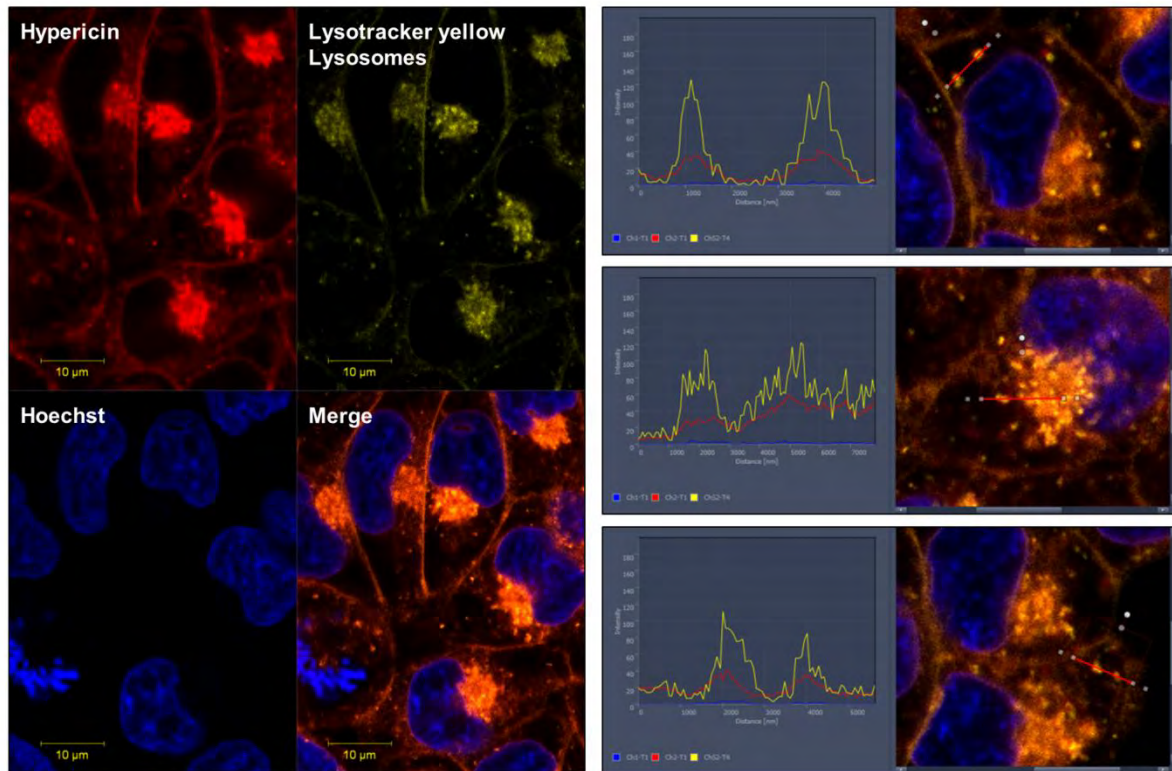


C



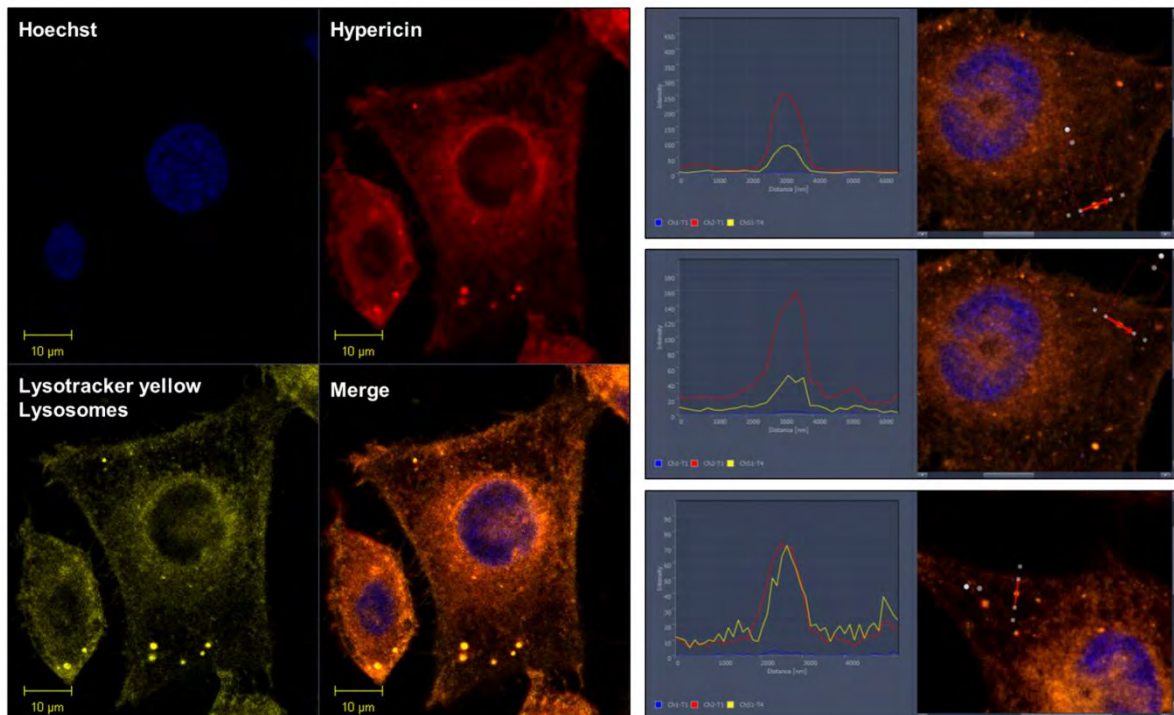
A

A375



B

501mel



C

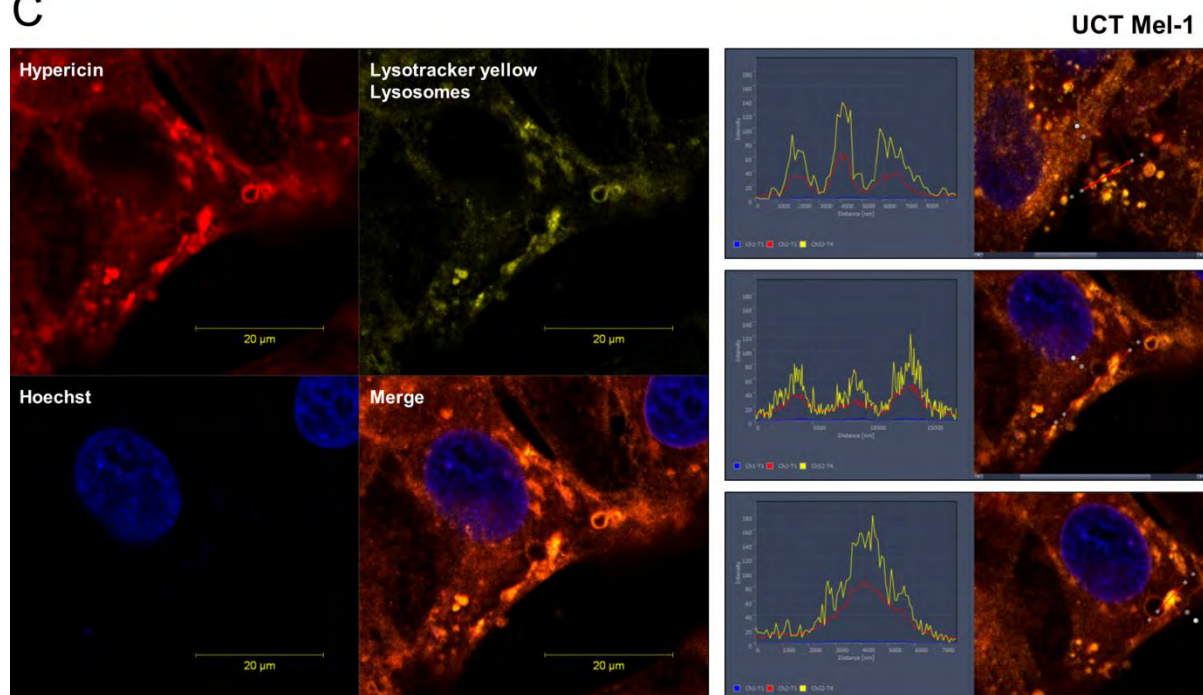


Figure 3.11: Intracellular localization of hypericin in relation to lysosomes. Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells stained with Lysotracker yellow. Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

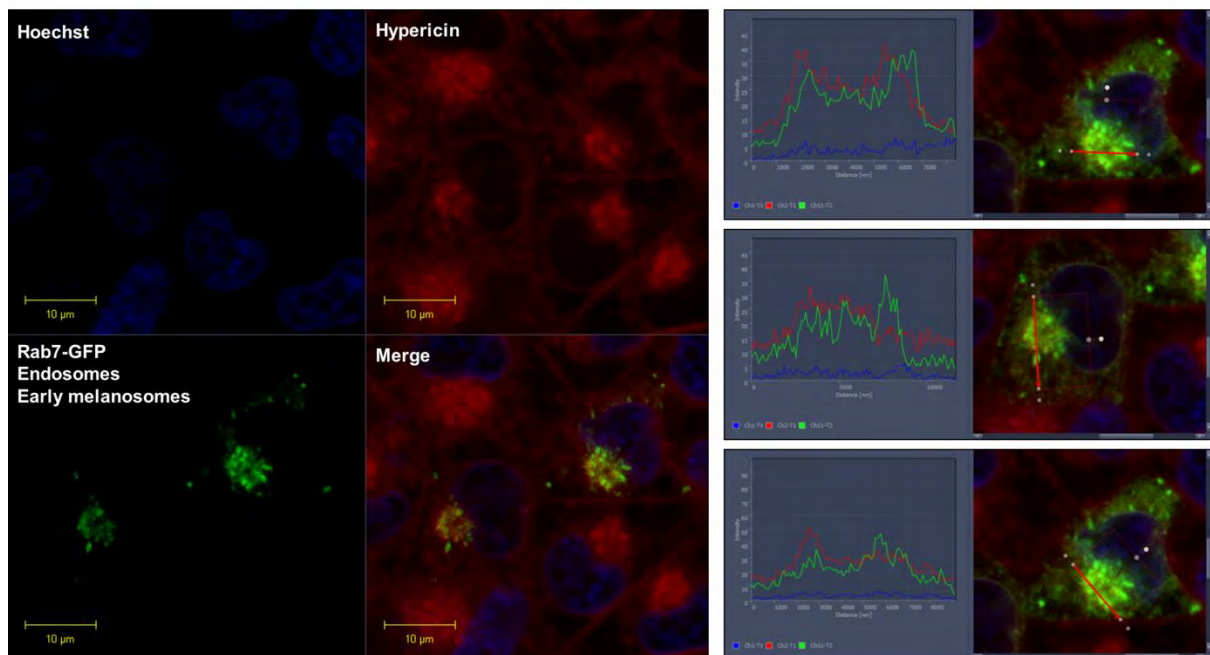
To visualise lysosomes, melanoma cells were exposed to Lysotracker yellow (Fig. 3.11). This fluorescent acidotropic probe labels acidic organelles in live cells. This includes lysosomes and possibly melanosomes as these are also acidic. Round, punctate lysosomes were present in all melanoma cells. In A375 cells these were predominantly found perinuclearly (Fig. 3.11A) whereas in 501mel and UCT Mel-1 lysosomes were distributed throughout the cell, especially on the periphery (Fig. 3.11B/C). Confocal profiles revealed convincing co-localization of hypericin with lysosomes in all melanoma cells.

To investigate melanosomal co-localization in more detail, melanoma cells were transfected with Rab7-GFP (Fig. 3.12), Rab27a-GFP (Fig. 3.13) and MyosinVa-GFP (Fig. 3.14). Rab7 is involved in microtubule-based transport between early and late endosomes, late endosomes and lysosomes and early and intermediate stage melanosomes^{284,293}. In the latter it facilitates the transport of tyrosinase and tyrosinase-related protein 1 (TYRP1) from the trans-golgi network to melanosomes. Rab27a is involved in the actin-dependent transport of mature melanosomes²⁹⁴. It localizes to the melanosomal membrane in its active GTP-bound state and acts as a receptor for the actin-dependent myosinVa motor protein, facilitated by the specific Rab27a effector melanophilin/Slac2a. Rab27a, melanophilin and myosinVa thus form a “molecular tripartite complex” that links mature

melanosomes to the actin network, transporting them to the distal parts of the dendrites in melanocytes²⁸⁵.

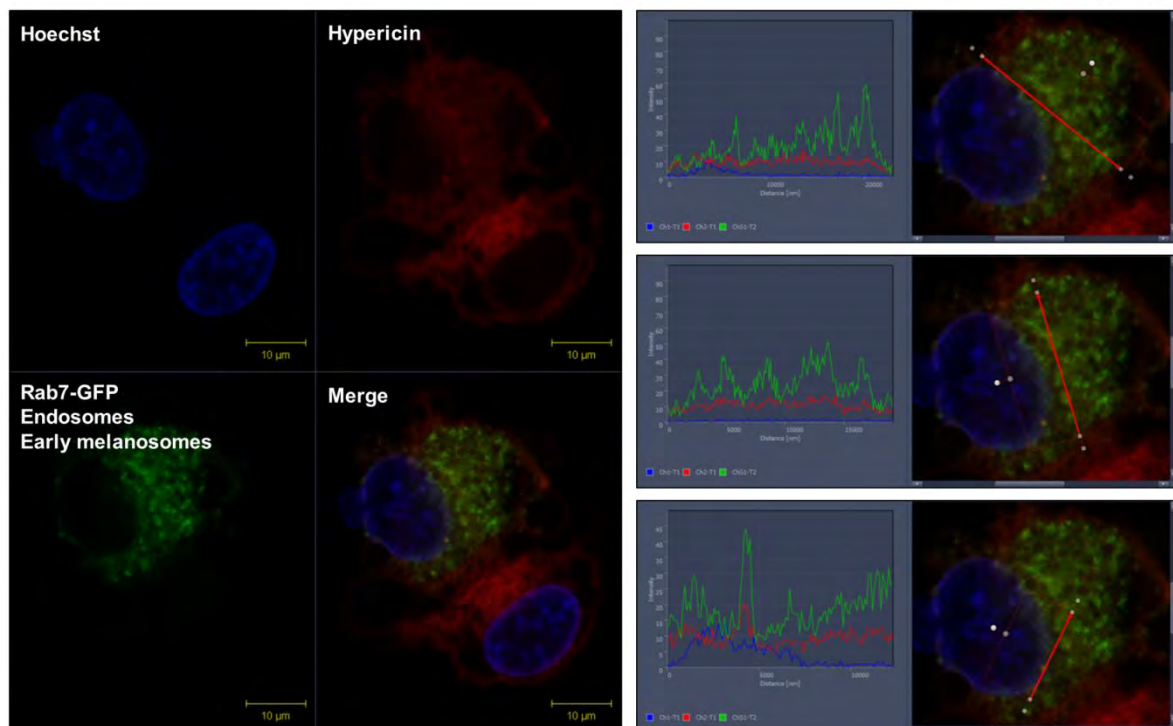
A

A375



B

501mel



C

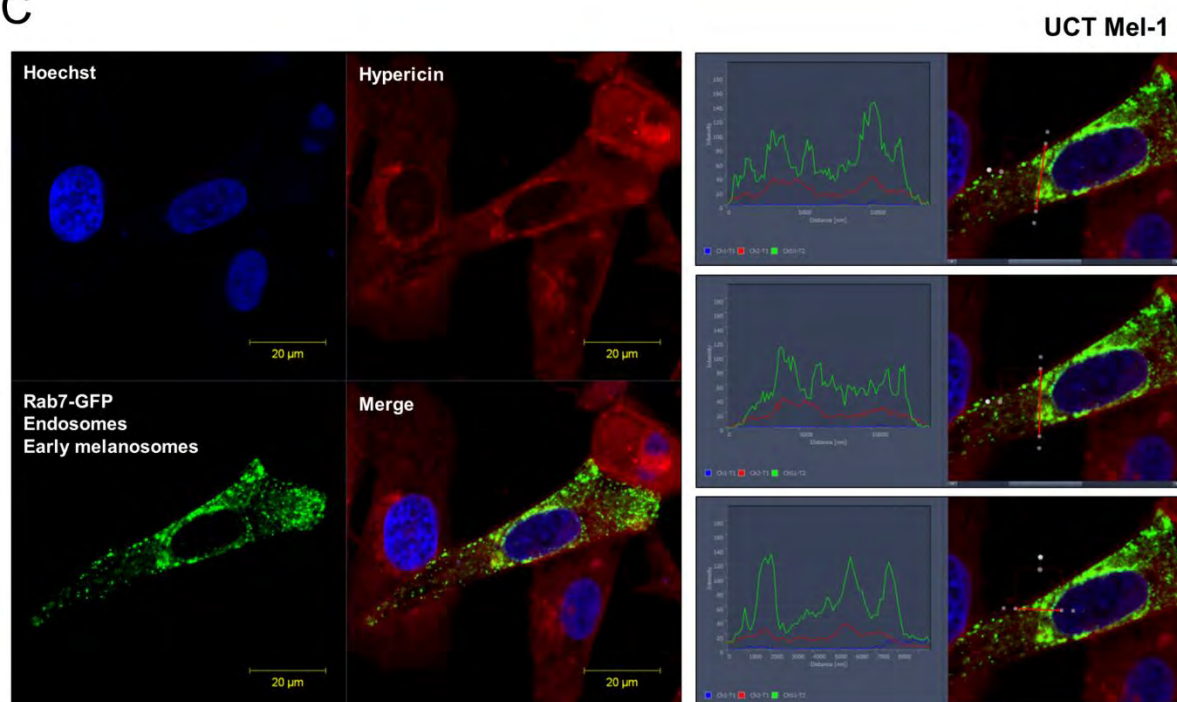
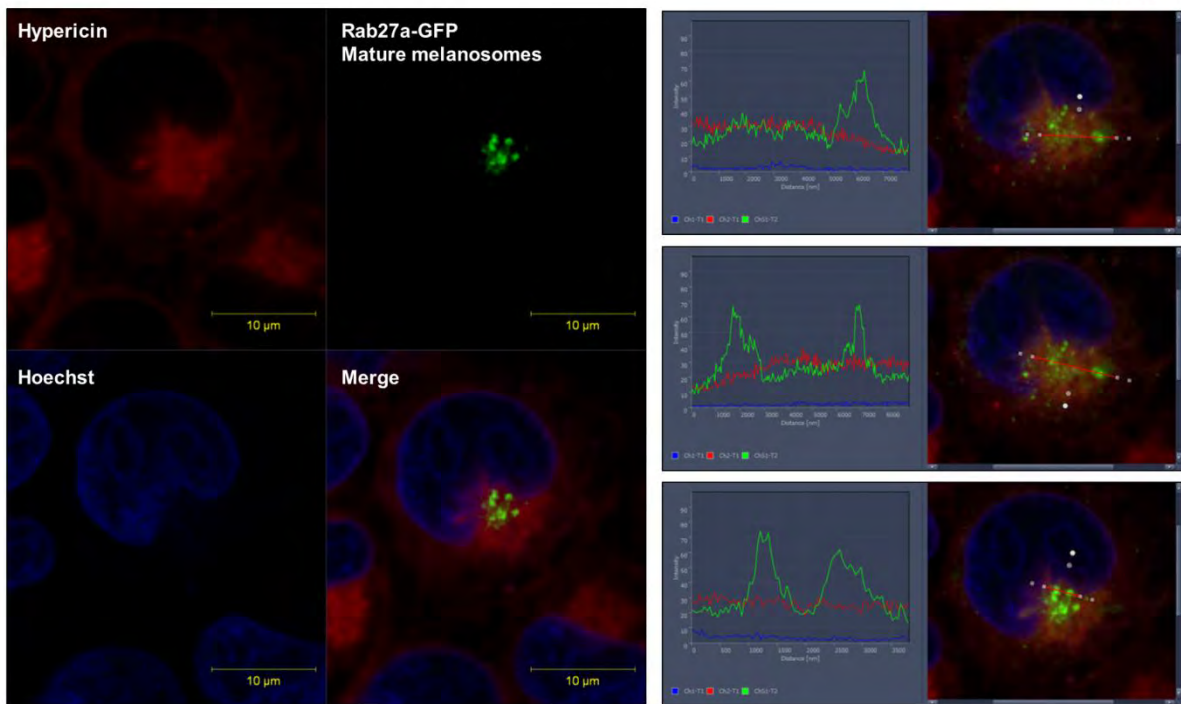


Figure 3.12: Intracellular localization of hypericin in relation to endosomes and early melanosomes (Rab7-GFP). Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells transiently expressing Rab7-GFP (green). Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

Transient expression of Rab7-GFP in melanoma cells revealed a circular, punctate staining perinuclearly in A375 melanoma cells with variable hypericin co-localization (Fig. 3.12A). The 501mel and UCT Mel-1 melanoma cells showed Rab7-GFP expression throughout the cell with variable hypericin co-localization in the former and no co-localization in the latter (Fig. 3.8B, C). A similar expression pattern was observed when cells were transfected with Rab27a-GFP, however profiles indicated no co-localization with hypericin (Fig. 3.13). Cells expressing MyosinVa-GFP revealed a comparable expression pattern to the other melanosomal markers, with variable hypericin co-localization in 501mel and UCT Mel-1 (Fig. 3.14B/C) and no co-localization in A375 melanoma cells (Fig. 3.14A).

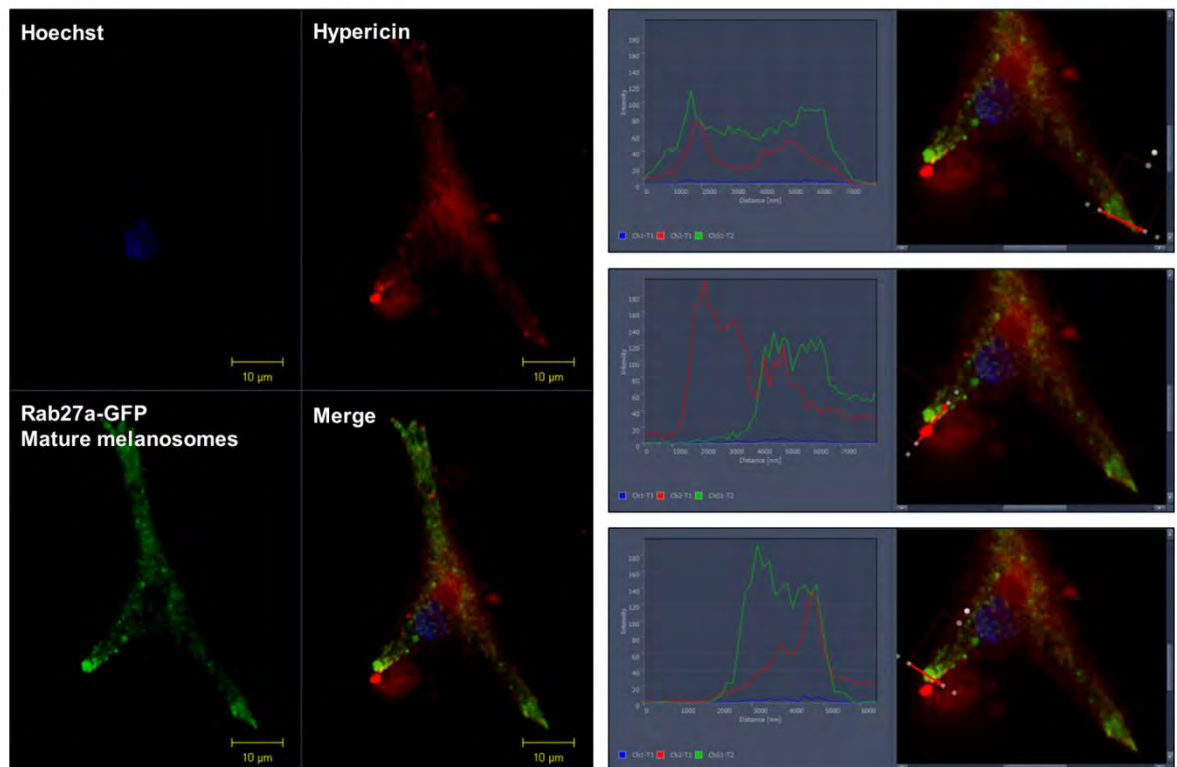
A

A375



B

501mel



C

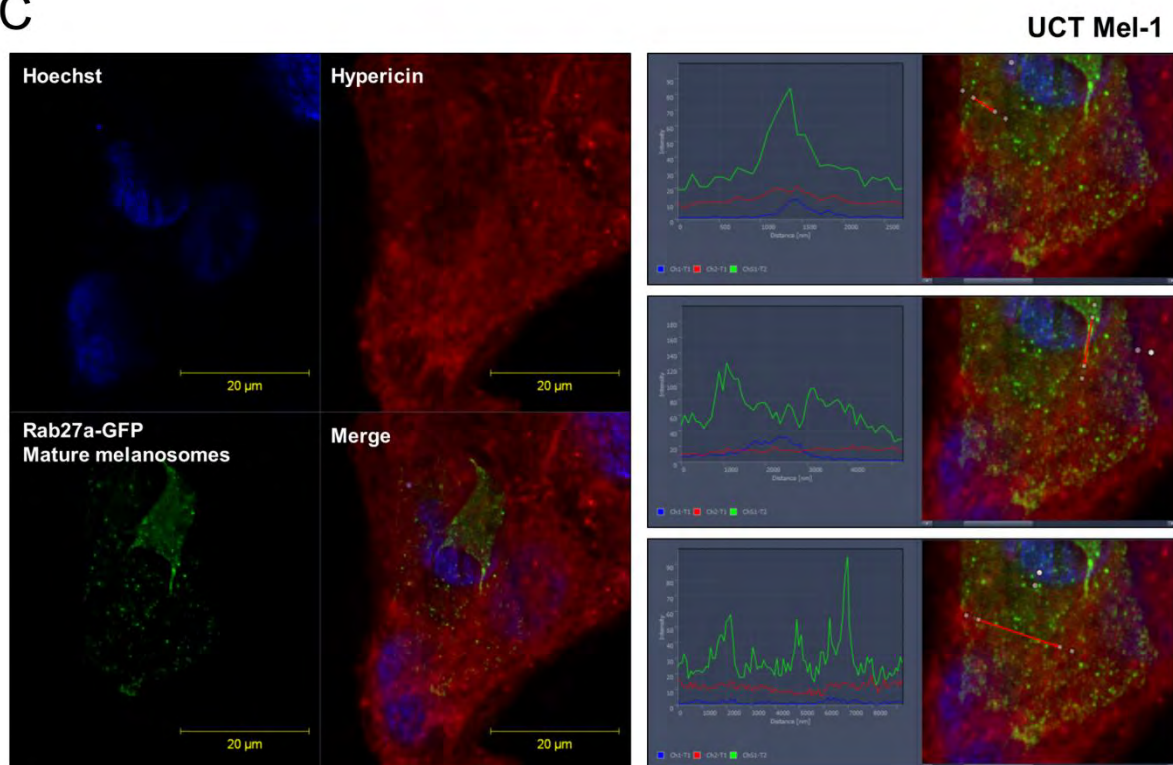
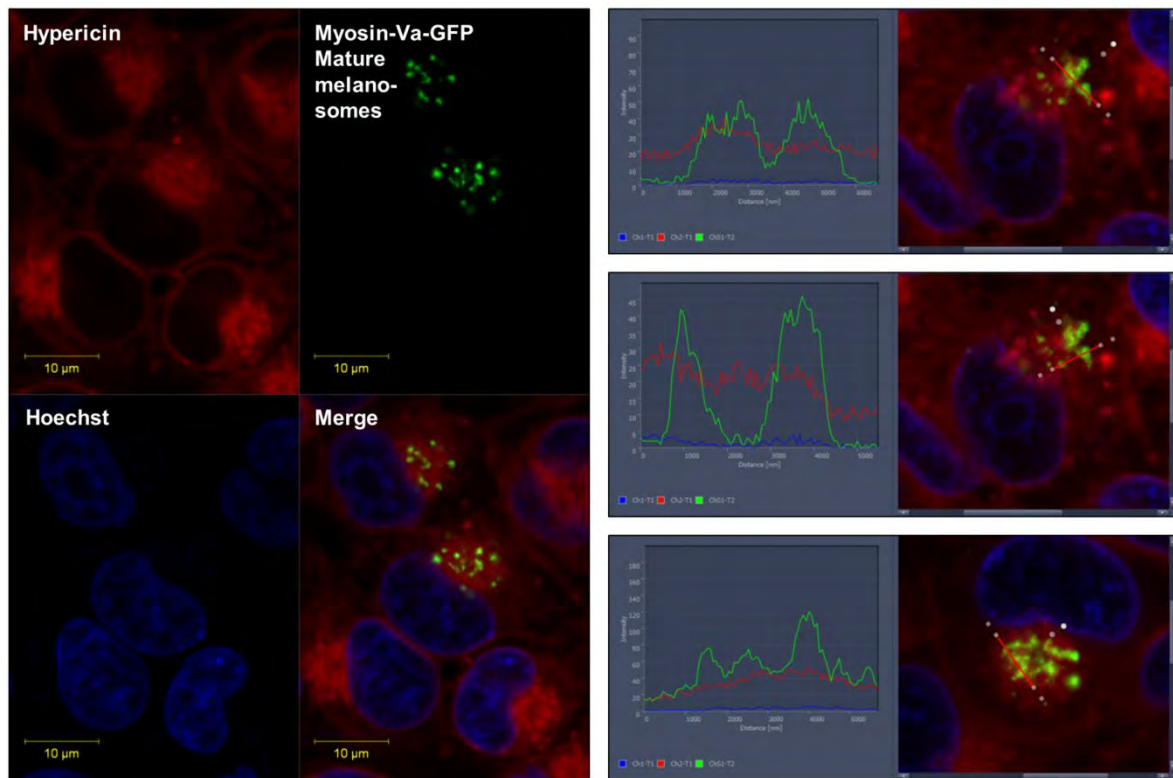


Figure 3.13: Intracellular localization of hypericin in relation to mature melanosomes (Rab27a-GFP). Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells transiently expressing Rab27a-GFP (green). Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

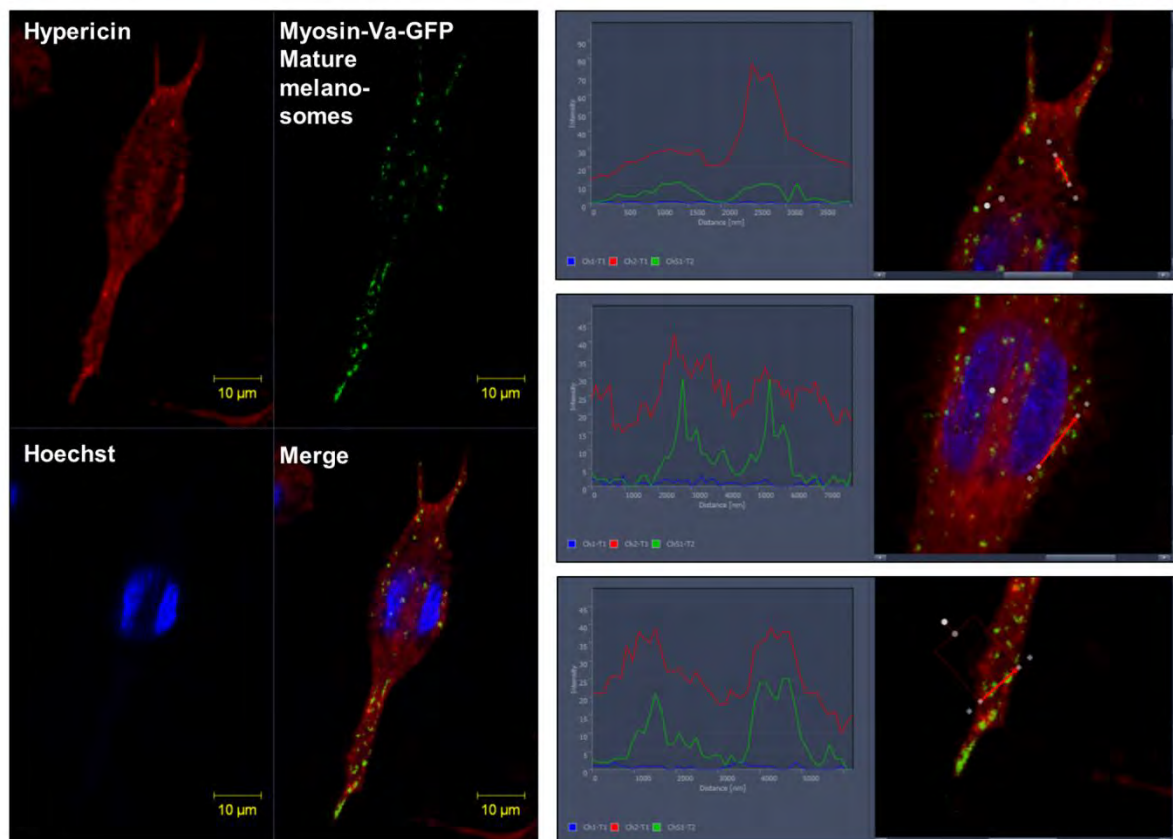
A

A375



B

501mel



C

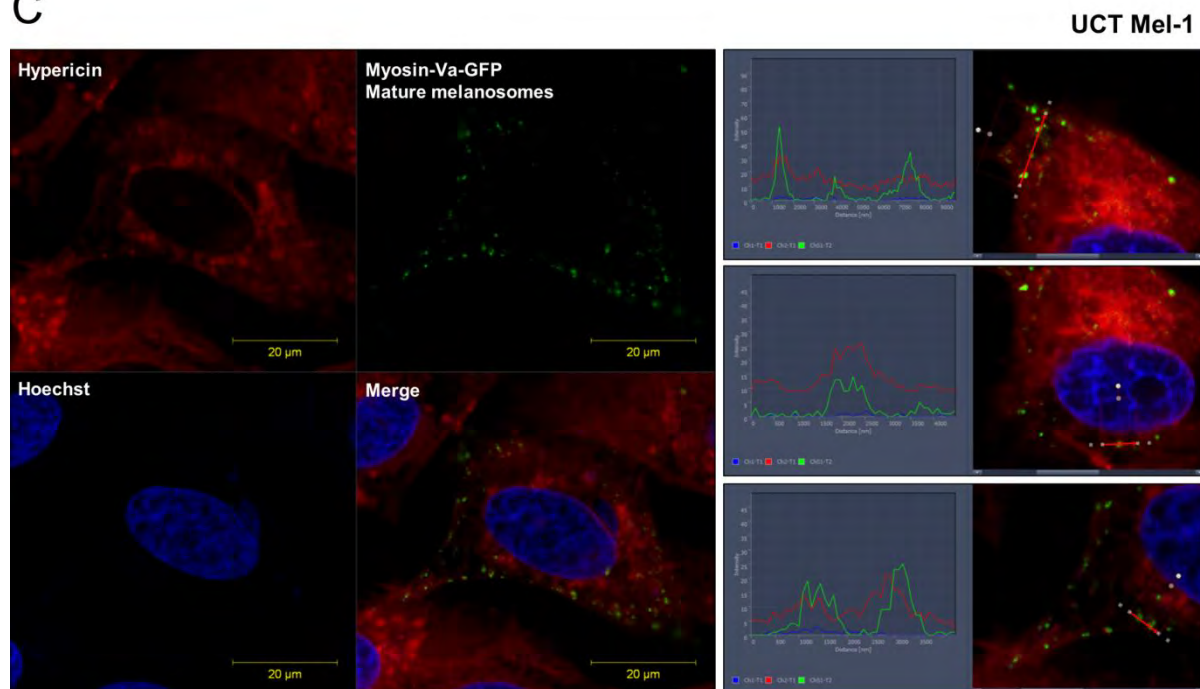


Figure 3.14: Intracellular localization of hypericin in relation to mature melanosomes (MyosinVa-GFP). Live fluorescent confocal microscopy images of (A) A375, (B) 501mel and (C) UCT Mel-1 cells transiently expressing MyosinVa-GFP (green). Cells were exposed to 3 μ M hypericin (red) for 4 h without light activation and nuclei were counterstained with Hoechst (blue). Profiles taken at different locations through the cell indicate co-localization of the fluorophores (right panel). A representative result is shown (n=3, scale bars: 10/ 20 μ m).

The intracellular localization of hypericin in melanoma cells is summarized in the table below (Table 3.1). It is worthy to note that hypericin did not co-localize with nuclei.

Organelle	A375	501mel	UCT Mel-1
Endoplasmic reticulum	partial	partial	partial
Mitochondria	partial	partial	partial
Lysosomes	+	+	+
Early melanosomes	partial	partial	-
Late stage melanosomes	-	partial	partial
Nuclei	-	-	-

Table 3.1: Intracellular localisation of hypericin. Intracellular organelles of A375, 501mel and UCT Mel-1 melanoma cells were visualised with confocal fluorescent microscopy using organelle-specific G/YFP-fusion proteins and dyes. Cells were exposed to 3 μ M hypericin for 4 hours without light activation. Profiles taken through the cells at different locations indicated co-localization of the fluorophores (+:co-localization, -: no co-localization, partial: not exclusive, n=3).

3.4 PART III: HYPERICIN-PDT INDUCED MELANOMA CELL DEATH

To investigate the mechanisms underlying the observed reduction in melanoma cell viability after hypericin-PDT treatment we chose to analyse several markers of cell death. These included: ultrastructural modifications, phosphatidylserine exposure and loss of cell membrane integrity, cell size and granularity/ pigmentation, intracellular hypericin post PDT and cell death protein expression.

3.4.1 ULTRASTRUCTURAL MODIFICATIONS

To investigate the initial response to light-activated hypericin we chose to analyse one unpigmented (A375) and one pigmented (UCT Mel-1) cell line ultrastructurally at 4 hours after treatment. We treated melanoma cells with a non-lethal dose (1 μ M) and the killing dose (3 μ M) of hypericin-PDT and visualised the resulting cellular ultrastructure with transmission electron microscopy (TEM).

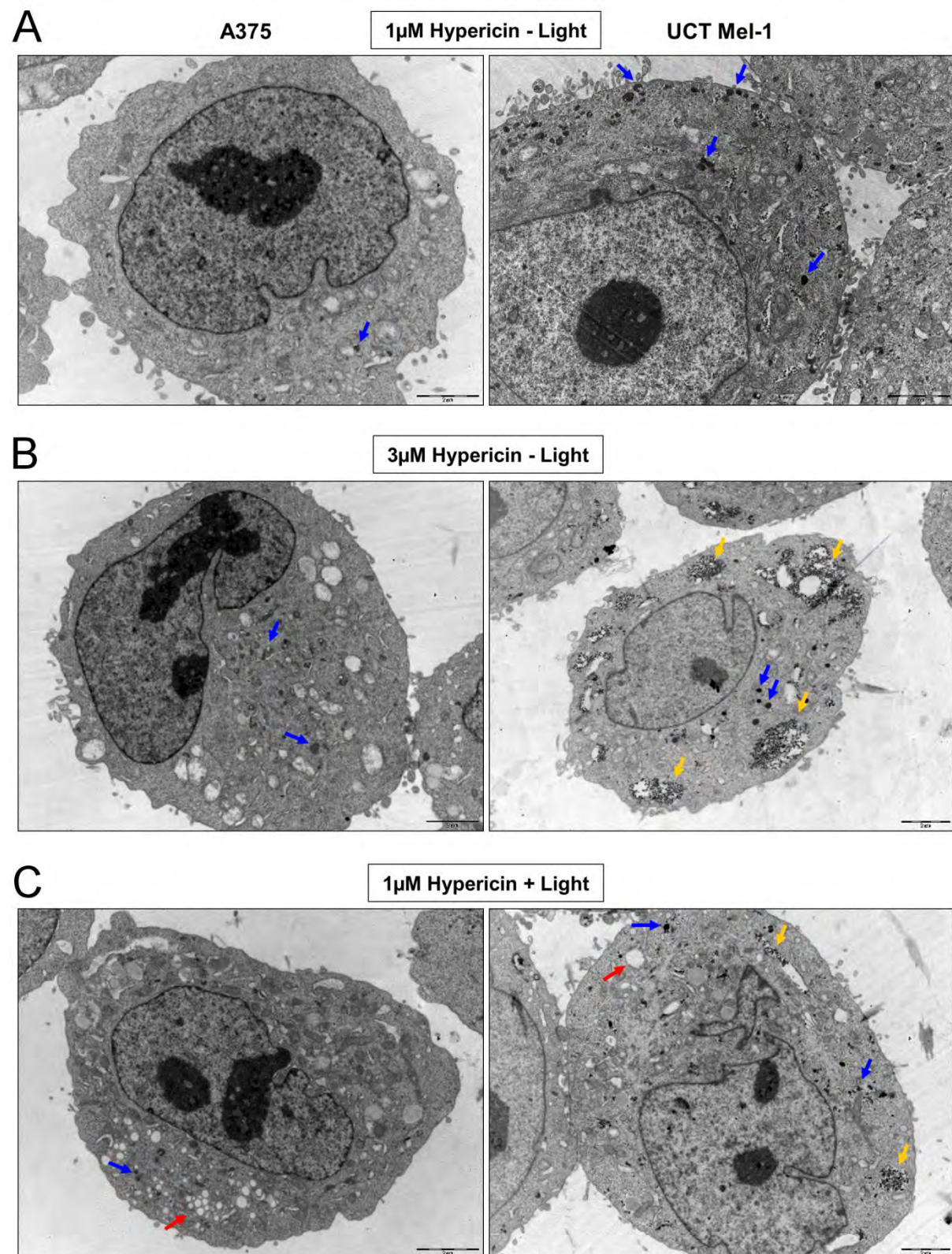
Treatments with 1- and 3 μ M sham-irradiated hypericin resulted in live cells with intact cell and nuclear membranes and no change to the ultrastructure of intracellular organelles (Fig. 3.15A/B). The UCT Mel-1 cells contained mostly mature (stage IV) and some early melanosomes (stage II, III), which tended to gather peripherally in the cells (Fig. 3.15A/B). In addition, large pools of glycogen were found in UCT Mel-1 (Fig. 3.15A/B), which was not observed in untreated cells (Fig. 3.2C). The A375 cells contained no glycogen aggregations and few mature melanosomes (Fig. 3.15A/B).

Light-activated 1 μ M hypericin treatment induced vacuolisation in both A375 (30-40 %) and UCT Mel-1 (20-30 %), with no change in cell and nuclear membranes or intracellular organelles (Table 3.2/Fig. 3.15C).

In contrast, light-activated 3 μ M hypericin treatment induced extensive vacuolisation, replacing most of the cytoplasm, in both A375 (>80 %) and UCT Mel-1 melanoma cells (70-80 %, Table 3.2/Fig. 3.15D). Numerous small and large vacuoles were found, the former predominantly aggregating perinuclearly and the latter along the periphery of the cells (Fig. 3.15D). Some small vacuoles contained electron dense material. The UCT Mel-1 contained glycogen aggregations (Fig. 3.15D), which were not found in A375 melanoma cells. Both cell lines revealed intact cellular ultrastructure with no change in cell or nuclear membranes or intracellular organelles (Fig. 3.15D).

Moreover, morphological features of apoptosis such as fragmentation of nuclei and formation of apoptotic bodies, or morphological features of necrosis including the swelling of intracellular

organelles and disintegration of the cell membrane³⁶, were not found in the melanoma cells at 4 hours after hypericin-PDT. Our findings are summarised in Table 3.2.



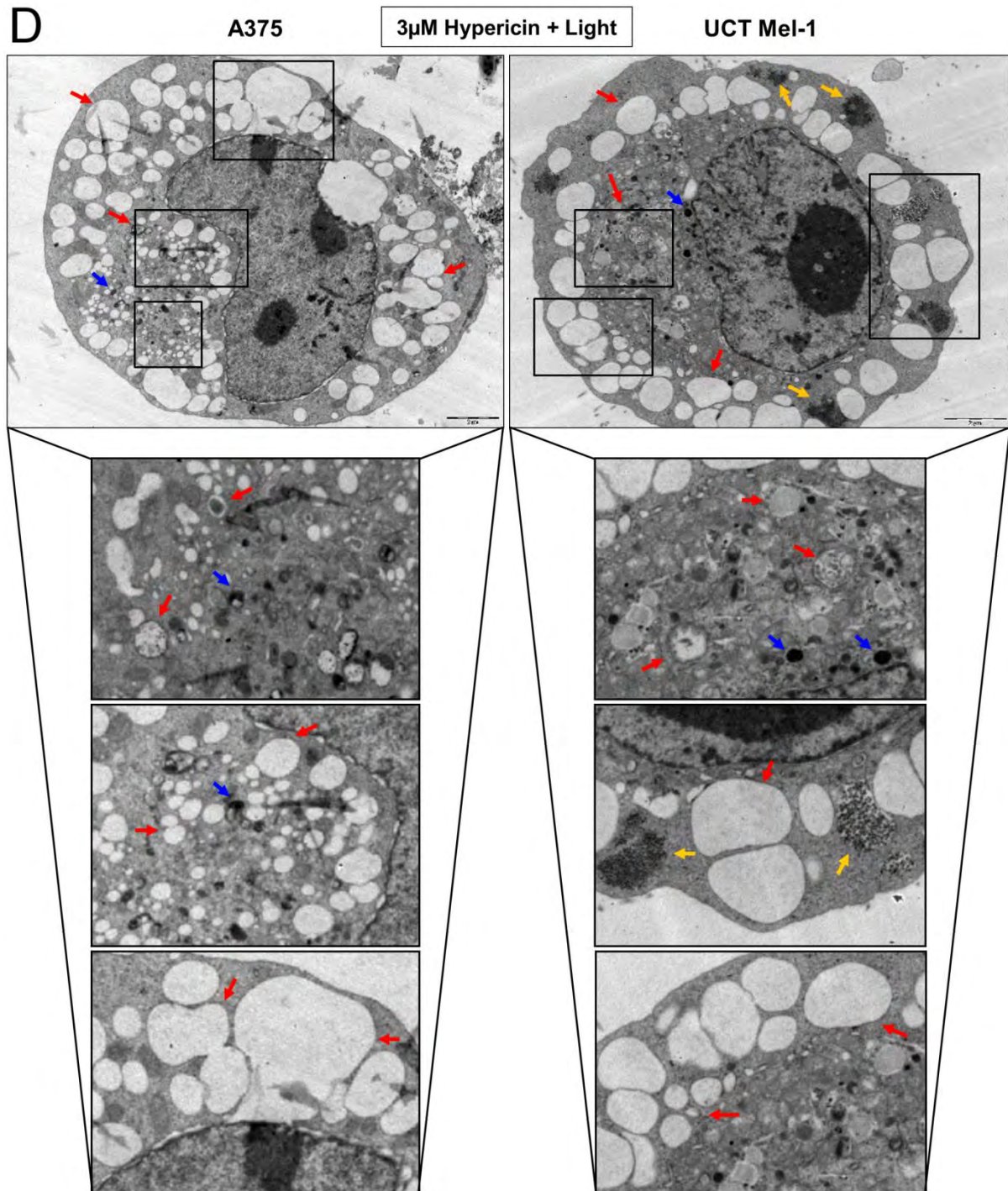


Figure 3.15: PDT induced ultrastructural changes. Transmission electron microscopy (TEM) images of A375 (left panel) and UCT Mel-1 (right panel) melanoma cells at 4h after treatment with (A) 1 μ M and (B) 3 μ M hypericin with sham-irradiation or (C) 1 μ M and (D) 3 μ M hypericin with light-activation (3 panels in D: magnified images taken from different areas in the cell, indicated by the rectangles). Yellow arrows indicate glycogen accumulation. Blue arrows show melanosomes filled with the electron dense pigment melanin. Red arrows show the presence of numerous vacuoles of different sizes. A representative result is shown (n=3, scale bar: 2 μ m).

Treatment	Unpigmented A375		Pigmented UCT Mel-1	
	Vacuolisation (%)	Glycogen accumulation	Vacuolisation (%)	Glycogen accumulation
Control –Light	0	-	0	-
1/3 μ M Hypericin –Light	5	-	5	+
1 μ M Hypericin +Light	30-40	-	20-30	+
3 μ M Hypericin –Light	>80	-	70-80	+++

Table 3.2: Hypericin-PDT induced ultrastructural changes. Melanoma cells were treated with 1 and 3 μ M hypericin with sham-irradiation or light-activation followed by transmission electron microscopy (TEM) analyses at 4 hours after treatment. Cellular vacuolisation (% area of cytoplasm) and glycogen accumulation (-: no glycogen, +: glycogen) was analysed. Approximately 50 cells were scored from resin sections for each sample and the scoring was determined by 2 independent viewers.

3.4.2 PS EXPOSURE & LOSS OF CELL MEMBRANE INTEGRITY

A change in cell membrane structure by surface exposure of phosphatidylserine (PS) is observed in early apoptotic cells²⁹⁵. This involves the translocation of PS from the inner side of the cell membrane to the outer leaflet, where it acts as an “eat me” signal for clearance by phagocytes. Annexin V is a phospholipid binding protein with high affinity for PS, labelling early apoptotic cells effectively²⁸⁸. It has however also been reported that early necrotic cells expose PS followed by macrophage clearance²⁹⁶. Using Annexin V in conjunction with a cell permeability dye (e.g. propidium iodide) enables the distinction between cells with exposed PS and those with compromised cell membranes. Loss of cell membrane integrity is a feature of necrotic cells²⁹⁷. Due to the red fluorescent nature of propidium iodide it is not the most suitable assay to use in conjunction with the red autofluorescent hypericin. We thus used the violet amine reactive viability dye (VIVID) to investigate loss of cell membrane integrity in our experimental system.

Melanoma cells were treated with the killing-dose of hypericin and analysed for PS exposure (Annexin V) and loss of cell membrane integrity (VIVID) at 30', 1, 4, 7 and 24 hours after treatment by fluorescent activated cell sorting (FACS). Resulting count vs fluorescent intensity plots showed one peak of fluorescence for Annexin V (Fig. 3.16 left panel) and VIVID but upon loss of membrane integrity, VIVID displays two peaks of fluorescence (Fig. 3.16 right panel). VIVID is an amine reactive dye that binds to amines on the outside of live cells (low intensity peak) and on the inside of cells with a compromised cell membrane (high intensity peak, Fig. 3.16 right panel). Cells treated with vehicle with sham-irradiation (control –light), vehicle with light-activation (control +light) and

hypericin with sham-irradiation (hypericin –light) displayed no externalization of PS and intact cell membranes (Fig. 3.15). Upon treatment with light-activated hypericin (hypericin +light) increasing Annexin V fluorescent intensity peaks were observed for all melanoma cells, indicating PS exposure (Fig. 3.16 left panel). The same treatment resulted in increasing VIVID fluorescent intensity peaks in all cells, evident by the second peak of higher fluorescence in the flow fluorocytometric histogram, revealing loss of cell membrane integrity (Fig. 3.16 right panel).

The Annexin V MFI analyses revealed a time-dependent increase in PS exposure after light-activated hypericin treatment in all melanoma cells, significantly different to controls (Fig 3.17). The highest Annexin V MFI was observed at 24 h after treatment, most pronounced in A375 (14.1 ± 1.5 fold, Fig. 3.17A) and similar in 501mel (7.2 ± 1.0 fold, Fig. 3.17B) and UCT Mel-1 (7.5 ± 1.3 fold, Fig. 3.17C), compared to that of the vehicle-treated, sham-irradiated control (Fig. 3.17 black bar).

The VIVID MFI analyses showed loss of 501mel cell membrane integrity after light-activated hypericin treatment at all time-points investigated, significantly different to the controls (Fig. 3.18B). Similarly, A375 cell membrane integrity was lost at all time-points after treatment, except for 4 hours (Fig. 3.18A). In contrast, UCT Mel-1 cell membrane integrity stayed intact up to 7 hours (Fig. 3.18C). These changes in cell membrane permeability were most pronounced at 24 hours for all cells (A375: 81.5 ± 9.4 , 501mel: 69.2 ± 11 and UCT Mel-1: 25.3 ± 6.1 fold compared to control, Fig. 3.18).

Comparing the cell lines with each other revealed a significantly higher A375 PS exposure compared to both 501mel and UCT Mel-1 at 24 hours after treatment (Fig. 3.19A). At the same time point, UCT Mel-1 cell membranes were significantly more intact than those of A375 and 501mel as shown by the VIVID results (Fig. 3.19B). Moreover, UCT Mel-1 loss of cell membrane integrity was significantly lower at 1 and 4 hours after treatment compared to that of 501mel cells (Fig. 3.19B).

A

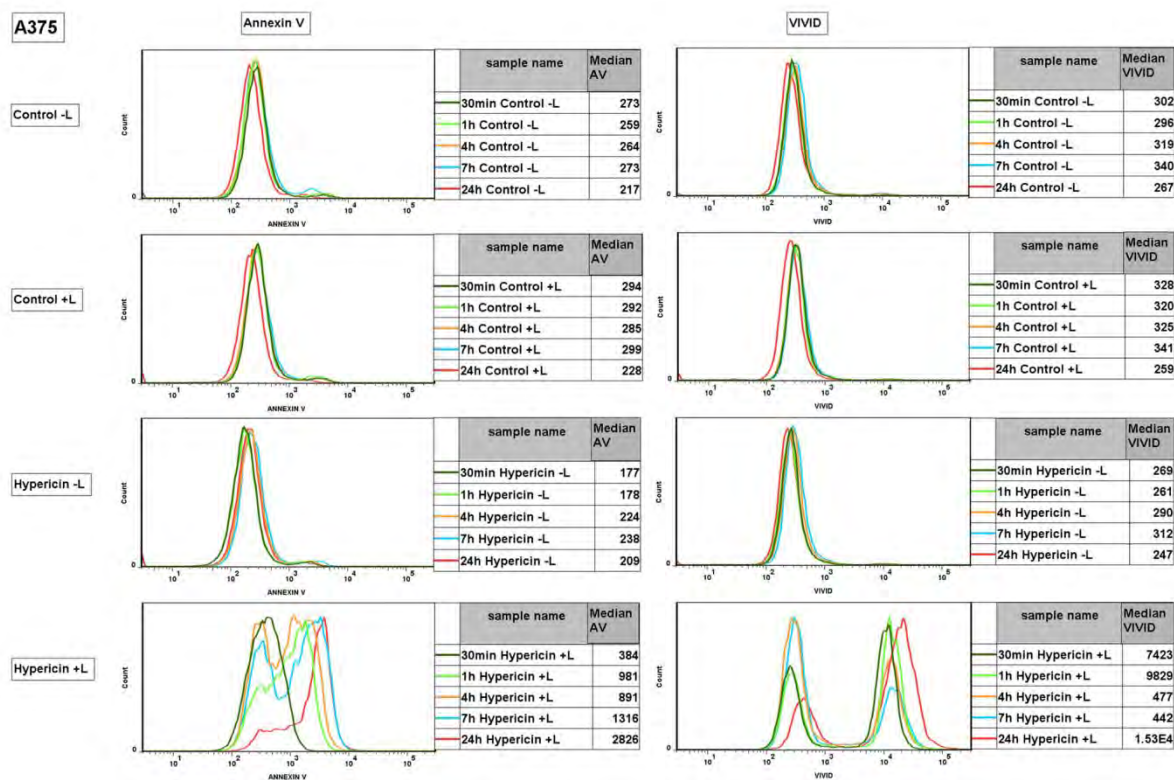
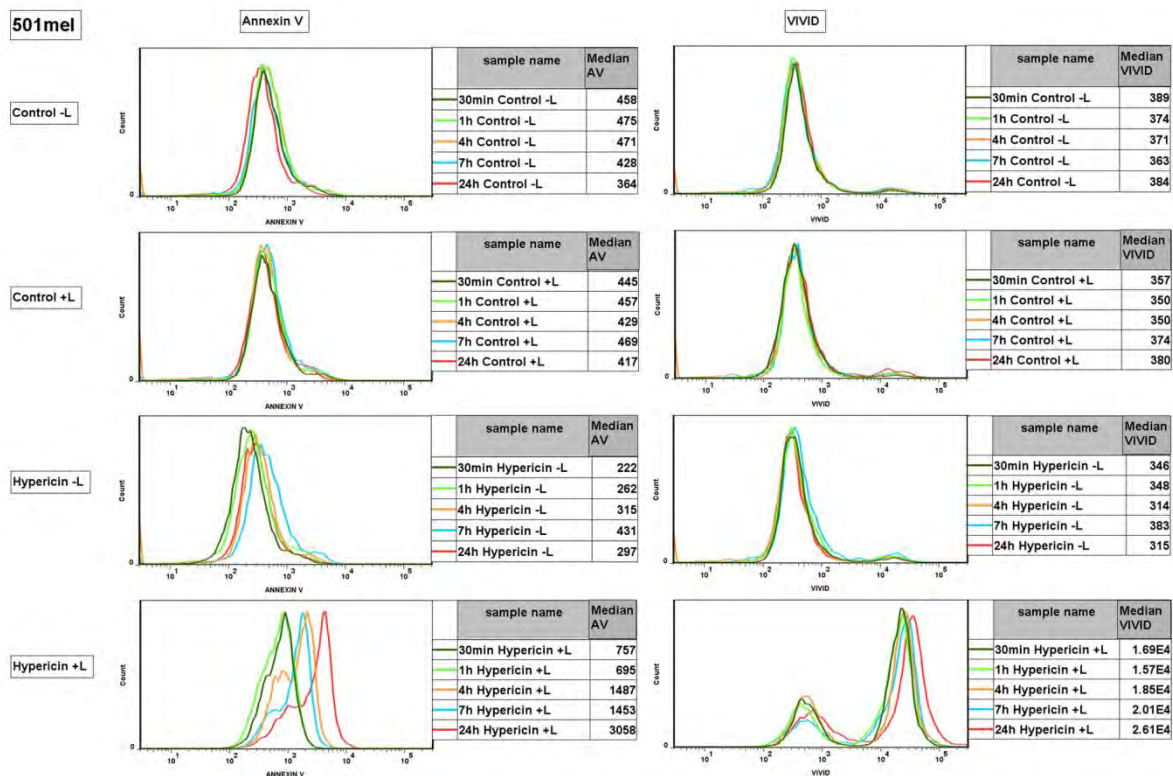


Figure 3.16: Flow fluorocytometric histograms of PDT induced cell death. (A) A375, (B) 501mel and (C) UCT count vs fluorescent intensity plots of Annexin V (phosphatidyl serine exposure, left panel) and VIVID (loss of cell membrane integrity, right panel) at 30', 1, 4, 7 and 24 h after treatment. A representative result is shown (n≥3, L: light).

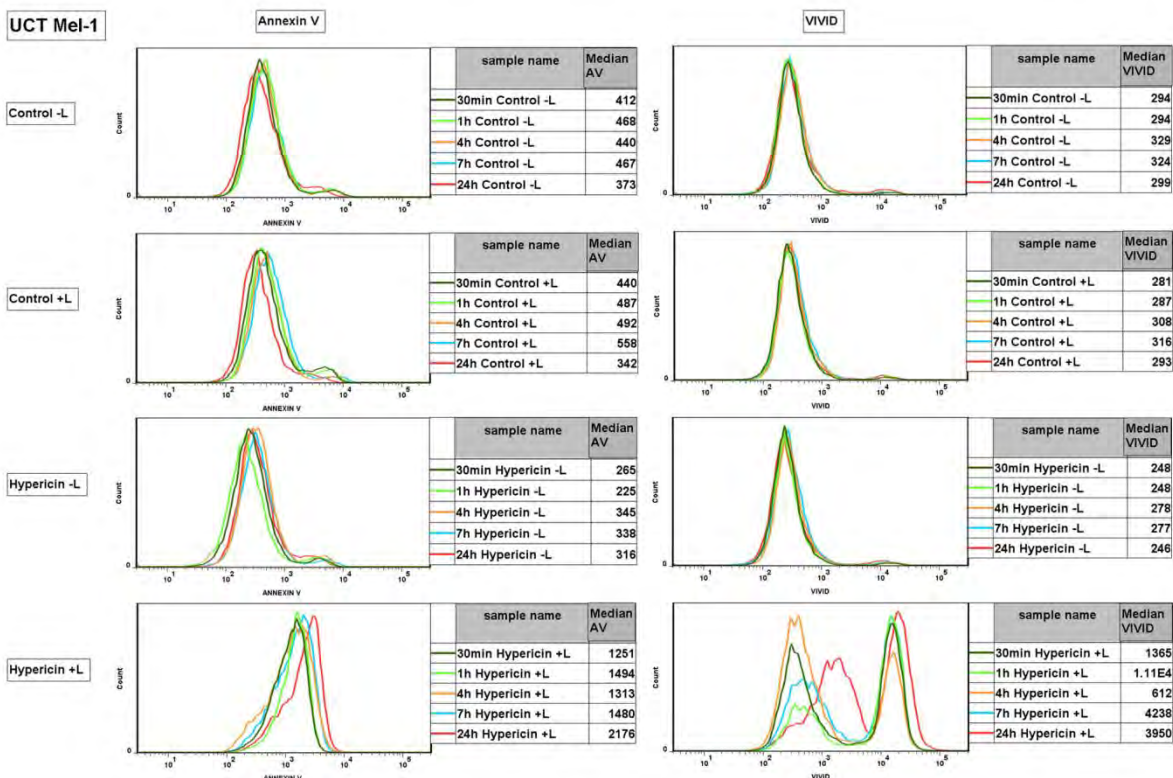
B

501mel



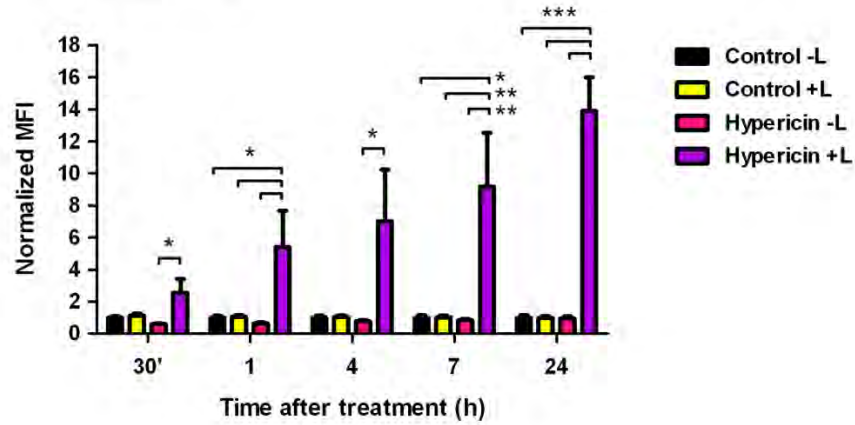
C

UCT Mel-1



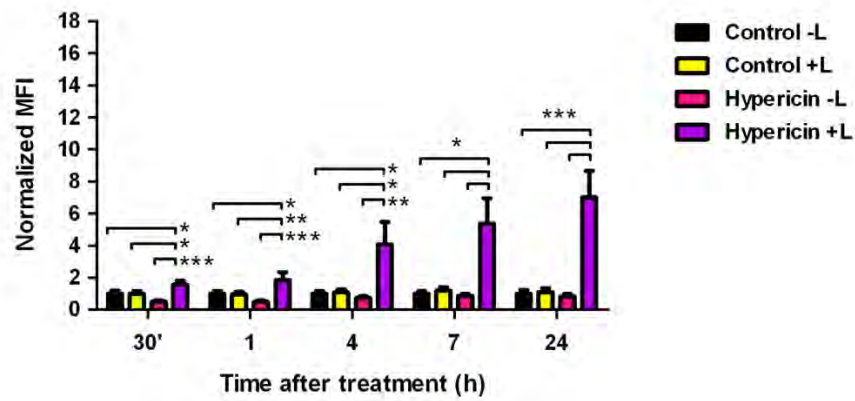
A

A375 Annexin V



B

501mel Annexin V



C

UCT Mel-1 Annexin V

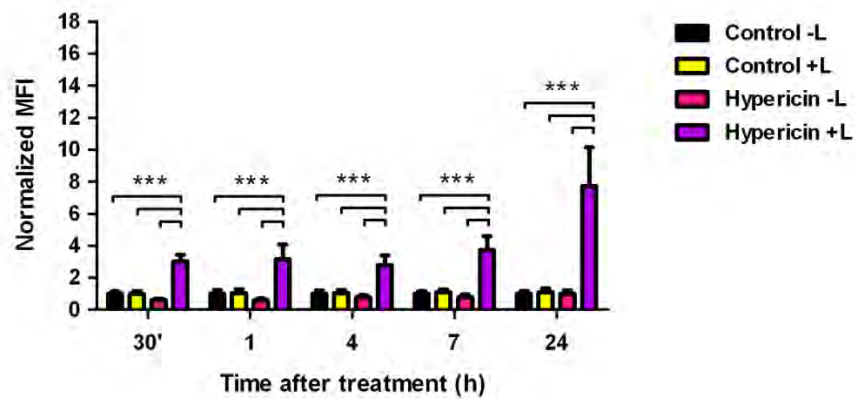


Figure 3.17: PDT induced phosphatidylserine exposure. (A) A375, (B) 501mel and (C) UCT Mel-1 Annexin V median fluorescent intensities (MFI) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment. Flow fluorocytometric data is shown as the median $\pm$ SEM ($n \geq 3$, *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, L: light).

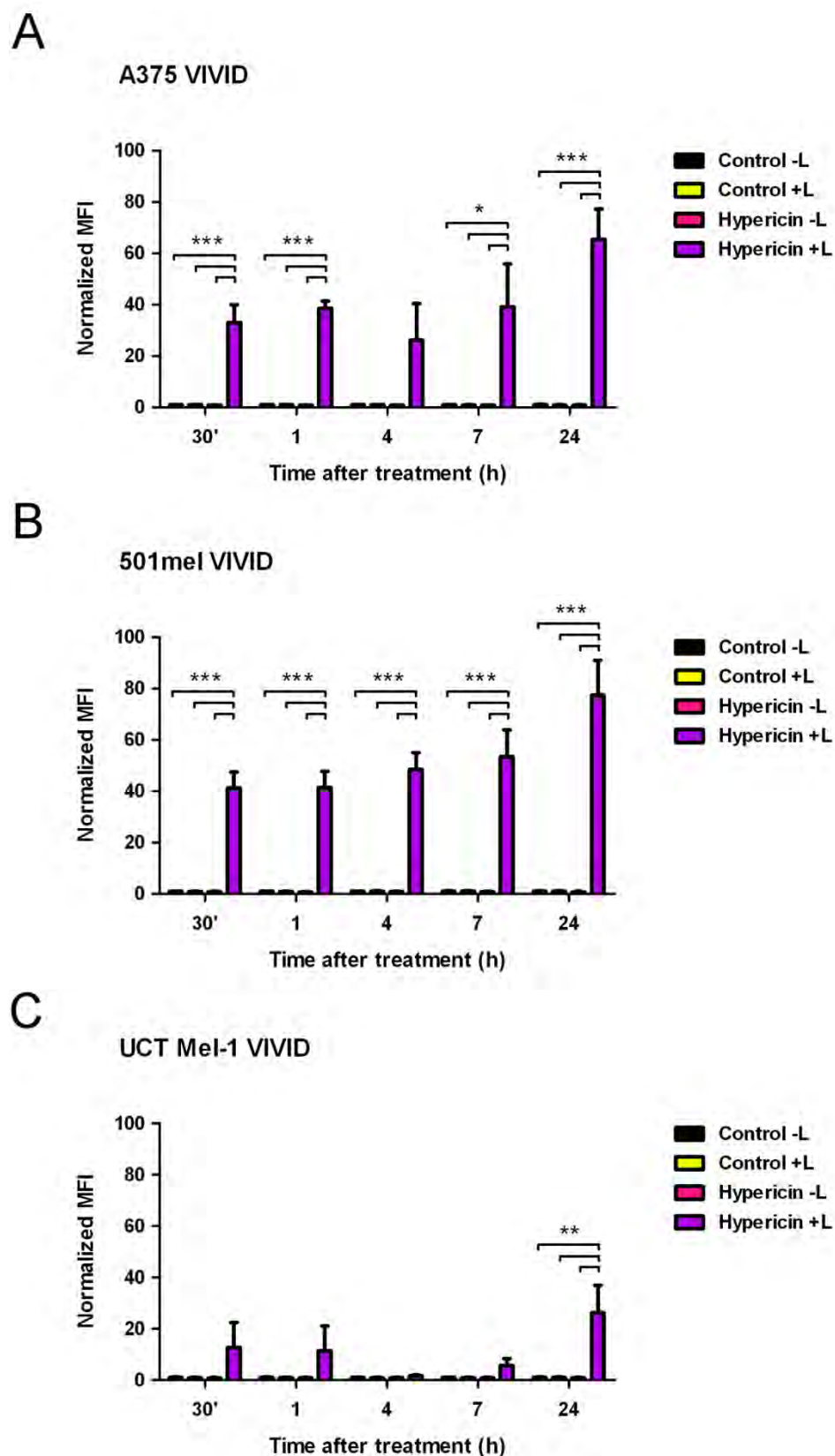
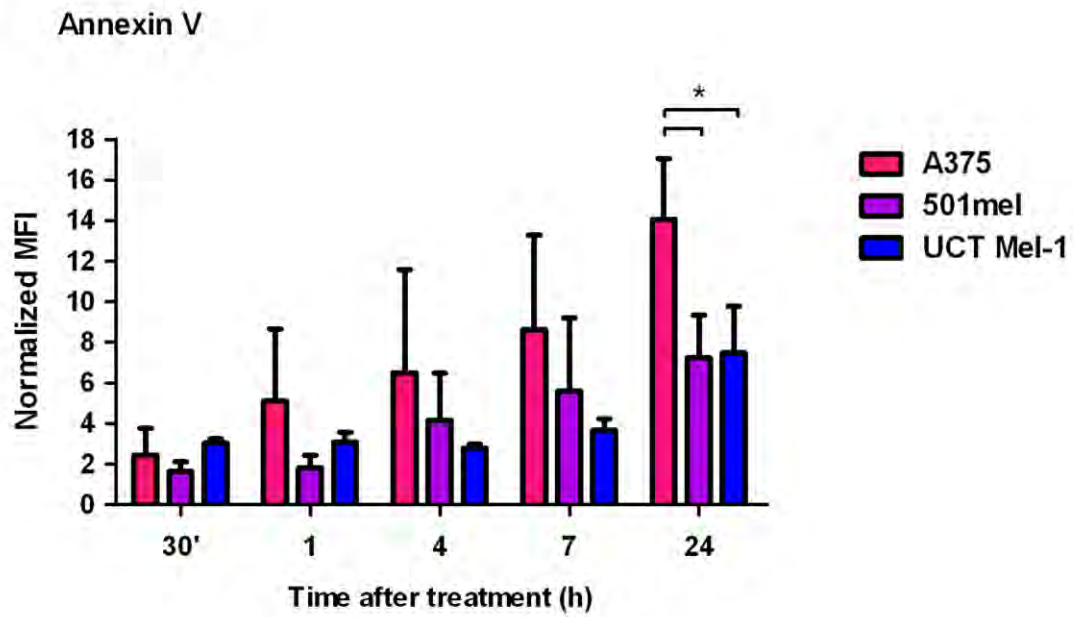


Figure 3.18: PDT induced loss of cell membrane integrity. (A) A375, (B) 501mel and (C) UCT Mel-1 VIVID median fluorescent intensities (MFI) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment. Flow fluorocytometric data is shown as the median $\pm$ SEM ($n \geq 3$, *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, L: light).

A



B

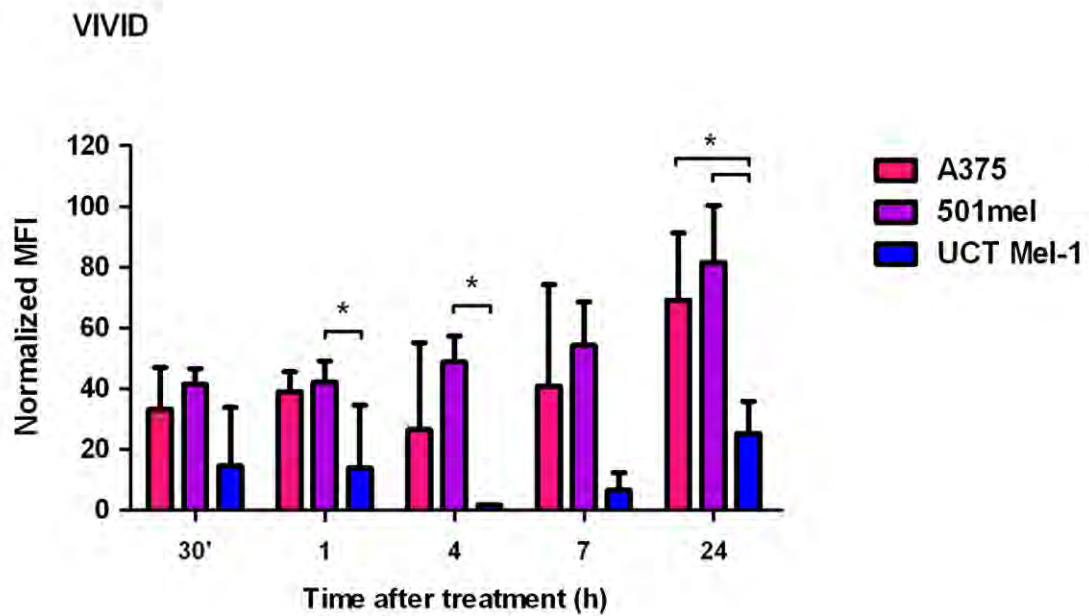


Figure 3.19: PDT induced cell death differences. (A) Annexin V (phosphatidyl serine exposure) and (B) VIVID (loss of cell membrane integrity) median fluorescent intensities (MFI) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment. Flow fluorocytometric data is shown as the median $\pm$ SEM ($n\geq 3$, *** $p<0.0001$, ** $p<0.01$, * $p<0.05$).

Flow fluorocytometric analyses of Annexin V versus VIVID furthermore enabled the identification of 4 different populations: live (Annexin V- VIVID-), early apoptotic (Annexin V+ VIVID-), early necrotic (Annexin V- VIVID+) and late apoptotic/ necrotic (Annexin V+ VIVID+) (Fig. 3.20).

Control treatments of vehicle with sham-irradiation (control –light), vehicle with light-activation (control +light) and hypericin with sham-irradiation (hypericin –light) all resulted in a live population (Annexin V- VIVID-) of approximately 95 % or more for A375 (Appendix F: Fig. A.F.1), 501mel (Appendix F: Fig. A.F.2) and UCT Mel-1 melanoma cells (Appendix F: Fig. A.F.3). The live population was approximately twice as high for UCT Mel-1 cells (Fig. 3.21C) compared to that of A375 (Fig. 3.20A) and 501mel (Fig. 3.20B), at all time-points investigated. A375 and 501mel live populations were similar to each other (Fig. 3.20A/B). These results suggest that UCT Mel-1 cells are more resistant to hypericin-PDT than A375 or 501mel cells.

Light-activated hypericin treatment resulted in similar cell death responses for all melanoma cell lines investigated (Fig. 3.20).

A375 melanoma cells displayed an initial early necrotic response (AV- VIVID+) at 30 minutes after treatment (63 ± 6 %), followed by a time-dependent decrease in the early necrotic population to 9 ± 4 % at 24 hours (Fig. 3.20A). Conversely, the late apoptotic/necrotic population (AV+ VIVID+) increased with time after treatment from an initial 13 ± 7 % at 30 minutes to 75 ± 12 % at 24 hours (Fig. 3.20A).

Treatment of 501mel cells with light-activated hypericin resulted in a predominantly early necrotic population (AV- VIVID+, Fig. 3.20B). This population was highest at 30 minutes after treatment (77 ± 6 %) with comparable values up to 7 hours. At 24 hours 501mel cells showed similar early necrotic (36 ± 14 %) and late apoptotic/necrotic (48 ± 14 %, Annexin V+ VIVID+) populations. At time points earlier than 24 hours the late apoptotic/necrotic population was low (4h: 7 ± 5 %, 7h: 19 ± 14 %, Fig. 3.20B).

UCT Mel-1 cells displayed an initial early necrotic response (41 ± 6 %, Annexin V- VIVID+) at 30 minutes after light-activated hypericin treatment (Fig. 3.20C) which decreased with time (24h: 16 ± 7 %). The late apoptotic/necrotic population (Annexin V+ VIVID+) was initially low (30min: 12 ± 2 %) and only increased by 24 hours (42 ± 6 %). At 24 hours UCT Mel-1 cells showed an early apoptotic population (Annexin V+ VIVID-) of 15 ± 5 % (Fig. 3.20C).

One common feature of the cell death response to hypericin-PDT for all melanoma cell lines investigated was minimal early apoptotic induction (Annexin V+ VIVID-). UCT Mel-1 displayed the

highest early apoptotic population with $15 \pm 5\%$ at 24 hours, followed by $9 \pm 5\%$ for A375 at 7 hours and $2 \pm 1\%$ for 501mel at 24 hours (Fig. 3.20).

It was clear from these sets of experiments that melanoma cells died by specific mechanisms involving the externalization of PS and loss of cell membrane integrity at specific times after hypericin-PDT treatment. A similar cell death response was observed for the different melanoma cells used in this study.

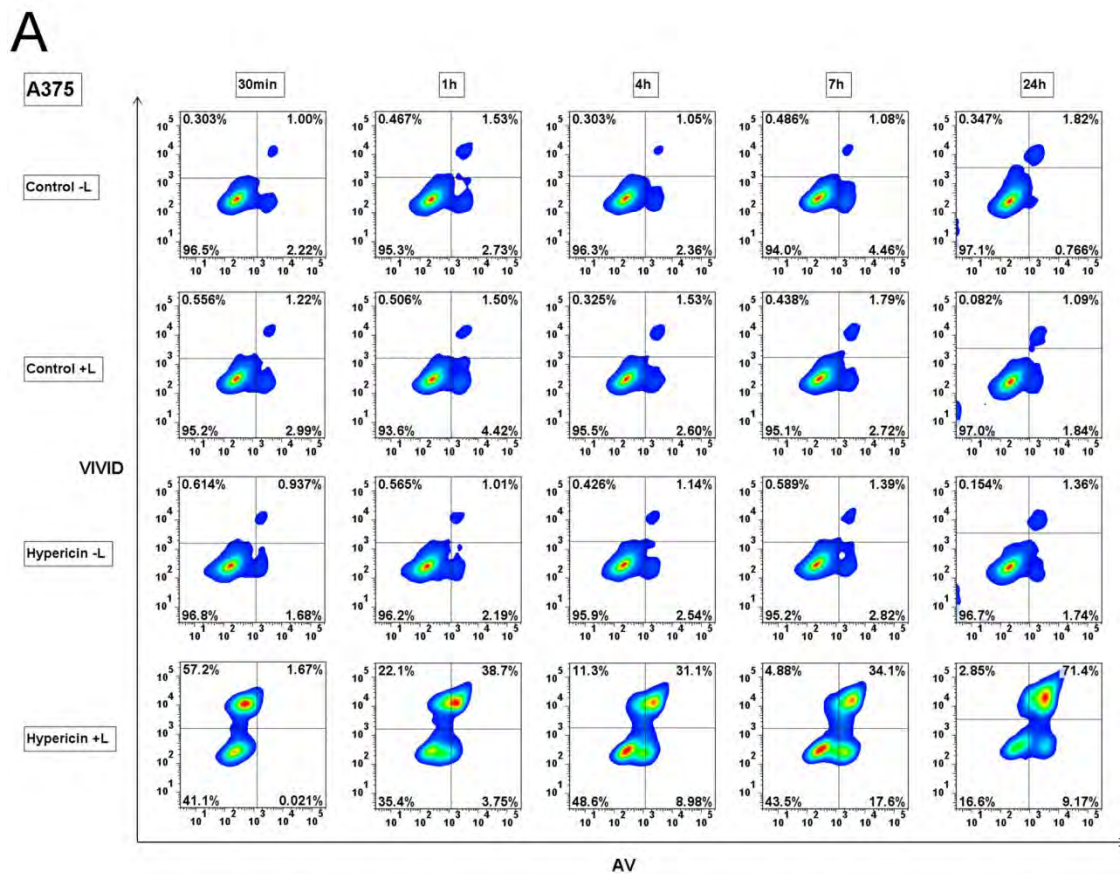
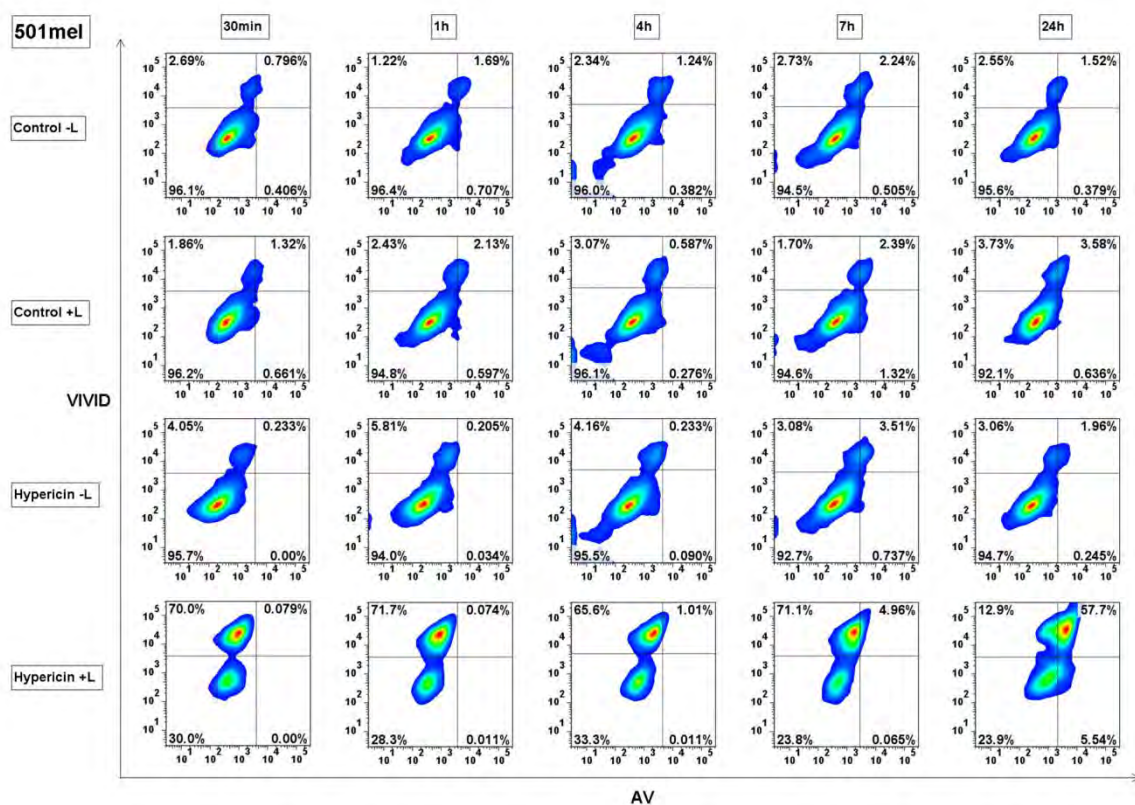
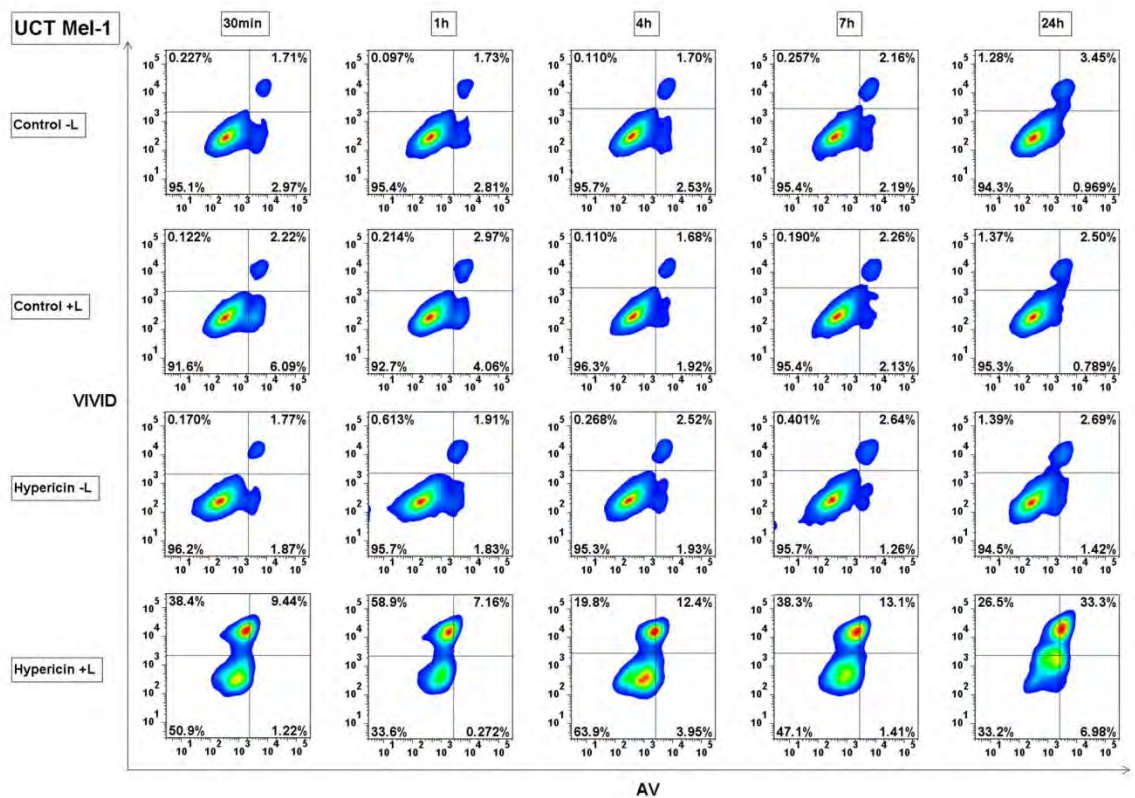


Figure 3.20: Flow fluorocytometric scatter plots of PDT induced cell death. (A) A375, (B) 501mel and (C) UCT Mel-1 percentage gated cells of 4 different populations labeled with Annexin V (AV, phosphatidyl serine exposure) and VIVID (loss of cell membrane integrity) at 30', 1, 4, 7 and 24 h after treatment. Lower left quadrant: AV- VIVID-, lower right quadrant: AV+ VIVID-, upper left quadrant AV- VIVID+ and upper right quadrant: AV+ VIVID+. One representative result is shown ($n \geq 3$, L: light).

B



C



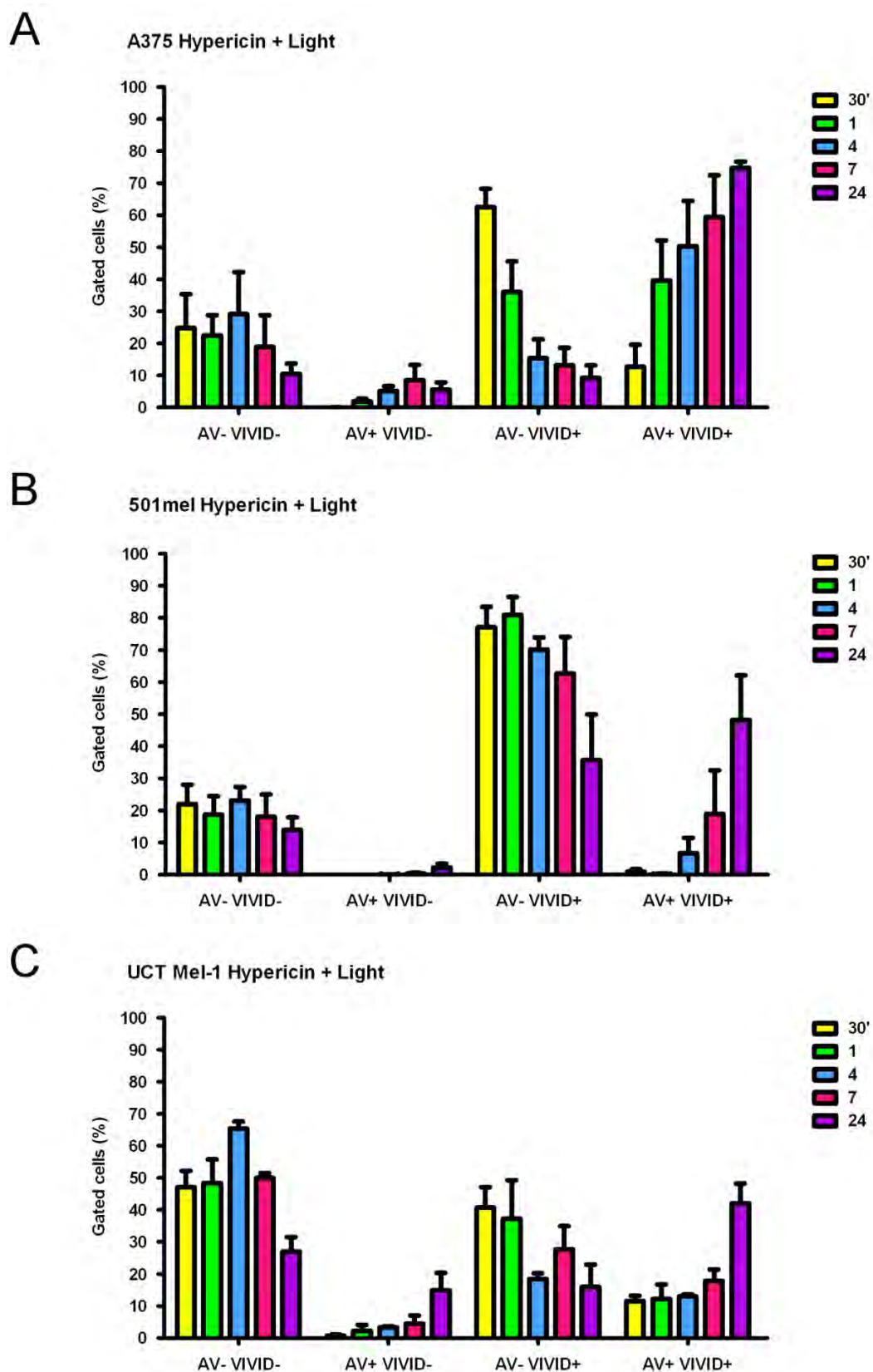


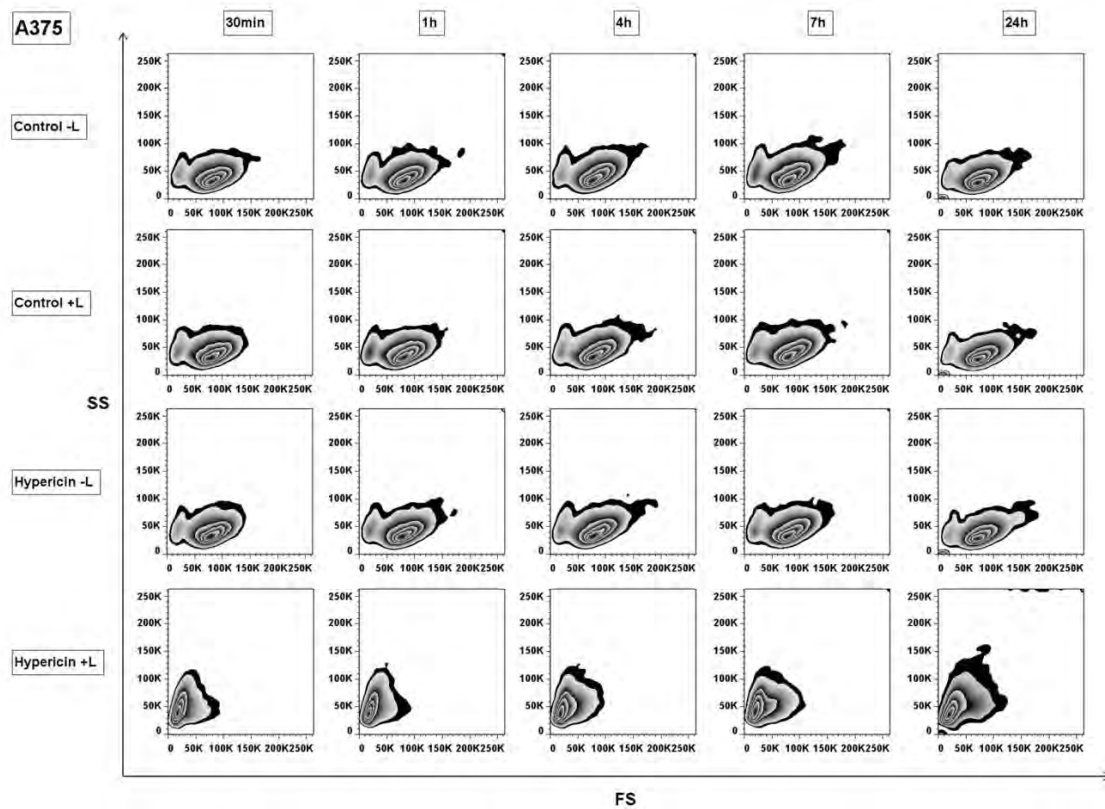
Figure 3.21: PDT induced cell death populations. (A) A375, (B) 501mel and (C) UCT Mel-1 percentage gated cells of 4 different populations labeled with Annexin V (phosphatidyl serine exposure) and VIVID (loss of cell membrane integrity): AV- VIVID-, AV+ VIVID-, AV- VIVID+ and AV+ VIVID+ at 30', 1, 4, 7 and 24 h after treatment. Data is shown as mean $\pm$ SEM of gated cells ($n \geq 3$).

3.4.3 CELL SIZE AND GRANULARITY/PIGMENTATION

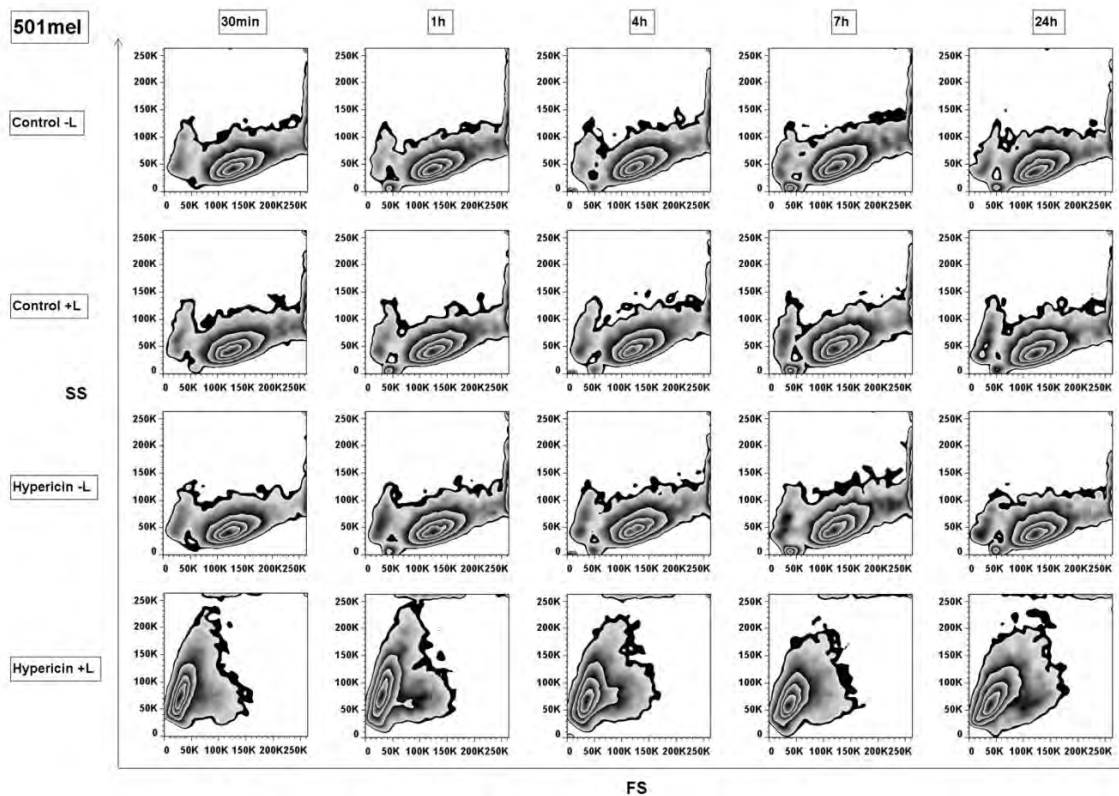
In addition to the fluorescence data acquired by flow cytometry, optical information known as forward scatter (FS) and side scatter (SS) is obtained based on the angle of light emitted from the cell²⁹⁸. FS provides information on the cell size and SS relates to cellular granularity. The resulting scatter FS-SS plot allows for visualisation of these parameters. These morphological characteristics are useful for investigating cell death²⁹⁹. Morphological alterations of dying cells includes shrinkage of apoptotic cells and swelling of necrotic cells³⁶. Investigating changes in cellular granularity is valuable in the context of melanoma as granularity indicates the pigmentation levels of the cell. This characteristic has been used in various experimental systems to identify cell populations with different pigmentation patterns^{300–303}. Melanoma cells have been shown to sequester chemotherapeutics in melanosomes and increase melanosomal biogenesis and export to circumvent cell death^{228,304,305}. It is thus of interest to identify if hypericin-PDT increases pigmentation of melanoma cells after treatment.

Flow cytometric analyses of FS and SS revealed different morphological characteristics of melanoma cells (Fig. 3.22). Vehicle-treated sham-irradiated control cells showed small FS and SS for A375, indicating a small size and granularity (Fig. 3.22A). In contrast, 501mel and UCT Mel-1 cells showed higher FS and SS, indicating larger cells and increased granularity (Fig. 3.22B/C). Treatments with vehicle and light-activation (control +light) or hypericin with sham-irradiation (hypericin –light) did not alter these morphological characteristics. Interestingly, light-activated hypericin treatment (hypericin +light) induced morphological changes in all melanoma cells (Fig. 3.22). For an enhanced visual presentation of these morphological changes we presented the flow cytometric data as histograms (Fig. 3.23). Normalization of the FS and SS medians to the vehicle-treated sham-irradiated control furthermore allowed for the quantification of cell size and granularity/pigmentation (Fig. 3.24/25).

A



B



C

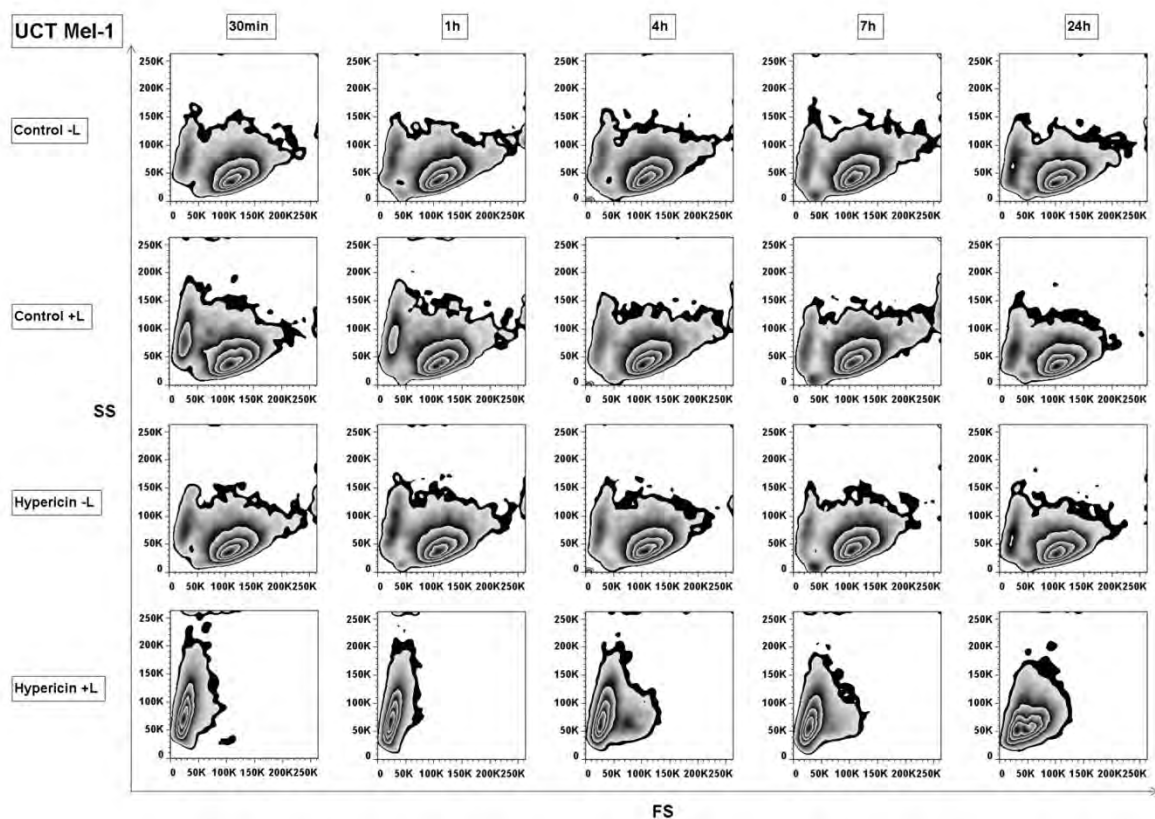
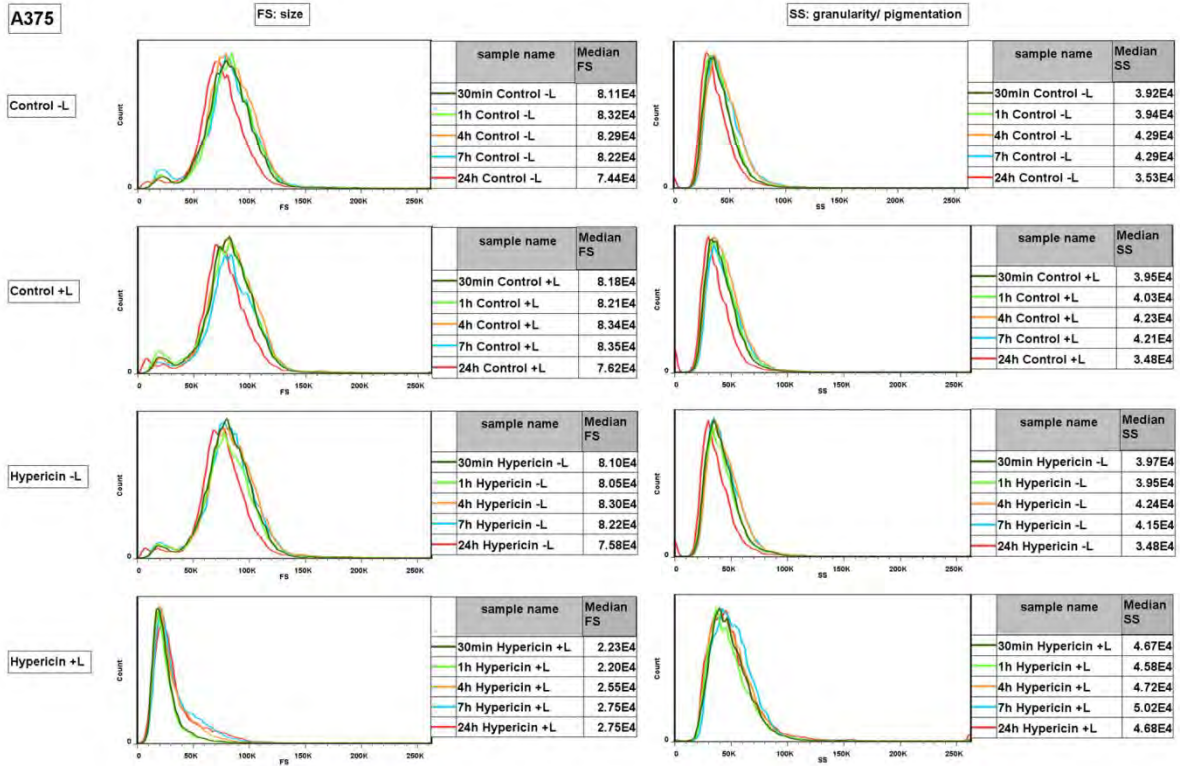


Figure 3.22: Flow cytometric scatter plots of PDT induced cell morphological changes. Forward scatter (FS) reflecting size and side scatter (SS) reflecting granularity/ pigmentation of (A) A375, (B) 501mel and (C) UCT Mel-1 cells at 30', 1, 4, 7 and 24 h after treatment. A representative result is shown (n≥3, L: light).

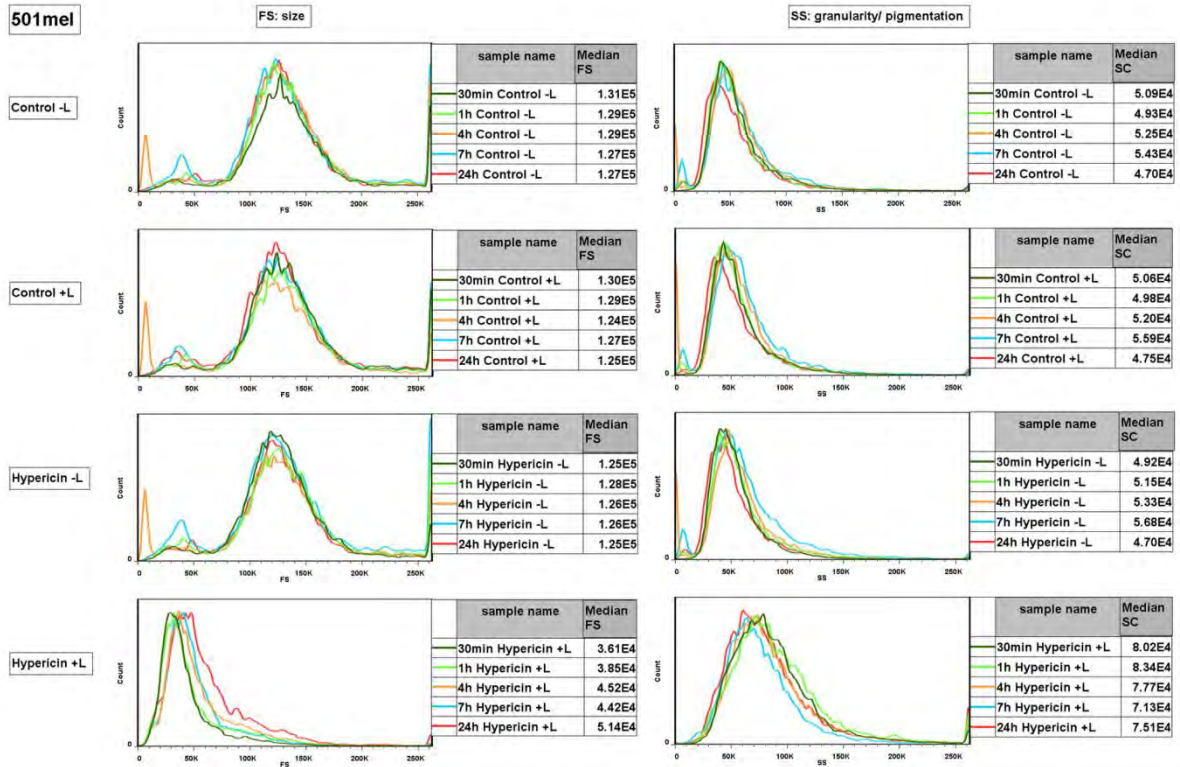
A

A375



B

501mel



C

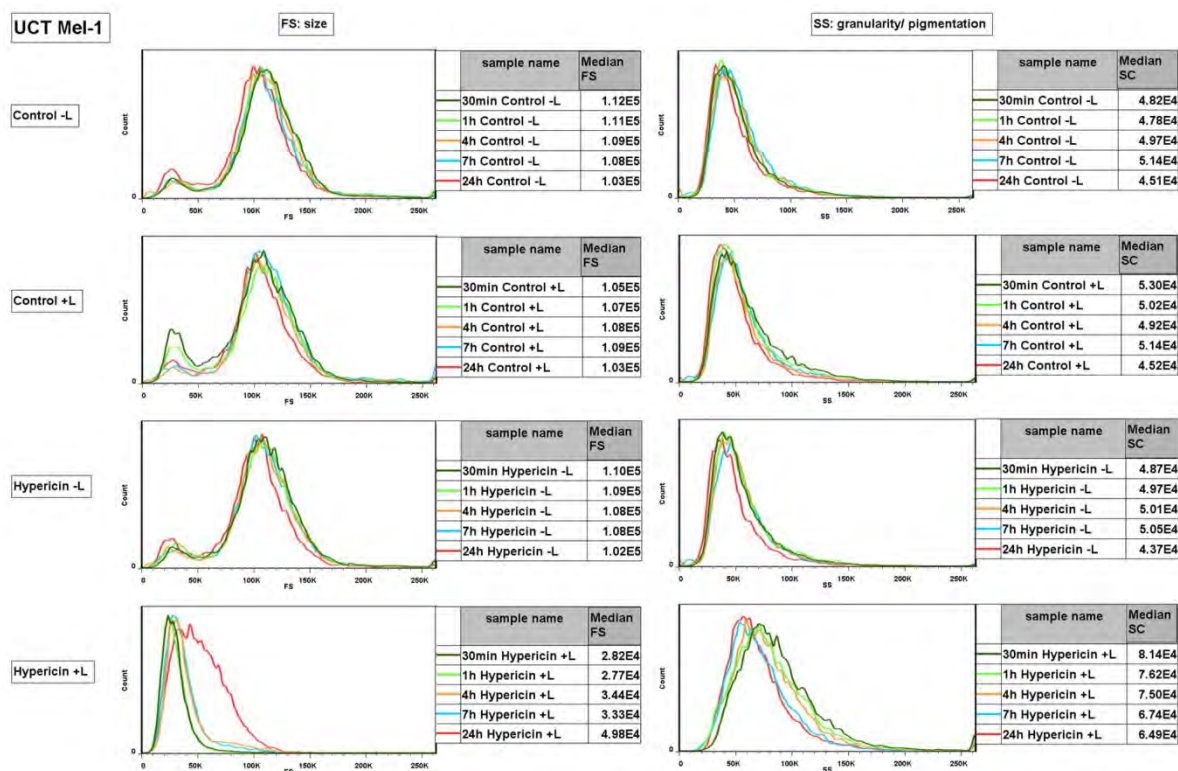


Figure 3.23: Flow cytometric histograms of PDT induced cell morphological changes. Forward scatter (FS) reflecting size and Side scatter (SS) reflecting granularity/ pigmentation of (A) A375, (B) 501mel and (C) UCT Mel-1 cells at 30', 1, 4, 7 and 24 h after treatment. A representative result is shown ($n \geq 3$, L: light).

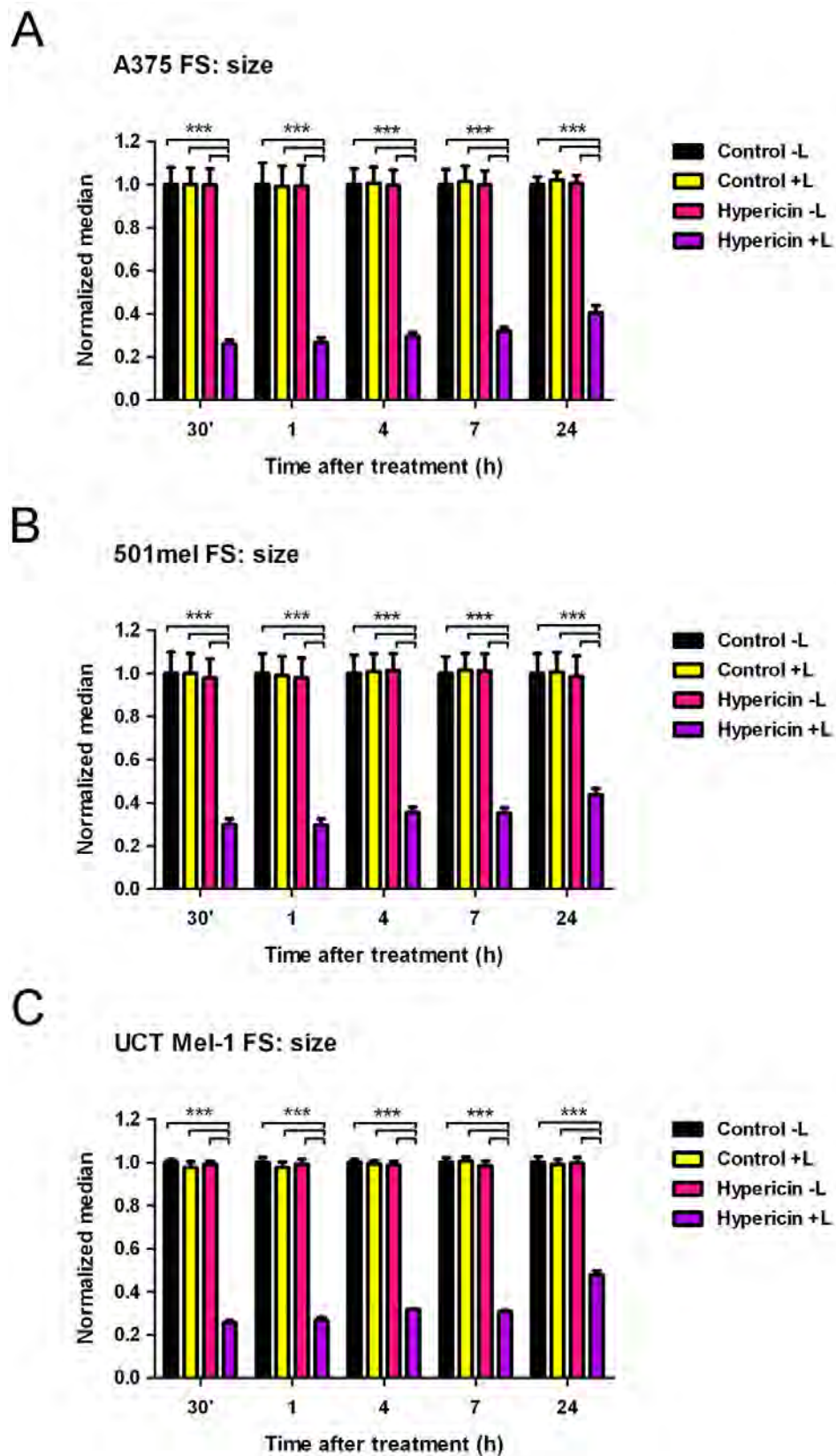


Figure 3.24: PDT induced changes in cell size. (A) A375, (B) 501mel and (C) UCT Mel-1 forward scatter (FS) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment. Flow cytometric data is shown as median $\pm$ SEM ($n\geq 3$, *** $p<0.0001$, L: light).

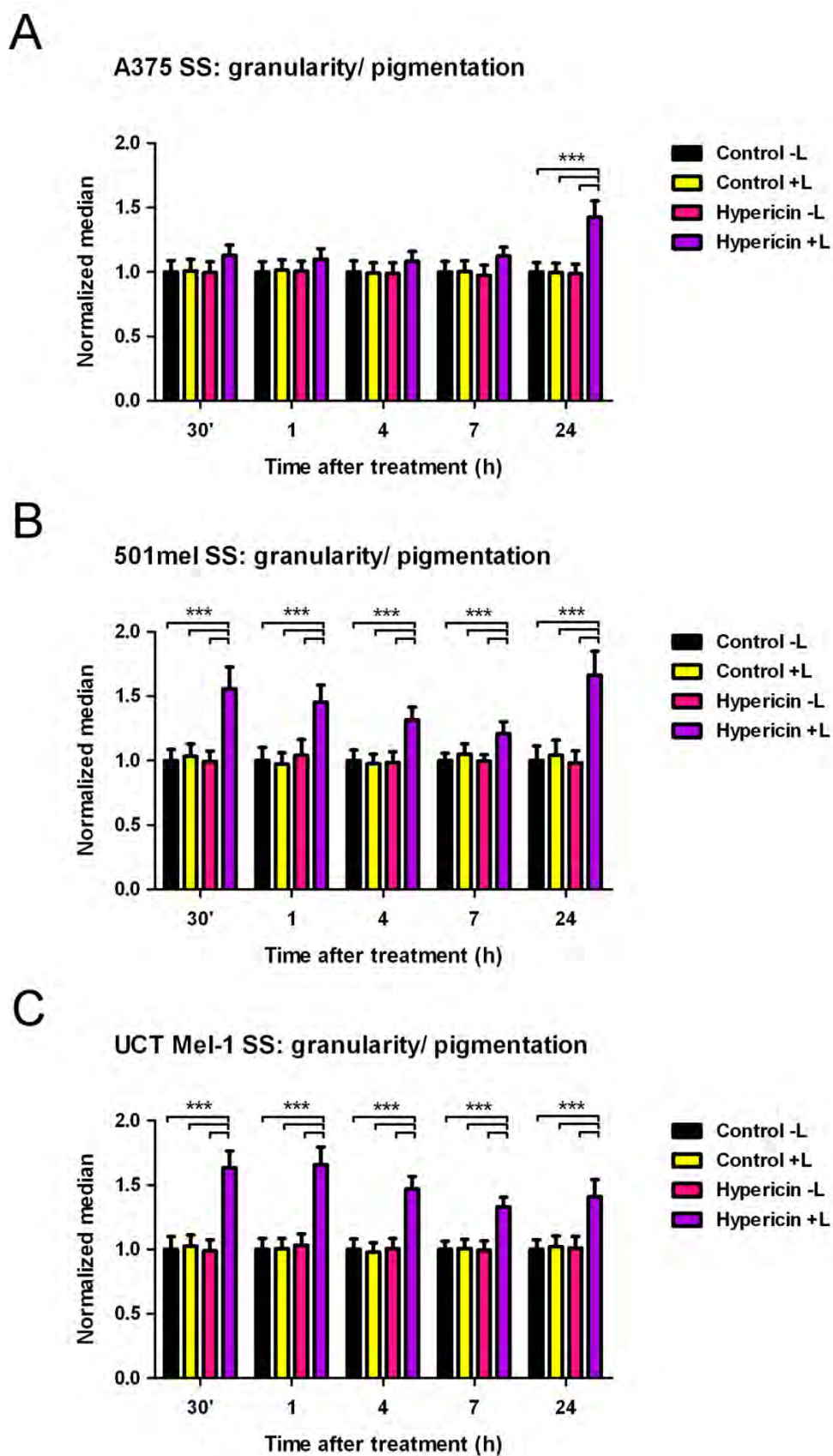


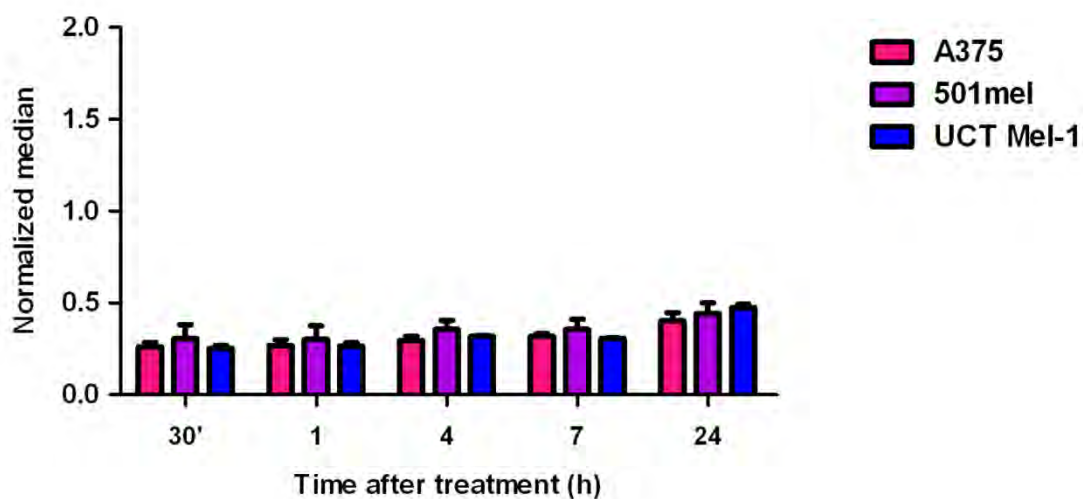
Figure 3.25: PDT induced changes in cell granularity/ pigmentation. (A) A375, (B) 501mel and (C) UCT Mel-1 side scatter (SS) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment. Flow cytometric data is shown as median $\pm$ SEM ($n\geq 3$, *** $p<0.0001$, L: light).

Light-activated hypericin treatment reduced melanoma cell size to approximately a third of that of the control cells, for all melanoma cell lines investigated at all time-points after treatment (Fig. 3.24). At 24 hours after treatment cells were slightly larger compared to the other time points but still significantly smaller than controls (A375: 0.40 ± 0.02 , 501mel: 0.44 ± 0.03 and UCT Mel-1: 0.48 ± 0.01 fold, Fig. 3.24). No significant difference in cell size was observed between the different melanoma cell lines (Fig. 3.26A).

A significant increase in granularity/pigmentation compared to the control was observed at all time-points after light-activated hypericin treatment of 501mel and UCT Mel-1 cells (Fig. 3.25B/C). This was most pronounced at 24 hours for 501mel (1.67 ± 0.05 fold) and at 1 hour for UCT Mel-1 (1.66 ± 0.04 fold). A375 granularity/pigmentation significantly increased at 24 hours to 1.42 ± 0.03 fold of the control, with no significant change at the other time-points investigated (Fig. 3.25A). The A375 cells were significantly less granular/pigmented compared to 501mel and UCT Mel-1 cells, at 30 minutes and 1 hour after treatment (Fig. 3.26B). The UCT Mel-1 cells were significantly more granular/pigmented than A375 cells at 1 hour after treatment (Fig. 3.26B). At 24 hours, 501mel cells were significantly more granular/pigmented than both A375 and UCT Mel-1 cells (Fig. 3.26B). These results indicate that melanoma cells responded to hypericin-PDT by increasing their granularity/pigmentation.

A

FS: size



B

SS: granularity/ pigmentation

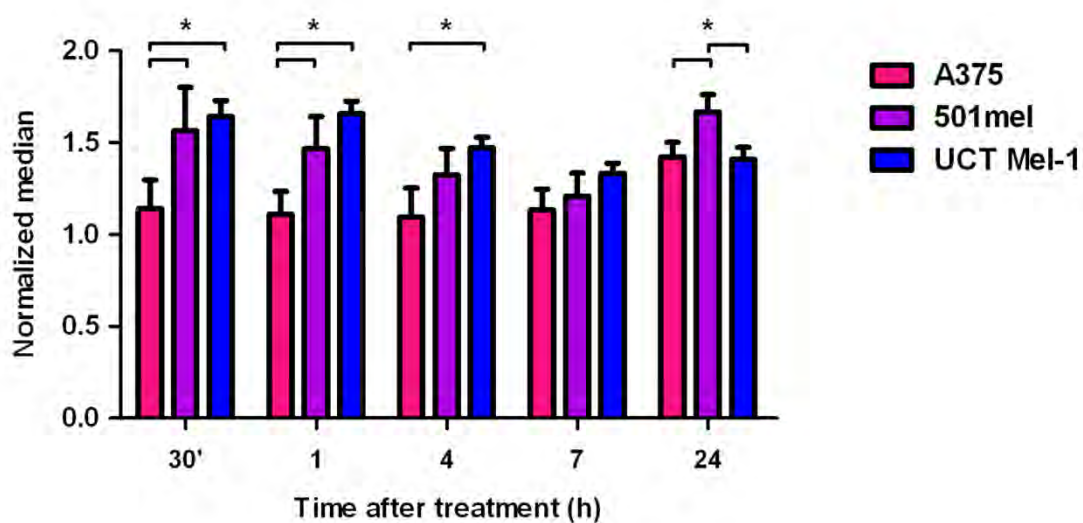


Figure 3.26: PDT induced cell morphological differences. (A) Forward scatter (FS) reflecting size and (B) side scatter (SS) reflecting granularity/ pigmentation at 30', 1, 4, 7 and 24h after treatment. Flow cytometric data is shown as median $\pm$ SEM normalized to the vehicle-treated, sham-irradiated control of each cell line ($n \geq 3$, * $p < 0.05$).

3.4.4 INTRACELLULAR HYPERICIN AFTER PDT

Melanoma cells have been shown to sequester the chemotherapeutic drug cisplatin in melanosomes in conjunction with increased melanosomal biogenesis and export in response to the therapy²²⁸. The involvement of potential melanosomal sequestration of hypericin was not in the scope of this study, however we were able to quantify intracellular hypericin post PDT. We analyzed the hypericin median fluorescent intensity (MFI) of the cells used in the FACS experiments.

Resulting histograms of hypericin MFI illustrate that cells not treated with hypericin (control -/+light) showed no fluorescence whereas cells treated with hypericin (hypericin -/+light) exhibited differential MFI at different times after treatment (Fig. 3.27). Normalization of MFI to the vehicle-treated, sham-irradiated control (control -light) allowed for the quantification of intracellular hypericin fluorescence in melanoma cells (Fig. 3.28). A time-dependent decrease of hypericin fluorescence for both the sham-irradiated (hypericin -light) and light-activated (hypericin +light) treatments were observed in all melanoma cells, significantly different to both the vehicle-treated, sham-irradiated (control -light) and vehicle-treated, light-activated control (control +light, Fig. 3.28). At 7 hours post PDT, hypericin fluorescence of A375 melanoma cells was significantly more in the light-activated (28.7 ± 3.3 fold) compared to sham-irradiated cells (16.8 ± 1.8 fold, Fig. 3.28A). At 24 hours, the same was observed for 501mel (hypericin +light: 40.1 ± 6.6 and hypericin -light: 15.4 ± 2.3 fold, Fig. 3.28B) and UCT Mel-1 cells (hypericin +light: 23.64 ± 2.6 and hypericin -light: 8.2 ± 1.0 fold, Fig. 3.28C).

The 501mel cells exhibited significantly higher hypericin fluorescence compared to that of the A375 cells at 1 hour after light-activated hypericin treatment (Fig. 3.29A). Sham-irradiated hypericin treatment resulted in significantly higher hypericin fluorescence in 501mel cells compared to that of the A375 cells at 1, 7 and 24 hours post PDT (Fig. 3.29B). No significant difference in hypericin fluorescence was observed between the 501mel and UCT Mel-1 cells (Fig. 3.29B).

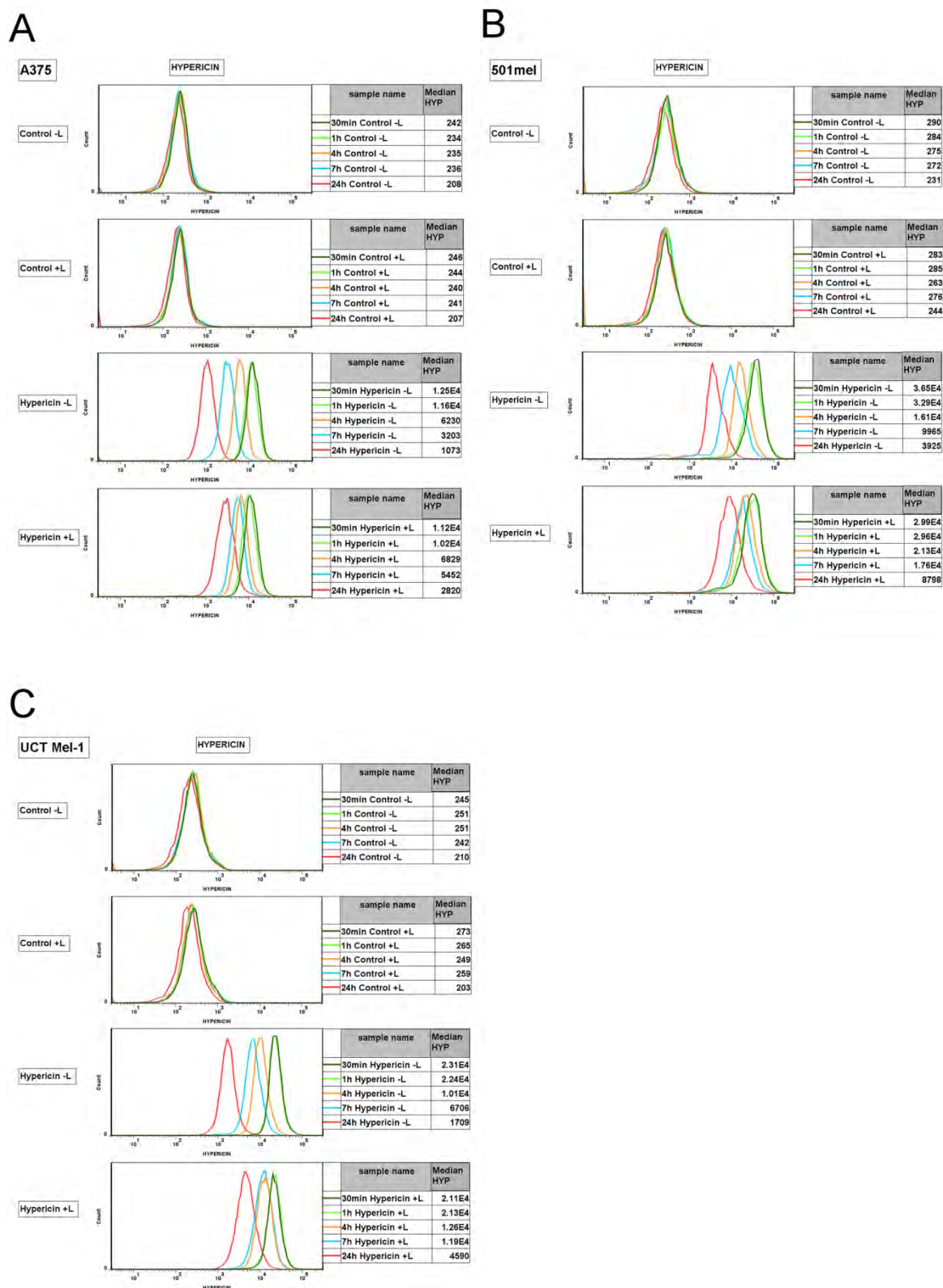
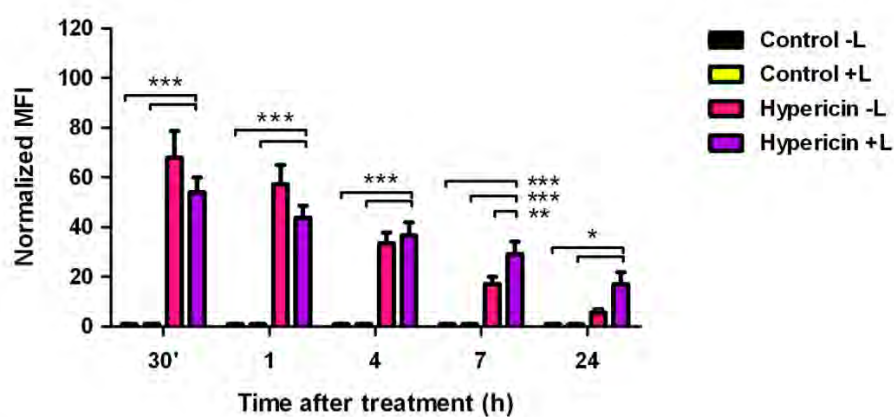


Figure 3.27: Flow fluorocytometric histograms of intracellular hypericin post PDT. Hypericin median fluorescent intensities (MFI) of (A) A375, (B) 501mel and (C) UCT Mel-1 melanoma cells at 30', 1, 4, 7 and 24 h after treatment. A representative result is shown ($n \geq 3$, L: light).

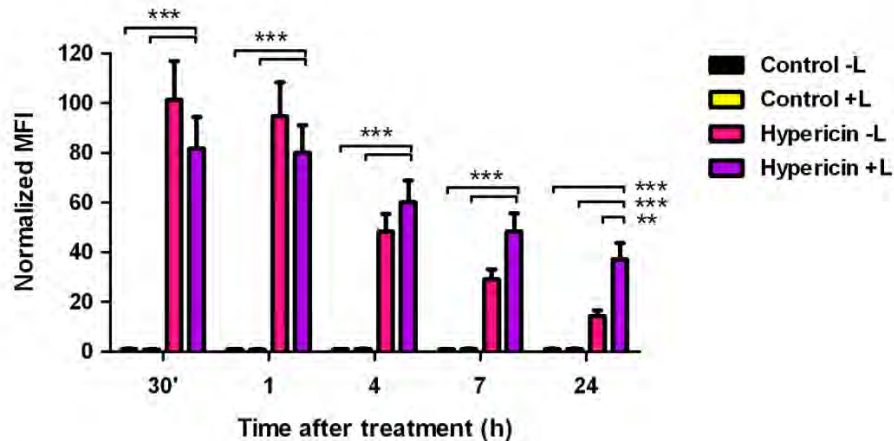
A

A375 Hypericin



B

501mel Hypericin



C

UCT Mel-1 Hypericin

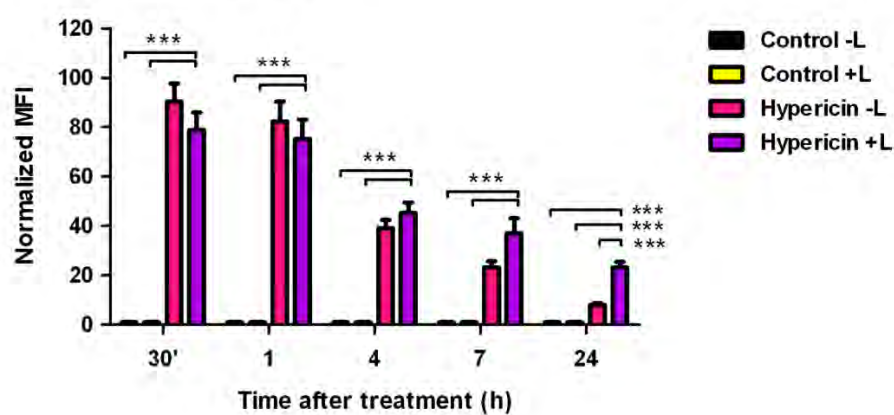
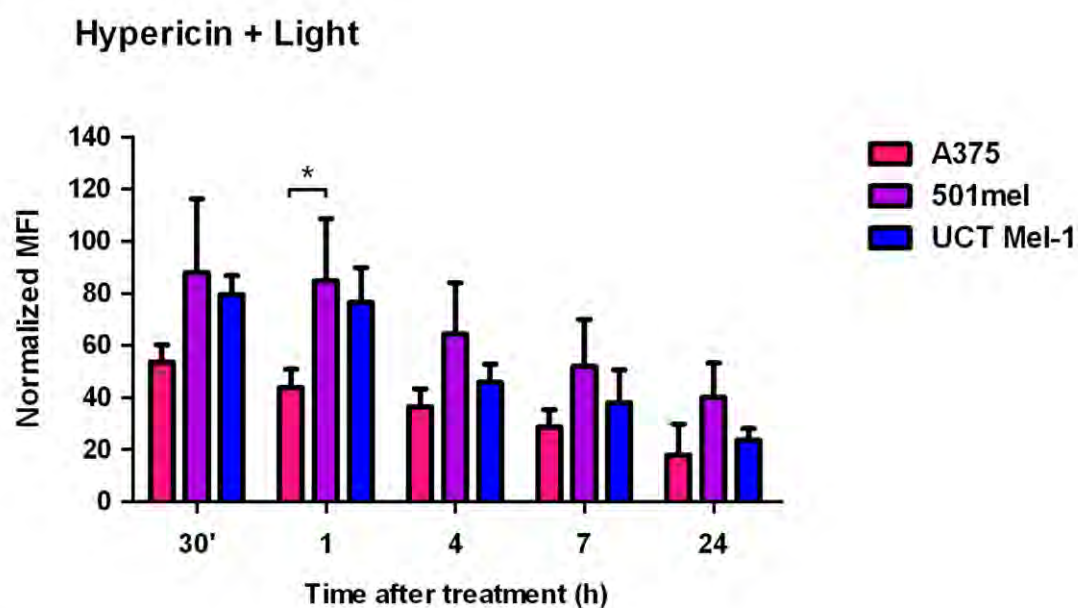


Figure 3.28: Intracellular hypericin post PDT. (A) A375, (B) 501mel and (C) UCT Mel-1 hypericin median fluorescent intensities (MFI) normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24 h after treatment (median±SEM, n≥3, ***p<0.0001, **p<0.01, *p<0.05, L: light).

A



B

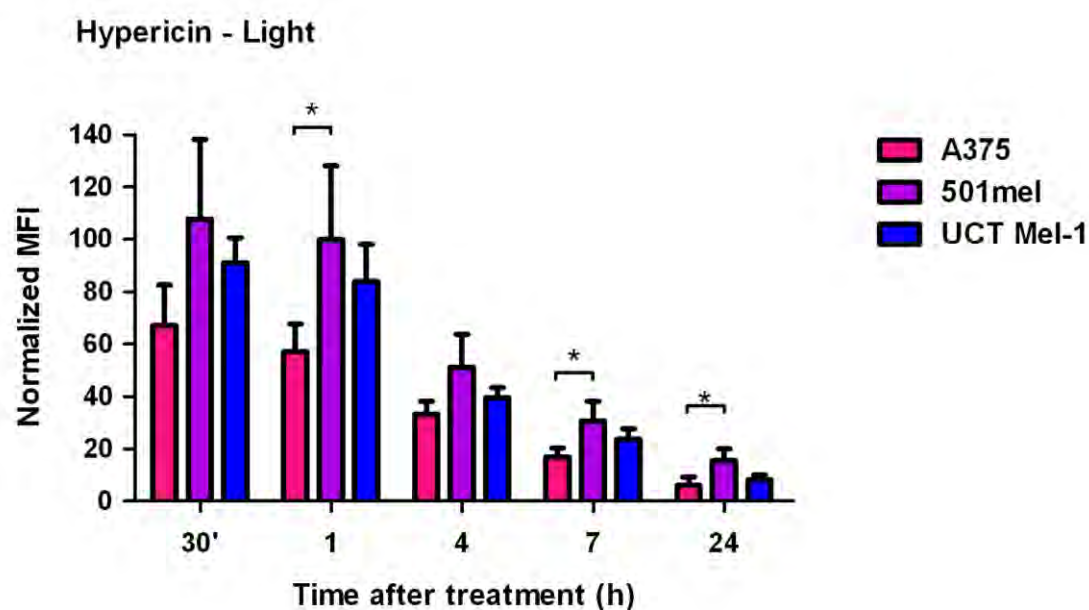


Figure 3.29: Intracellular hypericin differences post PDT. Cells were treated with hypericin and (A) light-activation or (B) sham-irradiation. Hypericin median fluorescent intensities (MFI) are shown, normalized to the vehicle-treated, sham-irradiated control at 30', 1, 4, 7 and 24h after treatment (median $\pm$ SEM, $n\geq 3$, * $p<0.05$).

Comparing hypericin fluorescence at different time points revealed a significant decrease in light-activated hypericin from 30 minutes to 7, 24 hours and from 1 to 24 hours for A375 melanoma cells (Appendix F: Fig. A.F.4A). In the same cells, sham-irradiated hypericin treatment (hypericin –light) resulted in a significant decrease from 30 minutes and 1 hour to 4, 7 and 24 hour time-points as well as from 4 to 24 hours. Light-activated hypericin fluorescence was not significantly different in 501melanoma cells at different time points post PDT (Appendix F: Fig. A.F.4B). Sham-irradiated hypericin however showed a significant decrease from 30 minutes and 1 hour to 4, 7 and 24 hours (Appendix F: Fig. A.F.4B). The UCT Mel-1 treated with light-activated hypericin presented a significant decrease in hypericin from 30 minutes and 1 hour to 4, 7 and 24 hours (Appendix F: Fig. A.F.4C). Sham-irradiated hypericin treatment of UCT Mel-1 revealed similar results as the light-activated treatment, with an additional significant hypericin decrease from 4 to 24 hours (Appendix F: Fig. A.F.4C).

It was clear from these experiments that intracellular hypericin was reduced with increasing time after hypericin-PDT treatment, independent of light activation.

3.4.5 CELL DEATH PROTEIN EXPRESSION

To shed more light on hypericin-PDT induced cell death mechanisms we further investigated the involvement of specific cell death proteins. These included: caspase 3 (CASP3), caspase 8 (CASP8), apoptosis inducing factor (AIF) and poly(ADP-ribose)polymerase 1 (PARP1).

A vital step in both the intrinsic and extrinsic caspase-dependent apoptotic cascade is the activation of the executioner CASP3⁴¹. The initiator caspase of the extrinsic apoptotic pathway is CASP8, which upon activation either activates CASP3 directly or indirectly through activation of the mitochondrial apoptotic pathway⁴³. The intracellular apoptotic pathway can however also be executed in a caspase-independent manner, through AIF⁴⁶. Poly(ADP-ribose)polymerase 1 (PARP1) is involved in both parthanatos with AIF⁵⁹ and apoptosis with caspases, as it contains a caspase cleavage site³⁰⁶.

Melanoma cells were treated with the killing concentration of light-activated hypericin followed by Western blot analyses at 1, 4, 7 and 24 hours after treatment, with specific cell death proteins. Resulting data was normalized to total protein used as a loading control and ratios to the vehicle-treated, sham-irradiated control were densitometrically quantified and presented (Fig. 3.30).

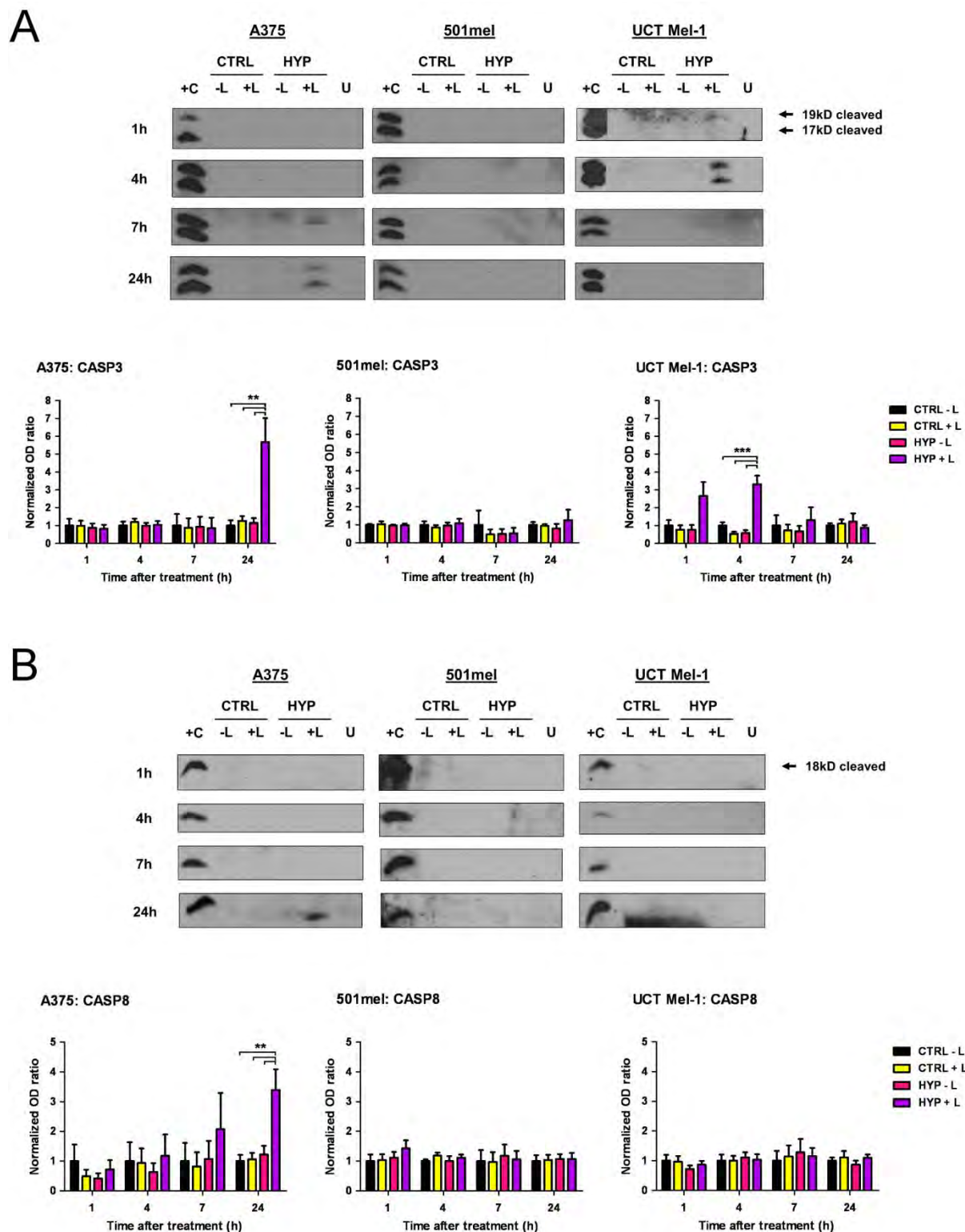
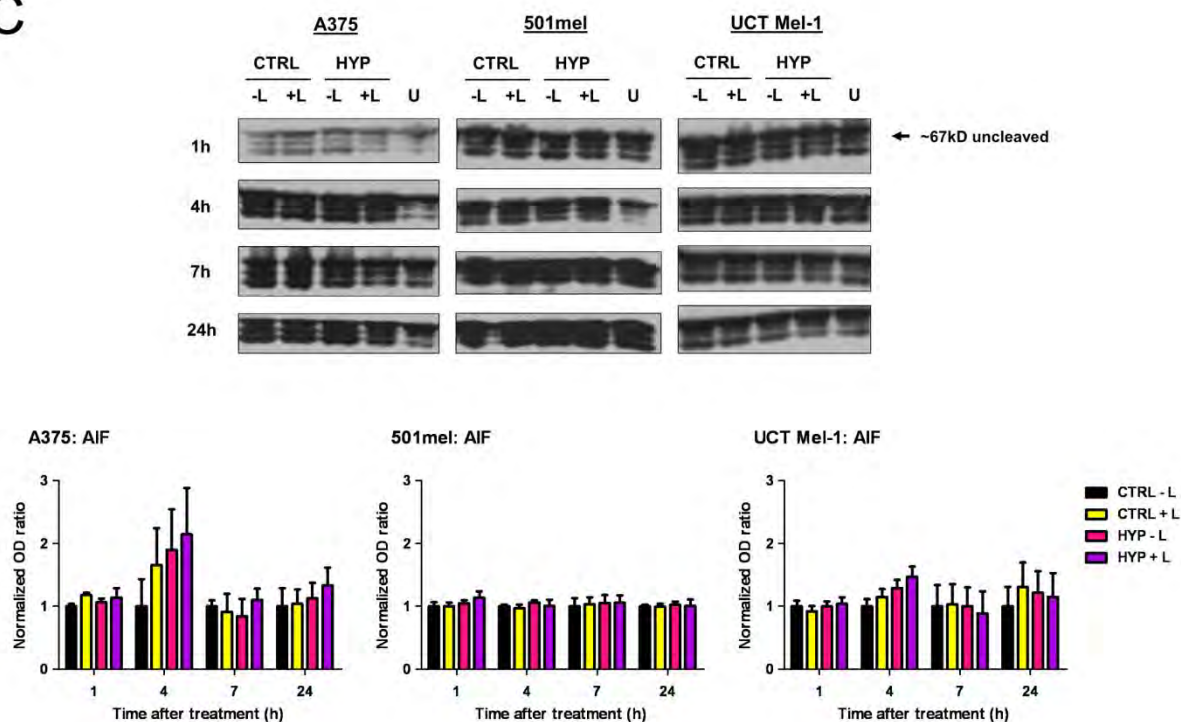
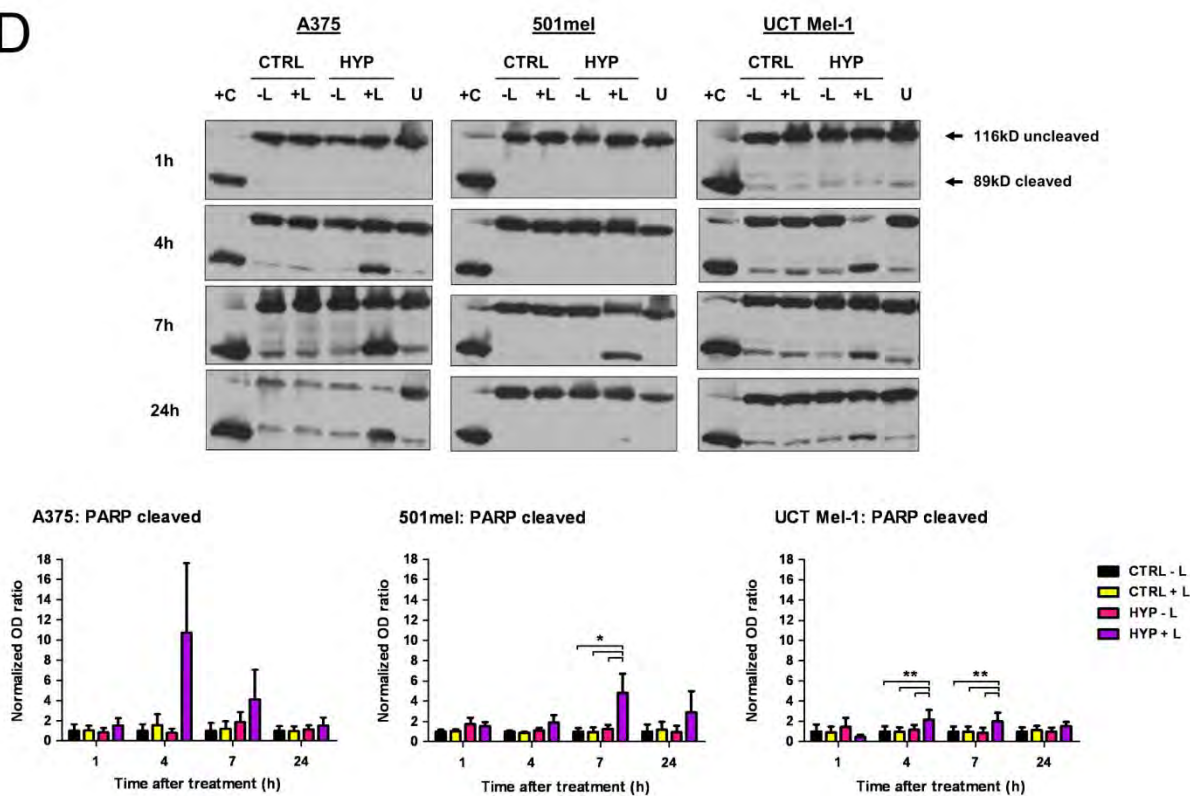


Figure 3.30: PDT induced cell death protein expression. (A) Caspase 3 (CASP3) (B) caspase 8 (CASP8), (C) apoptosis inducing factor (AIF), (D) poly(ADP-ribose)polymerase 1 (PARP1) Western blots analyses of whole cell lysates detected at 1, 4, 7 and 24 hours after treatment. A representative result of the same exposure is shown (n=3). The membrane was stained with Ponceau S to assess protein loading and the resulting optical density (OD) ratio was normalized to the untreated, sham-irradiated control (CTRL: control vehicle-treated, HYP: hypericin, +C: positive control, U: untreated non-irradiated, L: light).

C



D



Cleavage of CASP3 into two fragments of 17 and 19kDa activates the protease and subsequent apoptotic cascade⁴¹. Melanoma cells treated with doxorubicin showed CASP3 cleavage and were thus used as a positive control in our experimental system (Fig. 3.30A). A375 melanoma cells expressed cleaved CASP3 at 24 hours after treatment, 6.0 ± 1.9 fold significantly higher than the vehicle-treated, sham-irradiated control (Fig. 3.30A). At 4 hours, UCT Mel-1 showed CASP3 cleavage, 3.4 ± 0.3 fold significantly higher than the control (Fig. 3.30A). At 1 hour, UCT Mel-1 presented inconsistent CASP3 cleavage results, which were not significantly different to that of the control. In contrast to the other melanoma cells investigated, 501mel cells did not express cleaved CASP3 at any of the time points analyzed after hypericin-PDT (Fig. 3.30A).

Investigations into CASP8 cleavage of the active 18kDa fragment of the protein revealed its presence upon treatment of melanoma cells with doxorubicin used as a positive control (Fig. 3.30B). Light-activated hypericin treatment induced CASP8 cleavage at 24 hours in A375 melanoma cells, 3.45 ± 0.38 fold significantly higher than control (Fig. 3.30B). This was not observed at any other time points investigated in the A375 cells. Furthermore, light-activated hypericin treatment of 501mel and UCT Mel-1 did not induce CASP8 cleavage in these cells (Fig. 3.30B).

Apoptosis inducing factor (AIF) protein expression revealed a triplet of bands around 67kDa, corresponding to uncleaved, vital mitochondrial AIF (Fig. 3.30C). The cleaved, lethal form of AIF (57kDa) was not found in neither of the controls nor light-activated hypericin treated melanoma cells (Fig. 3.30C). The expression of vital AIF did not change significantly at any of the time points investigated after PDT in all melanoma cells (Fig. 3.30C).

Poly(ADP-ribose)polymerase 1 (PARP1) was found in both its inactive, uncleaved (116kDa) and active, cleaved (89kDa) fragments in melanoma cells after treatment (Fig. 3.30D). Cleavage of PARP after light-activated hypericin treatment was significantly higher than the control at 7 hours for 501mel cells (4.9 ± 1.4 fold) and at 4 (2.4 ± 0.4 fold) and 7 hours (2.2 ± 0.3 fold) for UCT Mel-1 cells. The A375 melanoma cells presented inconsistent results, especially at 4 hours after treatment; statistical analyses however showed that these were not significantly different to that of the control.

In summary, light-activated hypericin-PDT elicited a differential cell death response in melanoma cells (Table 3.3, Fig. 3.31). The A375 melanoma cells initiated extrinsic apoptosis at 24 hours post hypericin-PDT, evident by the activation of the initiator CASP8 and executioner CASP3. The intrinsic apoptotic cascade was activated in UCT Mel-1 melanoma cells at 4 hours after treatment with the activation of CASP3 and cleavage of PARP, inactivation of PARP was also found at 7 hours. The 501mel cells expressed cleaved PARP at 7 hours post PDT; however neither CASP3 or CASP8 were cleaved at any of the time-points investigated, suggesting a CASP3-independent PARP cleavage mechanism. The 501mel cells presented cleaved CASP3 after doxorubicin treatment, thus showing that these cells are able to undergo caspase-dependent apoptotic cell death. The lethal form of AIF was not induced by hypericin-PDT in the melanoma cell lines investigated.

Cell line	A375				501mel				UCT Mel-1			
Time (h)	1	4	7	24	1	4	7	24	1	4	7	24
CASP3	-	-	-	+	-	-	-	-	-	+	-	-
CASP8	-	-	-	+	-	-	-	-	-	-	-	-
AIF	-	-	-	-	-	-	-	-	-	-	-	-
PARP	-	-	-	-	-	-	+	-	-	+	+	-

Table 3.3: Cell death protein expression in response to hypericin-PDT. Melanoma cells were treated with 3 μ M light-activated hypericin and analyzed for protein expression of caspase 3 (CASP3), caspase 8 (CASP8), apoptosis inducing factor (AIF) and poly(ADP-ribose)polymerase 1 (PARP1) at 1, 4, 7 and 24 hours after treatment by Western blot analyses (-: not active, +: active).

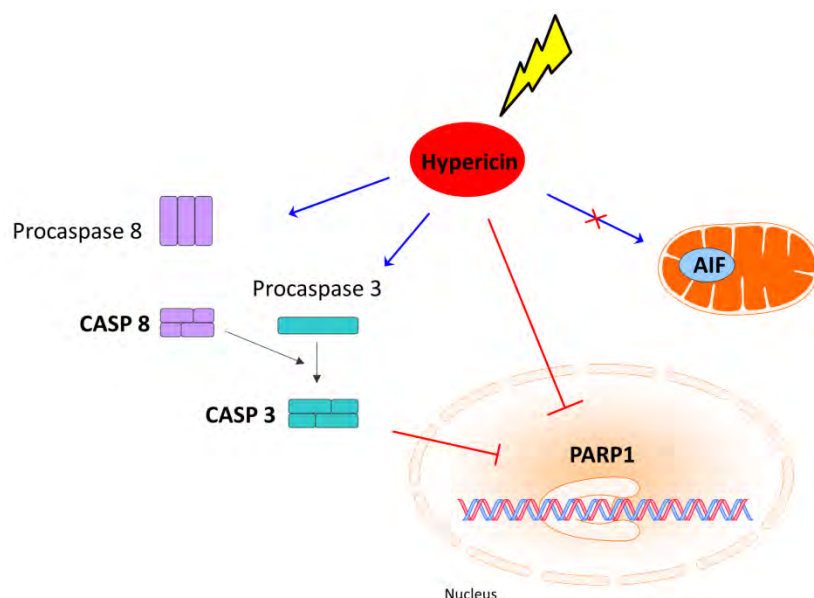


Figure 3.31: Melanoma cell death protein expression in response to hypericin-PDT. Extrinsic and intrinsic caspase-dependent apoptotic modes of melanoma cell death were observed, characterized by activation of caspase 8 (CASP8), caspase 3 (CASP3) and cleavage of poly(ADP-ribose)polymerase 1 (PARP1), as well as a caspase-independent apoptotic mode that did not involve apoptosis inducing factor (AIF).

CHAPTER 4: DISCUSSION

To shed more light on the response mechanisms of melanoma cells to hypericin-PDT *in vitro*, we embarked on a three-fold investigation. Firstly, the susceptibility of melanoma cells to hypericin-PDT was determined. Next, hypericin uptake and specific association with intracellular organelles was characterized and finally, melanoma cell death mechanisms were elucidated in response to the killing-dose of light-activated hypericin.

4.1 PART I: MELANOMA SUSCEPTIBILITY TO HYPERICIN-PDT

The “trinity” of PDT comprises a photosensitizer, light and molecular oxygen⁶. The choice of the light source in the clinic depends on various factors such as photosensitizer absorption, disease (size, location, accessibility and tissue characteristics) and cost⁶.

Hypericin absorbs light of both UVA and visible wavelengths (Fig. 1.1). Bernd et al. (1999) investigated the effect of hypericin on human keratinocytes, with light activation by UVA, UVB and visible light³⁰⁷. Both visible light and 1 J/cm² UVA induced a phototoxic reaction whereas UVB irradiation showed no effect.

The penetration of light into tissue increases with its wavelength (Fig. 1.3), therefore photosensitizers with absorption peaks in the red to deep red spectrum (600- 800 nm) offer tumour control in deeper tissues⁶. In this regard hypericin is not an ideal photosensitizer due to its minimal absorption of light of wavelengths above 600 nm (Fig. 1.10). Hypericin is able to absorb green/yellow light (495-570/570-590 nm) which allows for deeper penetration in tissue (~2 mm) compared to shorter wavelengths (~1 mm). Moreover, green/yellow light does not cause detrimental effects to cells and tissue. However, cutaneous melanoma is predominantly located in the epidermis (~50-150 µm) and upper dermis of the skin (~1 mm). Violet light (380-450 nm) and blue light (450-495 nm) are able to penetrate the epidermis (~50-150 µm) and upper dermis of the skin (~1 mm) respectively. Therefore, shorter wavelengths of light could be used for photodynamic therapy of cutaneous melanoma. Furthermore, as one of the factors influencing choice of light source for PDT is cost, sunlight-mediated PDT becomes an interesting avenue to explore for the activation of hypericin.

It has been shown in 3 randomized controlled studies that daylight-mediated PDT was an effective treatment for thin actinic keratosis²⁶⁻²⁸. Daylight-mediated PDT is nearly pain-free, more convenient for both patients and clinics and is especially suited for patients with large field cancerized areas, which can easily be exposed to daylight²⁹. It poses a particularly interesting avenue to explore for hospitals in developing countries, such as South Africa, where space is limited and budgets are inadequate.

UVA (320- 400 nm) comprises approximately 95 % of incident midday solar ultraviolet irradiation and penetrates skin much deeper than UVB (290- 320 nm)³⁰⁸. UVA is less mutagenic than UVB and absorption of UVA irradiation in endogenous chromophores frequently leads to the generation of ROS such as singlet oxygen^{308,309}. On the other hand, UVA can induce heme oxygenase-1 (HO-1) expression in cells resulting in an antioxidant and anti-inflammatory effect³¹⁰. The latter highlights that not all UVA induced effects are deleterious and potentially depend on the dose of exposure.

On the basis of the above outlined factors, including the effective activation of hypericin by UVA, the lower mutagenicity of UVA compared to UVB and the potential of sunlight-mediated PDT, we chose to use UVA to activate hypericin. A dose of 1 J/cm² UVA was employed based on its effective hypericin activation resulting in phototoxicity in experimental systems, including our own^{114,276,307}. This dose is below the minimal erythral dose (MED) baseline of 20 J/cm² UVA observed in the study by Beattie, P.E. et al³¹¹. Clinical PDT furthermore is able to use a non-cytotoxic light dose to visualise the tumour-localised photosensitizer, a method used effectively in the photodynamic diagnosis of cancer¹⁴², followed by the selective illumination of the tumour with cytotoxic light doses. This implies that the surrounding healthy tissue is minimally exposed to light.

Exposing melanoma cells to 1 J/cm² UVA resulted in melanoma cell viability not significantly different to that of the sham-irradiated control (Fig. 3.11/ 3.13). Sham-irradiation was employed in our system to consistently expose the control and treatment groups to the same environment. The irradiation time for the chosen UVA dose was 6 minutes and 10 seconds which is short and thus unlikely to contribute to deleterious effects. The cells were irradiated in phosphate-buffered saline (PBS) to ensure a physiological environment and effective photosensitizer activation without absorption of light by tissue culture media³¹². We solubilized hypericin in dimethylsulfoxide (DMSO) and found that the resulting vehicle concentration of 0.5% did not affect melanoma cell viability. The vehicle-treated, sham-irradiated control was thus used for normalization of the cell viability data.

Without light activation, varying concentrations of hypericin have been shown to negatively affect cancer cells through being cytostatic¹⁷¹, anti-metastatic¹⁷³, anti-angiogenic^{174,175}, anti-enzymatic¹⁷⁶ and survival pathway inhibitory^{120,168}. These responses were observed with high concentrations of hypericin (10 µM and more) and may thus be negligible at lower concentrations. We found a significant decrease in A375 melanoma cell viability after treatment with sham-irradiated 2.5 µM hypericin (84± 4 %, Fig. 3.5A). This however contradicted the results of the first sets of experiments in which a higher dose of 3µM sham-irradiated hypericin treatment resulted in an A375 cell viability of 113± 7 % (Fig. 3.3A). These inconsistent results could possibly be related to different batches of XTT reagent used for the assay as these experiments were performed at different times.

Furthermore, in contrast to the above mentioned studies, we found a significant increase in melanoma cell viability after sham-irradiated hypericin treatment compared to the control, in A375 (0.4 μ M), 501mel (1.5 and 2 μ M) and UCT Mel-1 melanoma cells (5 μ M, Fig. 3.3). Lower hypericin concentrations without light activation could thus have a stimulatory effect. This effect could also be due to an increase in cellular dehydrogenase and reductase activity, on which the cell viability assay is based on, or an increase in cell numbers. Initial investigations in our laboratory however showed correlation of cell numbers with cell viability obtained from the assay³¹³. Taken together and in alignment with the ideal photosensitizer characteristic of minimal dark cytotoxicity⁶, the sham-irradiated hypericin results obtained from this study are favorable for PDT.

Light-activated hypericin resulted in a significant increase in melanoma cell viability compared to the control, at lower treatment doses (Fig. 3.3/3.4). This was found for A375 (0.4 μ M) and 501mel (1 μ M) While the observed increase in melanoma cell viability at lower hypericin concentrations is not the desired outcome, it does point to the fact that future work needs to critically identify the correct dose, time of administration and therapeutic window after activation to ensure optimal effect. An increase in cell viability could be attributed to beneficial effects of low levels of reactive oxygen species (ROS) such as activation of the antioxidant response. A study by Hadjur et al. (1996) showed an increase in total SOD activity, a decrease in glutathione peroxidase (GSHPx) and catalase (Cat) activities and decreased GSH levels in unpigmented melanoma cells after light-activated hypericin treatment¹²⁷. The study furthermore showed that in pigmented melanoma cells GSHPx was unaffected, while Cat activity was increased. This shows that pigmentation might play a role in the antioxidant response to hypericin-PDT. However, this needs to be verified with a larger panel of cell lines of different pigmentation status. The activation of the antioxidant response in response to hypericin-PDT was not in the scope of our study; however our laboratory has found no significant differences in catalase activity between A375 and UCT Mel-1 and no significant differences in basal GSH levels in all the melanoma cell lines used in this study (Parker, A., Nsole Biteghe, F.A. unpublished data). Noteworthy however, these results are basal levels of antioxidants which do not reflect the response to PDT and are thus not comparable to the findings from the study by Hadjur et al. (1996)¹²⁷. The effect of hypericin-PDT on the antioxidant status of melanoma cells used in our system remains to be elucidated.

Higher treatment doses produced a phototoxic response in melanoma cells. A dose of 3 μ M light-activated hypericin was effective in significantly reducing cell viability to 50 % or less than the control, for all melanoma lines investigated (Fig. 3.3). The UCT Mel-1 cells were most resistant to this dose (50 $\pm$ 8 %) whereas A375 and 501mel cells displayed similar susceptibilities (A375: 32 $\pm$ 4 % and

501mel: 39 ± 5 %, Fig. 3.3). Higher doses up to 10 μ M light-activated hypericin revealed a further significant reduction in cell viability to 17 ± 0.8 % for A375, 14 ± 0.6 % for 501mel and 17 ± 1.2 % for UCT Mel- cells (Fig. 3.3). Lower doses than 3 μ M proved effective in reducing cell viability to less than 50% of the control only in A375 cells (2.5 μ M: 29 ± 4 %, Fig. 3.5A) and not the other melanoma cells used in our system (2.5 μ M: 501mel: 82 ± 3 % and UCT Mel-1: 68 ± 5 %, Fig. 3.5B/C). Hypericin-PDT has been shown to induce a dose-dependent cell death response, ranging from autophagy-related cell death and apoptosis at lower treatment doses and necrosis at higher treatment doses^{92,167–169}. Using a high treatment dose of 10 μ M hypericin resulted in a melanoma cell viability of approximately 15 %, suggesting a necrotic response, which is not suitable for cell death analyses due to the low numbers of cells.

We thus decided to employ 3 μ M hypericin activated with 1 J/cm² UVA as the killing-dose for further investigations into melanoma cell death mechanisms in response to hypericin-PDT.

4.2 PART II: HYPERICIN AND MELANOMA CELLS

In order for PDT to be effective, the cellular uptake and localization becomes important aspects to understand. We therefore investigated hypericin uptake and localization in melanoma cells before light-activation. An exposure of 3 μ M hypericin for 4 hours in serum-supplemented media without light-activation was chosen on the basis of our earlier studies¹¹⁴. This mimics photosensitizer uptake in the clinical setting in which it is given topically or intravenously, followed by a variable time of accumulation in cancerous tissue and subsequent activation by light⁶. We found significantly increased hypericin uptake in the A375 cells compared to the 501 and UCT Mel-1 melanoma cells (Fig. 3.7). In addition, no significant difference in hypericin uptake was evident between 501 and UCT Mel-1 cells.

This uptake assay protocol involved total cell extraction on the tissue culture dish by scraping the cells into an extraction buffer, followed by fluorescence measurement of hypericin and normalization to protein content. Recent work has demonstrated that hydrophobic photosensitizers, including hypericin, adhere to tissue culture plates¹⁵⁶, which could have implications relating to final assay values. However, all our cells were assayed simultaneously including the controls and thus even if the values are lower, the differences will still be evident after normalization to the controls. Moreover, the aim was not to establish an exact intracellular quantification of hypericin but rather to compare the hypericin uptake between the melanoma cells, for doing this, this assay served the purpose.

Another possible caveat with measuring hypericin fluorescence in melanoma cells, is the pigment melanin found in these cells. Light absorption of melanin increases in absorbance with decreasing wavelength, with very low absorbance at wavelengths higher than 450 nm³¹⁴. We used excitation wavelengths of 488 nm or 563 nm to assay for hypericin fluorescence in the uptake assay. This suggests that melanin may not affect the hypericin fluorescence measurements as melanin absorbs light minimally at 488 and 563 nm. Moreover, hypericin fluorescence did not correlate with pigmentation status as hypericin fluorescence was not significantly different in 501mel and UCT Mel-1, but the cells exhibited significantly different tyrosinase activity. This suggests that interference by melanin may be negligible.

A further limitation related to this assay is the extraction in an aqueous environment. Hypericin has been shown to form non-fluorescent high molecular weight aggregates in aqueous solutions, opposed to its distinct red fluorescence in organic solvents^{95,291}. We were however able to quantify hypericin fluorescence with this assay, which implies that not all hypericin was in an aggregated,

non-fluorescent state. This might not be a true reflection of the total hypericin fluorescence but, as with the first point, our interest was in the relative measure of hypericin uptake between cell lines and not an absolute intracellular quantification. An absolute intracellular hypericin quantification assay has previously been described¹³⁹ but was not used in this instance.

Hypericin has been shown to co-localize with various intracellular organelles such as the endoplasmic reticulum at low concentrations (125-150 nM) and long incubation times (16 h), eliciting an autophagy-related or apoptotic cell death response^{117,119,315}, whereas in other experimental systems (1,6, 24 h incubation, 500 nM hypericin) predominant lysosomal co-localization was found eliciting necrosis¹²⁴. These findings illustrate the effect of the intracellular localization of the hypericin on its mechanism of action. To investigate hypericin uptake and intracellular localization in melanoma cells, live confocal fluorescent microscopy with organelle-specific fluorescent fusion proteins and cellular dyes were used. Initial investigations were performed by fixing cells in 4 % paraformaldehyde after hypericin uptake, before visualization with confocal fluorescent microscopy. However, these experiments revealed a somewhat diffuse hypericin pattern inside the cells and although hypericin was found associated with specific organelles; the result was less than optimal “hazy” pattern. To circumvent this phenomenon we conducted further investigations with live cell imaging on the confocal fluorescent microscope. This approach resulted in a more “crisp” hypericin pattern upon visualization. The potential limitation of this setup is the activation of hypericin by the confocal laser which is 250 times stronger than the light-activation used in subsequent experimental setups. Despite this, the experimental system provided much clearer, defined intracellular localization information, which more closely related to the cells own natural environment. To minimize activation of hypericin in this experimental setting, cells were first scanned for the expression of the organelle-specific fluorescent fusion proteins with the respective laser which did not activate hypericin. As all cells took up hypericin but not all cells expressed the organelle-specific fluorescent fusion proteins, this approach was feasible and ensured minimal hypericin activation and cytotoxic effects.

Intracellular hypericin localization studies revealed that hypericin was taken up by all melanoma cells used in this study, with a characteristic red fluorescence upon excitation with the confocal fluorescent microscope laser of 562 nm (Fig. 3.8- 3.14). This indicated that hypericin was found as fluorescent monomers in the melanoma cells. Whether non-fluorescent hypericin aggregates were also present needs to be verified experimentally. Hypericin fluorescence in monomer form could be attributed to the incubation media which contained fetal bovine serum. This serum contains low- and high-density lipoproteins (LDL, HDL), which associate strongly with the lipophilic hypericin

resulting in the formation of red fluorescent momomers⁹⁶⁻⁹⁹. When associated to LDL/HDL, hypericin enters cells by receptor-mediated endocytosis resulting in lysosomal localization in various cell types^{96,97,117,124,128,135,316}. Furthermore, the association of hypericin with LDL enables the preferential uptake in tumor versus normal tissue *in vivo*, due to upregulated expression of LDL surface receptors on cancer cells²¹⁴. This is further supported by a study in which a comparison of cells with different expression levels of LDL surface receptors revealed a substantial increase of hypericin uptake in cells with elevated numbers of LDL receptors¹¹³. In addition, cells with elevated LDL receptor expression showed enhanced hypericin co-localization with lysosomes when the incubation medium contained LDL¹¹³.

Co-localization of hypericin with the cell membrane^{130,131}, golgi apparatus^{107,117,132,133}, mitochondria^{107,124,134} and the endoplasmic reticulum^{107,117,120,128,132,133} and lysosomes^{113,117,124,128,135} has been reported. Hypericin has only been associated with the nucleus after prolonged incubation time¹³¹ and hardly after short incubation times¹⁰³. The intracellular localization of hypericin is likely to depend on a multitude of factors such as the cell type, incubation time and presence of serum proteins.

Hypericin partially co-localized with the endoplasmic reticulum, mitochondria and the cell membrane in the melanoma cells used in our study. To investigate co-localization we used profiles of the fluorophores, taken through the cell at different locations. One drawback of this analysis is that by only analyzing along a single line in a cell one might miss the complete analysis, where there might have been co-localisation or “similar intensity profiles”. Even if profiles do not match exactly, a large overlap of signal was sometimes observed, visualized by the overlap of the two fluorophores (hypericin and the fluorophore of interest). If this was the case we thus concluded that a partial co-localization was found. Complete co-localization analysis in future studies will refine these observations.

An interesting phenomenon was furthermore found in 501mel cells in which upon visualization of the fluorophores some cells displayed a modification from the tubular mitochondrial network into a beaded appearance (Fig. 3.10). One could speculate that this was a result of hypericin activation by the confocal laser and concomitant ROS production in and around the vicinity of mitochondria leading to extensive mitochondrial alteration. Investigating profiles of the fluorophores indicated a partial co-localization of hypericin with the tubular and beaded mitochondria. How this partial co-localization of hypericin with mitochondria can produce such extensive alterations needs to be clarified in future studies.

Co-localization with lysosomes was evident in the melanoma cell lines used in our study (Fig. 3.11). In 501mel and UCT Mel-1 melanoma cells, hypericin presented the same fluorescent profile as LysoTracker, in all profiles taken at different locations through the cell. For A375 melanoma cells this was variable, some profiles indicated co-localization whereas others did not show the same profile pattern. LysoTracker is a fluorescent acidotropic probe labelling acidic organelles in live cells. This includes lysosomes and possibly melanosomes in pigmented cells as these are lysosomal-related organelles, a specialisation of the endocytic pathway^{227,317}.

To investigate the co-localization with melanosomes in more detail, we expressed both early and mature melanosomal markers in melanoma cells. These experiments showed variable co-localization of hypericin with Rab7-GFP in A375 melanoma cells only and not in 501mel or UCT Mel-1 cells (Fig. 3.12). Rab7 is involved in microtubule-based transport between early and late endosomes, late endosomes and lysosomes and early and intermediate stage melanosomes^{284,293}. In the latter it facilitates the transport of tyrosinase and tyrosinase related protein 1 (TYRP1) from the trans-golgi network to melanosomes. This indicated that in A375 cells, hypericin variably co-localized with the endocytic pathway as well as early stage melanosomes. Variable hypericin co-localization with the mature melanosomal marker myosinVa but not Rab27a was furthermore observed in 501mel and UCT Mel-1 but not in A375 melanoma cells (Fig. 3.13/14). Rab27a and myosinVa are both involved in melanosomal transport to the distal parts of dendrites of mature melanosomes²⁹⁴. Rab27a localizes to the melanosomal membrane in its active GTP-bound state and acts as a receptor for the actin-dependent myosinVa motor protein, facilitated by the specific Rab27a effector melanophilin/Slac2a, forming a “molecular tripartite complex” that links mature melanosomes to the actin network²⁸⁵. Differences in the co-localization results with these two mature melanosomal markers necessitate further verification. A possible explanation for these inconsistent results obtained with these mature melanosomal markers could be related to the molecular size of the GFP fusion protein which logically is bigger. Moreover, this could affect cellular interaction. To tease out the exact nature and location of hypericin co-localization with the melanosomal system, pharmacological inhibition or genetic ablation of proteins involved in the different melanosomal stages is an interesting avenue to explore for future studies.

It is worthy to note that hypericin only partially and not exclusively co-localized with melanosomal-specific proteins. This is an important finding as melanosomes have been associated with chemotherapeutic drug trapping, followed by increased melanosomal biogenesis and export from the cell^{228,248,249}. These studies revealed that drug-resistant melanoma cells treated with a sub-lethal dose of cisplatin showed the presence of predominantly stage II–III and III melanosomes.

Melanosomes at these stages are typically cigar-shaped with some nascent melanin deposition on proteinaceous fibrils, whereas stage IV melanosomes are typically round and completely filled with melanin²²⁷. Stage II–III and III melanosomes possess a greater capacity to trap cytotoxic drugs in the nascent melanin and are thus likely to be involved in drug resistance²²⁸. Moreover, treatment with the tyrosinase inhibitor phenylthiourea (PTU) induced the pharmacological modulation of melanosomes to stage II or II–III and significantly increased resistance to cisplatin by 12-fold²⁴⁹. On the contrary, investigations from our laboratory have shown that pre-treatment of UCT Mel-1 cells with PTU or kojic acid (another tyrosinase inhibitor) increased susceptibility to hypericin-PDT^{318,319}. This could imply that the shift in melanosomal biogenesis to stages II–III and III, used to sequester and export cytotoxic drugs, does not occur in response to hypericin-PDT in our system. The variable and non-exclusive co-localization of hypericin with melanosomes furthermore suggests that the melanoma cells investigated in this study may not use their melanosomal system for hypericin trapping.

Localization of hypericin in melanosomes could however be of advantage for cancer treatment with PDT. Melanogenesis comprises several cytotoxic melanin precursors and is thus confined to melanosomes³²⁰. Localization of hypericin in the melanosomal membrane followed by its activation by light could lead to ROS production and subsequent melanosome destruction, resulting in release of toxic melanin precursors into the cytoplasm. Moreover, the shift of melanosomal biogenesis to early stage melanosomes to trap and export cytotoxic drugs from the cell, amplifies the presence of toxic precursors in the cell. The drug-detoxifying system used by melanoma cells for chemotherapeutic drugs could thus pose a potential effective killing mechanism for PDT. Whether this is the case in our experimental system needs to be verified in future studies.

In our study, transmitted electron microscopy (TEM) images revealed round stage IV melanosomes, filled with the pigment melanin, in untreated and hypericin-PDT treated melanoma cells (Fig. 3.2C/3.15). These were evident in unpigmented A375 and pigmented UCT Mel-1 cells. In addition, all melanoma cells showed significantly higher levels of tyrosinase activity than the control MCF7 breast cancer cells (Fig. 3.2B). The UCT Mel-1 cells (25887 ± 744 cpm/120 μ g protein) showed significantly higher tyrosinase activity than 501mel (6286 ± 964 cpm/120 μ g protein) and A375 cells (5191 ± 811 cpm/120 μ g protein, Fig. 3.2B). These results illustrate the basal pigmentation characteristics of melanoma cells used in this study. Whether these basal pigmentation levels change in response to hypericin-PDT needs to be verified by future studies. In addition, investigations into the antioxidant response of the melanosomal system to PDT and the potential

drug trapping of hypericin in melanosomes of different stages are interesting factors to explore in future investigations.

It has also been reported that multi-drug resistant (MDR) cancers exhibit increased endocytosis and exocytosis, an enlarged lysosomal compartment and a marked increase in lysosomal number which enables the cells to sequester and secrete chemotherapeutic drugs effectively^{321,322}. Interestingly, lysosomal targeted photosensitizers have been shown to effectively circumvent MDR by lysosomal photodestruction, resulting in the reversion to the parental drug sensitivity³²¹. Similar results have been obtained with lysosomotropic agents such as chloroquine, which has been shown to reduce MDR⁷⁸. Whether the lysosomal co-localization of hypericin in melanoma cells used in this study is an active drug trapping effort or the product of LDL-mediated endocytosis of the hypericin-LDL complex needs to be unraveled; nonetheless the lysosomal co-localization is very favorable for effective cancer therapy.

Chen et al. (2009) have hypothesized that melanoma cells express several ABC transporters (e.g. ABCB5, ABCC2 and ABCC1) in melanosomes and diverse subcellular organelles of the endosomal pathway, constituting a buffer system that prevents nuclear or mitochondrial import of cytotoxic drugs²²⁸. Furthermore, the same transporters, especially ABCB5, might function as heterodimers on lysosomal and/or melanosomal membranes to trap cytotoxic melanin metabolites in those organelles, thereby protecting the cells from endogenous melanosomal cytotoxicity generated by late-stage melanosomes²²⁸.

Hypericin has been shown to increase the activity of the ABC transporters multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) in HT-29 colon cancer cells²⁰⁶. The same study showed increased intracellular hypericin upon pharmacological inhibition of these transporters thus suggesting their direct involvement in hypericin pharmacokinetics.

Investigating the involvement of ABC transporters in hypericin efflux from melanoma cells was not in the scope of our study; however we were able to quantify hypericin fluorescence in melanoma cells post PDT. We analysed hypericin median fluorescent intensities in the cells used for the FACS experiments. Our results showed that intracellular hypericin was reduced with increasing time after hypericin-PDT treatment in all melanoma cells (Fig. 3.28). We used the median and not the mean hypericin fluorescence for this quantification, thus more accurately reflecting the middle fluorescent value in the population. The observed decrease in hypericin fluorescence could be a result of various factors. Hypericin fluorescence could degrade in the cell. This might be attributed to the formation of high-molecular weight aggregates that are non-fluorescent. Light activation might influence the

fluorescence of hypericin as light-activated hypericin showed significantly higher fluorescence than sham-irradiated cells. Hypericin might be actively pumped out by the activity of ABC transporters and/ or sequestration into melanosomes/ lysosomes followed by active efflux from the cell. Alternatively hypericin might be diluted out due to cell division at least at later time points of the analyses. Which of these factors influence the intracellular hypericin dynamics in melanoma cells post hypericin-PDT remains to be elucidated in future investigations.

Another discussion point is the clinical range of the photosensitizer and whether a dose of 3 μM hypericin is plausible to obtain in a clinical setting. Clinically, 3 μM hypericin is calculated to be of the order of 1.513 mg/kg body weight which would render it a lower efficacy than current clinically related experiments, where 8 μM is being used which is equivalent to 4 mg/kg body weight^{143,323}. Other studies show uses of 5 mg/kg hypericin^{191,324} which is equivalent to hypericin concentrations of the order of 10 μM . Our concentrations would therefore have to be increased but as this was a cellular based project; our guide was the original cytotoxicity curves which showed that higher concentrations (up to 10 μM , see Fig. 3.3) completely destroyed the cells without leaving the option to investigate cell death mechanisms.

4.3 PART III: HYPERICIN-PDT INDUCED MELANOMA CELL DEATH

Hypericin phototoxicity has been shown to induce several cell death mechanisms in various experimental systems, ranging from apoptosis to necrosis and autophagy-related cell death^{92,167,168}. Hypericin-PDT has been shown to reduce melanoma cell viability^{114,126,127,221,273,274,325}, however reports on the induction of specific cell death mechanisms are, to the best of our knowledge, limited to one study from our laboratory¹¹⁴. Understanding the dynamics of cell death is crucial for effective therapy and is therefore pertinent to investigate in more detail.

This project analysed several cell death markers over a time-course after hypericin-PDT treatment. These analyses included: ultrastructural modifications, phosphatidylserine (PS) exposure and loss of cell membrane integrity, cell size and granularity and activation of cell death proteins.

4.3.1 ULTRASTRUCTURAL MODIFICATIONS

To investigate the initial response to light-activated hypericin we chose to analyse one unpigmented (A375) and one pigmented (UCT Mel-1) cell line ultrastructurally at 4 hours after treatment. Resulting transmission electron microscopy revealed extensive cytoplasmic vacuolisation upon treatment with the killing dose (Fig. 3.15D). Moreover, cell and nuclear membranes and cytoplasmic organelles were intact and exhibited normal morphologies (Fig. 3.15D). We found activation of cell death proteins at 24 hours in A375 and 4 and 7 hours in UCT Mel-1 cells, it is thus not surprising that the apoptotic morphological features of nuclear fragmentation and apoptotic body formation, endpoints of the apoptotic cascade³⁶, were not visible at 4 hours after treatment.

The observed vacuoles were of variable sizes. Some contained electron-dense material, found in the perinuclear area of the cell. These vacuoles could possibly represent autophagosomes. Autophagy has been observed in response to hypericin-PDT in a variety of both normal and cancerous cells^{11,117,207,209}. To verify the involvement of autophagy further investigations are needed. These could include assays to determine the conversion of microtubule-associated protein light-chain 3 (LC3) in the formation of autophagosomes and the visualisation and quantification of autophagosomes with LC3-GFP and confocal fluorescent microscopy as well as techniques investigating autophagic flux³²⁶.

Most vacuoles however did not contain electron-dense material and were found on the periphery of the cell, especially in the UCT Mel-1 cells. In A375 vacuoles of all sizes were found throughout the cell. These vacuoles could also represent swollen endoplasmic reticulum. In the case of co-localization of hypericin with the endoplasmic reticulum (ER), producing ROS in the vicinity of this

organelle, this hypothesis seems feasible. However, we did not find co-localization with the ER but with lysosomes in melanoma cells used in our study. The lysosomal photosensitizer NPe6 has been shown to induce extensive vacuolisation in hepatoma cells^{327,328}. These vacuoles were identified as an accumulation of late stage endosomes due to photodestruction of lysosomes, preventing flux from endosomes to lysosomes. Whether the observed vacuoles in melanoma cells post PDT represent swollen late endosomes remains to be identified.

Recently a new model for the externalization of phosphatidylserine (PS) has been proposed involving the internalization of the cell membrane as cells shrink during apoptosis, forming intracellular vesicles²⁹⁵. These vesicles are then trafficked back to the cell surface in a Ca^{2+} -dependent manner, exposing PS on the cell surface²⁹⁵. The vacuolisation could thus also represent vesicles implicated in the externalization of PS, as melanoma cells showed PS exposure at all time-points investigated post hypericin-PDT.

The exact nature of the extensive vacuolisation in response to hypericin-PDT in both pigmented and unpigmented melanoma cells is an interesting avenue to explore in future studies.

An interesting finding was the presence of glycogen accumulations in pigmented UCT Mel-1 but not untreated UCT Mel-1 cells or unpigmented A375 melanoma cells in response to hypericin-PDT (Fig. 3.15). Metabolically, circulating glucose is normally taken up by the liver and muscle cells where it is converted into glycogen and stored. Intracellularly, however, levels of glycogen are controlled by the activity of the glycogen synthase kinase (GSK) enzyme. GSK inhibits glycogen synthesis and glucose uptake by cells³²⁹. It can therefore be inferred that GSK inhibition causes an increase in glycogen accumulation. In addition, downregulating GSK3 β in B16 mouse melanoma cells and normal human melanocytes leads to activation of the Wnt signalling pathway and increased levels of differentiation-associated markers in these cells such as increased tyrosinase activity and melanin synthesis³³⁰. Increased glycogen accumulation in hypericin-treated pigmented melanoma cells may be a result of GSK3 β inhibition, for which at present there is no evidence. Moreover, activation and/or inhibition of GSK3 β in melanoma has been linked to different modes of cell death as well as stress of the endoplasmic reticulum^{331–333}, which could account for the difference in the modes of cell death in these melanoma cells¹¹⁴. The involvement of GSK3 β has been documented in response to PDT^{334–336} and its exact role in the response of melanoma cells to hypericin-PDT is an interesting future investigation.

4.3.2 PS EXPOSURE & LOSS OF CELL MEMBRANE INTEGRITY

To investigate the cell death response to hypericin-PDT over a range of time-points after treatment (30', 1, 4, 7 and 24 h) we chose to quantify phosphatidylserine (PS) exposure and loss of cell membrane integrity by fluorescent activated cell sorting analyses (FACS).

A common feature of apoptosis is the change in cell membrane structure by surface exposure of PS³³⁷. Externalized PS contributes to the recognition and subsequent removal of apoptotic bodies by phagocytes, it has also been reported that early necrotic cells exposed PS followed by macrophage clearance²⁹⁶. Annexin A5 (Annexin V) is an anionic lipid binding protein, widely used to detect apoptotic cells *in vitro*^{288,338} and is currently being explored as a non-invasive imaging tool for determining the efficacy of diseased tissue treatments, such as cancer³³⁹. Certain cell lines however undergo apoptosis with very little PS exposure and it was recently shown that this phenomena is related to the mechanistic basis of PS exposure²⁹⁵.

Different models for the mechanistic basis of PS exposure have been proposed, with the most widely accepted model suggesting activation of the enzyme scramblase, capable of randomizing PS across the cell membrane³⁴⁰. Other studies suggest Ca^{2+} -induced trafficking of lysosomes to the cell membrane, a process inhibited by the lysosomotropic agent chloroquine³⁴¹ or a role of the ATP-binding cassette (ABC) transporter ABC1³⁴². Recently another two-step model has been proposed resulting in the externalization of PS²⁹⁵. This involves the internalization of the cell membrane as cells shrink during apoptosis, forming intracellular vesicles. These vesicles are then trafficked back to the cell surface in a Ca^{2+} -dependent manner. This study furthermore showed that the second step is markedly diminished in cell lines with diminished apoptotic PS exposure²⁹⁵.

Using Annexin V in conjunction with a cell permeability dye (e.g. propidium iodide) enables the distinction between cells with exposed PS and those with compromised cell membranes. Loss of cell membrane integrity is a feature of necrotic cells²⁹⁷. It has however also been reported that cells undergoing apoptosis displayed an early uptake of propidium iodide followed by a condensation and then a fragmentation of their nuclei³⁴³. Due to the red fluorescent nature of propidium iodide (Ex: 535nm, Em: 617nm³⁴⁴) it is not the most suitable dye to use in conjunction with hypericin as this photosensitizer also fluoresces red Ex: 548/591, Em:594/642^{105,163}). We thus employed the violet amine reactive viability dye (VIVID) to investigate loss of cell membrane integrity in our experimental system using flow cytometry.

Melanoma cells treated with 1 J/cm² UVA or sham-irradiated hypericin displayed no PS exposure and the cell membranes remained intact (Fig. 3.16), confirming the non-cytotoxic nature of these PDT

components by themselves and verifying our cell viability experiments. In contrast, administering both components together induced a cytotoxic cell death response (Fig. 3.16).

Analysing Annexin V and VIVID median fluorescent intensities (MFI) after treatment enabled us to quantify and statistically analyse these two cell death markers compared to the sham-irradiated, vehicle-treated control cells. Light-activated hypericin induced a significant, time-dependent increase in PS exposure in all melanoma cells, most pronounced at 24 hours post PDT (Fig 3.17). Moreover, a significant loss of membrane integrity was found for 501mel at all time-points investigated and in A375 at all time-points except at 4 hours after PDT (Fig. 3.18). UCT Mel-1 cells on the other hand exhibited intact cell membranes up to and including 7 hours after treatment (Fig. 3.18C).

Another way to analyse the flow fluorocytometric data is by creating dot blots of Annexin V (AV) versus VIVID. This enables the identification of 4 different populations of cells: live (AV- VIVID-), early apoptotic (AV+ VIVID-), early necrotic (AV- VIVID+) and late apoptotic/ necrotic (AV+ VIVID+) (Fig. 3.19). Literature describes a lag period between PS exposure (AV+) and disintegration of the plasma membrane (VIVID+) in apoptotic cells whereas in necrotic cells these events coincide^{345,346}. During apoptosis, membrane changes leading to PS exposure are rapid and thus the population shifts from live (AV- VIVID-) to early apoptotic (AV+ VIVID-). PS exposure furthermore leads to loss of membrane integrity resulting in a shift of the population to late apoptotic/ necrotic (AV+VIVID+). During necrosis, PS exposure coincides with permeabilization of the plasma membrane. The population thus moves immediately from live (AV- VIVID-) to late apoptotic/ necrotic (AV+ VIVID+). This can be explained by the artifact of Annexin V labeling due to passive diffusion of this dye into a disintegrating plasma membrane.

Control treatments resulted in a live population of approximately 95 % or more for all melanoma cells (Appendix F: Fig. A.F.1/2/3). Interestingly, 24 hours after light-activated hypericin treatment the least number of live cells was found whereas at 4 hours the highest number of live cells was observed (Fig. 3.21). The live population was consistently higher for UCT Mel-1 cells compared to that of A375 and 501mel, at all time-points investigated (Fig. 3.21). The A375 and 501mel live populations were similar to each other. These results corroborate the cell viability data which showed a 50 ± 8 % UCT Mel-1 cell viability compared to 32 ± 4 % for A375 and 39 ± 5 % for 501mel after treatment with the killing dose (Fig. 3.4). Melanoma pigmentation levels could possibly explain these different results in susceptibility to hypericin-PDT. Tyrosinase activity was approximately 4-fold higher in UCT Mel-1 (25887 ± 744 cpm/120 μ g protein) compared to that of A375 (5191 ± 811 cpm/120 μ g protein) and 501mel cells (6286 ± 964 cpm/120 μ g protein, Fig. 3.2B). In addition, white

pellets are found for A375 and 501mel cells, whereas UCT Mel-1 cell pellets were pigmented (Fig. 3.2A). As melanin is indeed different in these cells, the susceptibility differences could be attributed to a filtering effect, absorbing the UV light and thereby preventing hypericin activation. Another possible protective role could be the antioxidant effects of melanin^{225,347}. A study by Bustamante et al (1993) showed the protective role of melanin, able to scavenge ROS and inhibit lipid peroxidation³⁴⁷. Another possibility is inherent differences in the basal antioxidant systems of the different melanoma cell lines. This was not in the scope of this study, however investigations in our laboratory have shown no significant differences in catalase activity for A375 and UCT Mel-1 and no significant differences in basal GSH levels in all three cell lines (Parker, A., Nsole Biteghe, F.A. unpublished data). Basal levels of other members of the antioxidant response and the effect of hypericin-PDT on the antioxidant status of melanoma cells used in our experimental system remain to be elucidated.

Light-activated hypericin treatment resulted in an initial early necrotic population (AV- VIVID+) for all melanoma cells, most pronounced in 501mel (Fig. 3.21). This early necrotic population (AV- VIVID+) decreased with time after treatment, whereas the late apoptotic/ necrotic population (AV+ VIVID+) increased (Fig. 3.20). Minimal early apoptotic populations (AV+ VIVID-) were found in all cell lines, with the highest found in UCT Mel-1 at 24 hours (15± 5%, Fig. 3.21C). It is interesting that we found an early necrotic population of cells that was positive for VIVID and negative for Annexin V, as literature describes PS exposure and permeabilization of the plasma membrane coinciding with necrosis, resulting in an immediate shift from live (AV-VIVID-) to late apoptotic/ necrotic (AV+ VIVID+)^{345,348}. The subsequent shift from an early necrotic population to a late apoptotic/ necrotic population positive for both markers suggests that Annexin V binding in this double-labelled population might be an artefact due to loss of cell membrane integrity. To verify whether Annexin V binding is an artifact or a genuine apoptotic “eat-me” signal, studies investigate whether Annexin V positive cells can be phagocytosed^{345,348}. It has however also been shown that early necrotic cells exposed PS followed by macrophage clearance²⁹⁶, indicating that Annexin V binding is not an artefact due to cell membrane permeabilization during necrosis. The double-labelled population could furthermore represent regulated necrosis, as double-labelling of Annexin V and propidium iodide has been shown to be a hallmark of cells undergoing AIF-mediated necroptosis in response to nitrosourea MNNG induced DNA alkylating damage^{53–55,349}.

U87 glioma cells have shown double labeling of Annexin V and propidium iodide in response to hypericin-PDT³⁵⁰, indicating that this finding might not be restricted to our experimental system.

Other studies have labeled cells with Annexin V alone and shown PS exposure after treatment^{170,351–354}.

One of the proposed mechanistic models for PS exposure involves the internalization of the cell membrane as cells shrink during apoptosis, forming intracellular vesicles. These vesicles are then trafficked back to the cell surface in a Ca^{2+} -dependent manner²⁹⁵. Investigating cell shrinkage after hypericin-PDT was thus of interest to us.

4.3.3 CELL SIZE AND GRANULARITY/ PIGMENTATION

Flow cytometric analyses obtains optical information known as forward scatter (FS) and side scatter (SS), based on the angle of light emitted from the cell²⁹⁸. Light scattered at a small angle is called FS and provides information correlating with cell size. Light that scatters off the cell at a 90° angle is called SS and provides an indication of the granularity of the cell.

Morphological alterations of dying cells includes shrinkage of apoptotic cells and swelling of necrotic cells³⁶. Accordingly these morphological characteristics have been used to investigate cell death^{299,355–358}. A shift from a live high FS to a low FS dead population, with either no change in SS or a shift from low SS to high SS dead population has been observed in these studies. However, this shift has been documented for both apoptotic and necrotic cells^{343,359}, and can thus not be used as a sole discriminate marker between these modes of cell death.

Changes in cellular granularity (SS) could further indicate changes in pigmentation levels of melanoma cells. This characteristic has been used in various pigmented experimental systems to identify cell populations with different pigmentation patterns^{300–303}. In line with melanosomes as drug trapping and export organelles²²⁸ and the antioxidant potential of the pigment melanin³⁴⁷ we investigated hypericin-PDT induced changes in SS.

Our results showed a decrease in melanoma cell size to less than half of the control at all times after hypericin-PDT treatment for all cell lines (Fig. 3.26). This change in size was accompanied by an increase in granularity to approximately half of the control in 501mel and UCT Mel-1 cells (Fig. 3.27). In A375 the change in granularity occurred only at 24 hours after treatment, suggesting that the change in SSC reflects a change in pigmentation and is not linked to the change in size (FSC). However, this speculation needs to be tested experimentally with quantifications of melanoma pigmentation levels post PDT, using assays such as the tyrosinase activity assay^{279,360}. In line with the recently proposed mechanistic model of PS exposure involving cellular shrinkage²⁹⁵, hypericin-PDT treated melanoma cells might initiate an apoptotic mode of cell death. It has however also been

shown that cells shrink during necrosis^{343,359}. To shine more light on melanoma cell death induced by hypericin-PDT we decided to investigate the expression of specific cell death protein markers which enable a more refined distinction of the suicidal mechanisms.

4.3.4 CELL DEATH PROTEIN EXPRESSION

Melanoma cells were treated with the killing concentration of light-activated hypericin followed by Western blot analyses at several time-points after treatment (1, 4, 7 and 24 h). We used total protein as a loading control as this method exhibits the following advantages: it is fast and requires fewer steps and consumables, thus allowing for the assessment of the quality of electrophoresis and Western transfer before committing to costly and time-consuming Western blots^{289,290,361}. Furthermore, it has been shown, at least under the investigated circumstances, that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin did not accurately reflect differences in protein loading^{289,361}. For the above mentioned reasons we normalized the optical density of the protein of interest, obtained after immunoblotting of the membrane, to the respective optical density of the nitrocellulose membrane stained for total protein. The resulting ratio was furthermore normalized to the vehicle-treated, sham-irradiated control of the respective experiment.

A vital step in both the intrinsic and extrinsic caspase-dependent apoptotic cascade is the activation of the executioner caspase 3 (CASP3)⁴¹. Cleavage of CASP3 into two fragments of 17 and 19 kDa activates the suicidal protease and subsequent apoptotic cascade. At 4 hours after treatment CASP3 cleavage was found in UCT Mel-1, whereas in A375 melanoma cells this event occurred at 24 hours (Fig. 3.33B). The positive control used in these experiments indicated that the 501mel melanoma cells used in our study are able to cleave CASP3, however this was not found after hypericin-PDT treatment.

The involvement of CASP3-mediated cell death in response to hypericin-PDT has been documented in various other experimental systems^{123,200,206,220,362–368}. The only published report on CASP3 involvement in melanoma cells, to the best of our knowledge, originates from our earlier work¹¹⁴. This preliminary investigation revealed elevated levels of caspase 3,7 activity at 4 hours post hypericin-PDT in unpigmented melanoma cells (UCT Mel-3), however no change was found for pigmented melanoma cells (UCT Mel-1). This contradicts the finding from our current study in which we show CASP3 cleavage at the same time point after treatment. These differences might be explained by a potential lag between the cleavage of CASP3 and its activity as these assays measured different parameters.

The initiator caspase of the extracellular apoptotic pathway, caspase 8 (CASP8), activates CASP3 directly or indirectly through activation of the mitochondrial apoptotic pathway⁴³. To determine whether the observed CASP3 cleavage is a product of the activation of the extra- or intra-cellular apoptotic cascade we thus investigated CASP8 cleavage. Light-activated hypericin treatment resulted in CASP8 cleavage at 24 hours in A375 melanoma cells (Fig. 3.33C). This was not found at other time points after treatment or in the other cell lines investigated. The positive control confirmed CASP8 cleavage in both 501mel and UCT Mel-1 therefore illustrating the ability of these cells to execute apoptosis via the extracellular pathway.

Hypericin-PDT mediated CASP8 activation has been shown in Jurkat T-lymphocytes²¹⁶ and nasopharyngeal carcinoma cells²¹⁷, however this was not observed in a variety of both murine and human cancer cell lines^{200,218–220} and CASP8 inhibition had no effect on HeLa cervical carcinoma cells apoptosis²⁰⁰. These findings indicate that cell death mechanisms in response to hypericin-PDT may depend on the cell type investigated and experimental system employed.

The intrinsic apoptotic cascade is initiated by mitochondrial outer membrane permeabilization (MOMP), followed by release of intermembrane space proteins, activating mitochondrial-mediated apoptosis. MOMP occurs due to the action of pro-apoptotic proteins, such as Bax and Bak, and Bax/Bak has been shown to be a mediator in hypericin-PDT induced cell death as Bax^{-/-} Bak^{-/-} double knockout (DKO) cells died in a caspase-independent fashion¹¹⁷. Re-expression of Bax in DKO cells abrogated this and switched the caspase-independent mode back to mitochondrial-mediated apoptosis¹¹⁷. The latter was mainly, but not entirely, dependent on intrinsic caspase-mediated apoptosis as it was only partially inhibited by caspase antagonists¹¹⁷. This suggested the release of other toxic mitochondrial intermembrane space proteins (such as AIF) along with cytochrome c (CYTC) from mitochondria after MOMP, orchestrating caspase-independent apoptosis. Photoactivated hypericin has furthermore been shown to induce AIF translocation from mitochondria to nuclei in HT-29 adenocarcinoma cells, resulting in condensed and fragmented apoptotic nuclei³⁶⁹. To investigate the involvement of an AIF-dependent, caspase-independent mode of cell death we thus determined AIF expression in melanoma cells post hypericin-PDT.

Treatment with hypericin-PDT did not induce the expression of the cleaved, lethal AIF fragment in melanoma cells in our study (Fig. 3.33A). These investigations were performed on total cell extracts and not cytosolic and nuclear fractions used in other studies^{54,369}, nevertheless the total cell extract contains all fractions and thus should predictably show all fragments of AIF present in the cell. As we did not find the lethal AIF fragment in our system we did not further investigate its expression in

different cellular fractions. A potential improvement in future studies may be to fractionate the samples into nuclear and cytosolic compartments to investigate expression levels.

Poly(ADP-ribose)polymerase 1 (PARP1), is a DNA damage repair enzyme involved in cell death. In response to DNA damage PARP-1 is activated to facilitate its repair, a process which generates PAR polymer via the utilization of nicotinamide adenine dinucleotide (NAD⁺)⁵². Overactivation of PARP-1 has toxic consequences to the cell due to NAD⁺ and ATP depletion and accumulation of the mitochondriotoxic polymer PAR which favors mitochondrial membrane potential dissipation and release of the lethal fragment of AIF with subsequent chromatinolysis, culminating in a regulated necrotic mode of cell death, called parthanatos⁵². During apoptosis, PARP1 is cleaved to ensure adequate ATP levels for apoptotic completion³⁰⁶. Cleavage of PARP1 by caspases results in a 89 and 24kDa fragment, the latter irreversibly binds to DNA strand breaks, thereby inhibiting DNA repair enzymes (including PARP1) and attenuating DNA repair³⁷⁰. Cleavage of PARP1 can however also take place by the action of various other suicidal proteases, including calpains, cathepsins, granzymes and matrix metalloproteinases³⁷⁰. PARP cleavage has been documented in various cell lines in response to hypericin-PDT treatment^{123,200,206,364,365,369}.

Hypericin-PDT induced PARP cleavage in UCT Mel-1 (4 and 7 h) and 501mel (7 h) after treatment (Fig. 3.33D). In the former cell type this cleavage could be mediated by CASP3 as this was found correlatively in its cleaved, active form at 4 hours. However, CASP3 cleavage was not observed at 24 hours, indicating that this might not be the only suicidal protease, capable of cleaving PARP in our experimental system. Similarly, in 501mel cells, CASP3 cleavage was not observed in response to hypericin-PDT. No significant PARP cleavage was found in A375 melanoma cells; however CASP8 and CASP3 cleavage was found at 24 hours after treatment and thus PARP cleavage might occur at a later time-point not investigated in this study.

To tease out the exact involvement of the suicidal proteases mediating PARP cleavage in our experimental system, a pharmacological inhibition approach of the different proteases in conjunction with hypericin-PDT treatment might prove valuable in future investigations. Involvement of cathepsin suicidal proteases is particularly interesting as we found hypericin co-localization with lysosomes in melanoma cells. Light-activation of hypericin could produce reactive oxygen species (ROS) in the vicinity of the lysosomal membrane, a potent inducer of lysosomal membrane permeabilization (LMP). Following LMP, cathepsins are released into the cytoplasm inducing changes in mitochondria (MOMP) and nuclei (DNA fragmentation), resulting in cell death⁷⁰⁻⁷⁴. Cathepsin release and induction of mitochondrial mediated apoptosis has been reported with the lysosomal-localizing photosensitizer N-aspartyl chlorin e6 in human lung adenocarcinoma cells³⁷¹. An

increase in cathepsin D has been reported after hypericin-PDT in nasopharyngeal, bladder and colon cancer cells³⁵¹ and a cathepsin B-dependent, hypericin-induced hypoxia-inducible factor 1 α (HIF 1- α) canabolism has been found in glioblastoma, renal cell carcinoma and in non-malignant retinal pigment epithelial cells¹⁸⁸. These studies demonstrate the potential involvement of cathepsins in hypericin-PDT mediated cell death, an interesting avenue for future exploration in the context of melanoma.

Taken together the investigations into specific cell death proteins verify the induction of apoptosis by hypericin-PDT in melanoma cells (Fig. 3.31). The extrinsic apoptotic cascade, mediated by CASP8 and CASP3, occurred in A375 melanoma cells at 24 hours after treatment. Intrinsic apoptosis was found in UCT Mel-1 cells at 4 and 7 hours, mediated by CASP3 and PARP1. Induction of apoptosis by cleavage of PARP1 was furthermore evident in 501mel cells at 7 hours after treatment; however PARP1 cleavage was not mediated by CASP3. Involvement of PARP1 cleavage by other members of the caspase family and suicidal proteases such as cathepsins and calpains remains to be elucidated in the future. Caspase-independent apoptosis or parthanatos mediated by AIF was not observed in our experimental system.

These results suggest that the phosphatidylserine (PS) exposure in conjunction with the loss of cell membrane integrity observed in our flow cytometric studies are not an artefact of PS labelling on the inside of compromised cell membranes but rather a product of the activation of the apoptotic cascade. These cells might represent late stage apoptotic cells in which both PS are exposed and cell membranes are compromised, as the processing time for the FACS experiments comprised 4 hours at 4°C. In contrast, the protein extraction for Western blotting took a total of 30 minutes and thus more accurately reflects the post-treatment time. It line with this thought, we found a very low population of cells expressing PS only (15 $\pm$ 5% maximum), without loss of cell membrane integrity, illustrating the late-, rather than early apoptotic nature of cells in the FACS assays.

CHAPTER 5: CONCLUSION & FUTURE DIRECTIONS

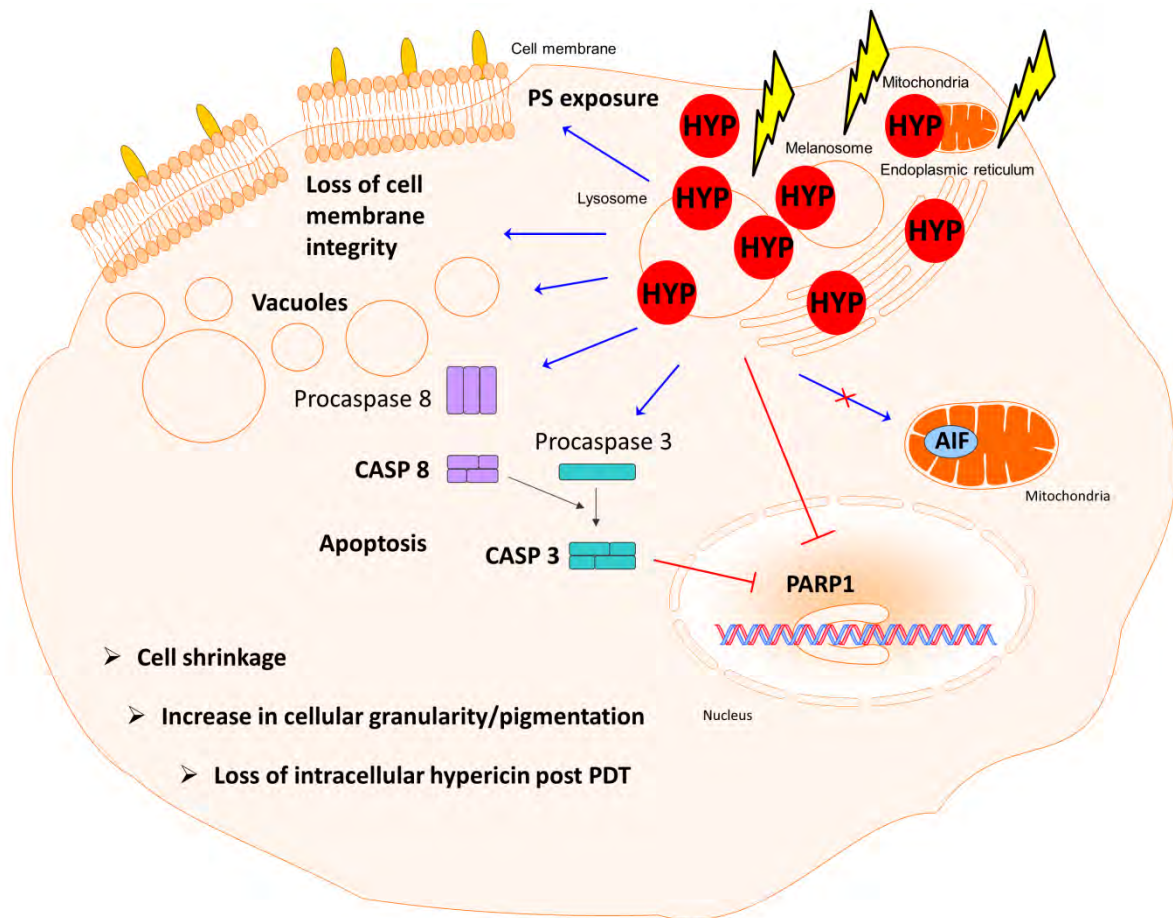


Figure 5.1: Melanoma response mechanisms to hypericin-PDT. (HYP: hypericin, AIF: apoptosis inducing factor, CASP3: caspase 3, CASP8: caspase 8, PARP1: poly(ADP-ribose)polymerase 1 and PS: phosphatidylserine).

In summary, this study demonstrated the effectiveness of hypericin-PDT in killing both unpigmented and pigmented melanoma cells by the induction of apoptosis (Fig. 5.1). Extrinsic and intrinsic caspase-dependent apoptotic modes of melanoma cell death were observed, as well as a caspase-independent apoptotic mode that did not involve apoptosis inducing factor. In addition, loss of cell membrane integrity as well as externalization of phosphatidylserines was found and it remains to be elucidated if these events are late stage apoptotic effects or accompany caspase activation.

Extensive cytoplasmic vacuolisation in both unpigmented and pigmented melanoma cells was revealed early after treatment with concomitant formation of glycogen aggregations in the latter cell type. The exact nature of these vacuoles and the possible involvement of the pigmentary system in the formation of glycogen aggregations, possibly through engagement of the enzyme glycogen synthase kinase (GSK3 β), are interesting factors for future investigations.

We furthermore showed hypericin co-localization with lysosomes and variably with melanosomes, the pigment producing organelles. We also observed cellular shrinkage accompanied by an increase in granularity/ pigmentation in the melanoma cells as well as an intracellular reduction of hypericin post PDT. Whether melanoma cells use melanosomes/ lysosomes to sequester and export hypericin from the cell and/ or increase the production of the pigment melanin to counteract PDT remains to be investigated. The use of hypericin hydroquinone is an interesting avenue to explore in this context, as it could act as a de-pigmenting agent as well as a photosensitizer, with the additional advantage of absorbance in the red spectrum offering deeper light activation in the tumour.

The involvement of the lysosomal localisation of hypericin and its potential action on cell death induction is another exciting future endeavour. Teasing out the possible involvement of the cathepsin suicidal proteases in lysosomal mediated cell death and the engagement with the apoptotic pathway will be an interesting exploration in this context.

Last but not least, translation of hypericin mediated photodynamic therapy into a three-dimensional skin model with melanoma invasion is of particular interest to us, to further simulate the natural environment of this aggressive cancer and thereby enable the identification of enhanced treatment options. For these investigations, different melanoma cell lines could be used with radial- and vertical growth phase characteristics, to furthermore test the efficacy of this photomedicine on the different stages of melanoma progression.

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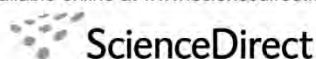
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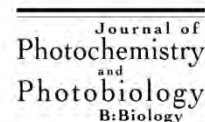
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Hypericin phototoxicity induces different modes of cell death in melanoma and human skin cells

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Abstract

Hypericin, the major component of St. John's Wort, absorbs light in the UV and visible ranges whereupon it becomes phototoxic through the production of reactive oxygen species. Although photodynamic mechanisms (i.e. through endogenous photosensitizers) play a role in UVA phototherapy for the treatment of skin disorders such as eczema and psoriasis, photodynamic therapy employing exogenous photosensitizers are currently being used only for the treatment of certain forms of non-melanoma skin cancers and actinic keratoses. There are few reports however on its use in treating melanomas. This *in vitro* study analyses the phototoxic effect of UVA (400–315 nm) – activated hypericin in human pigmented and unpigmented melanomas and immortalised keratinocytes and melanocytes. We show that neither hypericin exposure nor UV irradiation alone reduces cell viability. We show that an exposure to 1 μ M UVA-activated hypericin does not bring about cell death, while 3 μ M activated hypericin induces a necrotic mode of cell death in pigmented melanoma cells and melanocytes and an apoptotic mode of cell death in non-pigmented melanoma cells and keratinocytes. We hypothesize that the necrotic mode of cell death in the pigmented cells is possibly related to the presence of melanin-containing melanosomes in these cells and that the hypericin-induced increase in reactive oxygen species leads to an increase in permeability of melanosomes. This would result in toxic melanin precursors (of an indolic and phenolic nature) leaking into the cytoplasm which in turn leads to cell death. Hypericin localisation in the endoplasmic reticulum in these cells shown by fluorescent microscopy, further support a disruption in cellular processing and induction of cell death. In contrast, this study shows that cells that do not contain melanosomes (non-pigmented melanoma cells and keratinocytes) die by apoptosis. Further, using a mitochondrial-specific fluorescent dye, we show that intracellular accumulation of hypericin induces a mitochondrial-associated caspase-dependent apoptotic mode of cell death.

This work suggests that UVA is effective in activating hypericin and that this phototoxicity may be considered as treatment option in some cases of lentigo maligna or lentigo maligna melanoma that are too large for surgical resection.
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Keywords: Hypericin; Photodynamic therapy; Melanocyte; Keratinocyte; Melanoma; Apoptosis

1. Introduction

Photodynamic therapy (PDT) is an emerging, promising treatment modality in the field of dermatology. It involves the light-induced destruction of cells or target tissue which

has been sensitized to light by administration of a photosensitizing agent [1]. Light irradiation of the photosensitizer, in the presence of molecular oxygen, leads to the photogeneration of reactive oxygen species (ROS), which ultimately kills the target cells [2,3]. This therapy has been approved for treatment of non-melanoma skin cancers such as basal cell carcinoma (BCC) and Bowen's disease. For other non-malignant conditions such as psoriasis and acne, it remains experimental [3,4]. In general, PDT is not

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Melanomas display increased cytoprotection to hypericin-mediated cytotoxicity through the induction of autophagy

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Abstract

Photodynamic therapy (PDT) as a regime for melanoma is of limited success due to factors such as the efficacy of the photosensitizer used, penetration depth and the presence of pigment. We characterised a pigmented and an unpigmented melanoma cell line with respect to their phenotypes. Cell viability was assessed after exposure to hypericin, a UVA-activated photosensitizer. Exposure to 3 μ M activated hypericin induced a cytoprotective (autophagic) response from both cell lines. However, the pigmented cells accumulated a large amount of glycogen in their cytoplasm. We hypothesise that the treatment induces an initial cytoprotective response through autophagy, but with increased stress results in a different mode of cell death in pigmented melanoma cells from unpigmented cells. These results indicate that hypericin-PDT could be an adjuvant therapy for melanoma.

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Keywords: Hypericin; Photodynamic therapy; Melanoma; Autophagy; Apoptosis

1. Introduction

Melanoma, the leading cause of skin cancer deaths worldwide, is a highly malignant tumor that develops from transformed melanocytes (Garbe and Blum, 2001; Oppermann et al., 2005). Its potential to metastasize early leads to poor prognoses for disseminated disease (Helmbach et al., 2001). Current first-line therapy for treating melanoma is surgical resection followed by episodes of radiation and/or chemotherapy. Due to melanoma's intrinsic resistance to radiation and chemotherapeutic drugs, however, better therapeutic modalities are needed (Soengas and Lowe, 2003), one being photodynamic therapy (PDT). PDT involves the light-induced destruction of cells or target tissues which have been sensitized to light by a photosensitizing agent (Kiesslich et al., 2006). Although this therapy has been approved for treatment of non-melanoma skin cancers, such as basal cell carcinoma

(BCC), Bowen's disease and other non-malignant conditions (e.g. psoriasis and acne; Dolmans et al., 2003; Sibata et al., 2001), it is usually not recommended for the treatment of squamous cell carcinoma (SCC) and cutaneous malignant melanoma due to the relatively high recurrence rates and metastatic potential (Braathen et al., 2007).

Melanosomes are membrane-bound organelles found in melanocytes and melanoma cells that protect these cells against the harmful effects of toxic intermediates produced during melanin synthesis (Yamaguchi et al., 2005). Hypericin, a second generation photosensitizer and a member of the penanthroperylene quinone family (Fig. 1), is a major constituent of the plant *Hypericum perforatum* (St John's Wort) and acts as a potent light-activated photosensitizer in numerous *in vitro* and *in vivo* systems (Schmitt et al., 2006; Van de Putte et al., 2005). Due to its hydrophobic nature, hypericin passively crosses all cell membranes. Activating it with light, or UV-A, in the presence of molecular oxygen leads to the generation of reactive oxygen species (ROS) and these ultimately kill the cells (Milanesio et al., 2001; Dolmans et al., 2003). In addition, hypericin accumulates in the endoplasmic

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New Drugs

Combating melanoma: The use of photodynamic therapy as a novel, adjuvant therapeutic tool

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ABSTRACT

Metastatic malignant melanoma remains one of the most dreaded skin cancers worldwide. Numerous factors contribute to its resistance to hosts of treatment regimes and despite significant scientific advances over the last decade in the field of chemotherapeutics and melanocytic targets, there still remains the need for improved therapeutic modalities. Photodynamic therapy, a minimally invasive therapeutic modality has been shown to be effective in a number of oncologic and non-oncologic conditions. Using second-generation stable, lipophilic photosensitizers with optimised wavelengths, PDT may be a promising tool for adjuvant therapy in combating melanoma. Potential targets for PDT in melanoma eradication include cell proliferation inhibition, activation of cell death and reduction in pro-survival autophagy and a decrease in the cellular melanocytic antioxidant system. This review highlights the current knowledge with respect to these characteristics and suggests that PDT be considered as a good candidate for adjuvant treatment in post-resected malignant metastatic melanoma. Furthermore, it suggests that primary consideration must be given to organelle-specific destruction in melanoma specifically targeting the melanosomes – the one organelle that is specific to cells of the melanocytic lineage that houses the toxic compound, melanin. We believe that using this combined knowledge may eventually lead to an effective therapeutic tool to combat this highly intractable disease.

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Introduction

Despite extensive research and clinical trials, the prognosis and survival of metastatic melanoma remains dismal. Early detection of localized melanoma may be cured through surgery however there is no therapy for metastatic melanoma or melanoma with metastatic potential. In addition, recurrence rates of resected melanoma remain high. Because melanoma is inherently resistant to traditional forms of chemotherapy and radiotherapy,¹ various strategies have been developed for treatments which include immunotherapy e.g. interleukin-2 (IL-2),² radiotherapy³ biochemotherapy^{4–7} and gene therapy.^{2,8} A limited number of these therapies have progressed to human clinical trials but their outcomes remain negligible. One promising therapy is high-dose interferon (IFN) alpha-2b therapy which has just recently been approved as the only adjuvant therapy for melanoma approved by the US Food and Drug Administration.⁹ Despite convincing evidence of improved disease-free survival associated with this therapy, the overall survival remains negligible or very small.^{10–12} In addition, a number of melanoma-specific and melanoma-associated tumor antigens such as gp100, MART-1 and MAGE3

have been cloned¹³ and the hope is that these potential antigens may be developed to stimulate tumor-specific T cells to eliminate melanoma cells.¹⁴ Despite these advances, there remains the need for the development of novel and effective approaches to treat melanoma and this review explores the possibility of using photodynamic therapy (PDT) as an adjuvant therapy alone or in combination with current therapeutics to combat melanoma.

Grim statistics

Melanoma accounts for 4% of all dermatologic cancers but remains responsible for 80% of deaths from skin cancer with the average patient diagnosed with disseminated metastases surviving for an average of 5 years (Cancer facts and figures, 2003, Atlanta, American Cancer Society, 2003). According to the World Health Organization (WHO) melanoma skin cancer has been increasing over the past decades with a global estimation of 132,000 melanoma-related skin cancers reported to occur each year. The equivalent figures for Europe does not make better reading with current estimates at 15–20 per 100,000 people predominating in the 20–35 year old age group in Caucasians.¹⁵ South Africa, next to Australia, has one of the highest incidences of malignant melanoma in the world. Reliable statistics for South Africa are lacking, however currently an estimate figure for the South African Cape

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The Menace of Melanoma: A Photodynamic Approach to Adjunctive Cancer Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Metastatic malignant melanoma (MMM) remains one of the most dreaded skin cancers worldwide. Numerous factors contribute to its resistance to hosts of treatment regimes and despite significant scientific advances over the last decade in the field of chemotherapeutics and melanocytic targets, there still remains the need for improved therapeutic modalities. Photodynamic therapy (PDT), a minimally invasive therapeutic modality has been shown to be effective in a number of oncologic and non-oncologic conditions. Using second-generation stable, lipophilic photosensitizers with optimised activation wavelengths, PDT may be a promising tool for adjuvant therapy and even pre-treatment in combating melanoma. Potential targets for PDT in melanoma eradication include cell proliferation inhibition, activation of cell death and reduction in pro-survival autophagy, a decrease in the cellular melanocytic antioxidant system and a disruption in the endogenous multi-drug resistant (MDR) cellular machinery. This chapter highlights the current knowledge with respect to these characteristics and suggests that PDT be considered as a good candidate for adjuvant treatment in post-resected malignant metastatic melanoma. Furthermore, it suggests that primary consideration must be given to organelle-specific destruction in melanoma specifically targeting the melanosomes – the one organelle that is specific to cells of the melanocytic lineage that houses the toxic compound, melanin. We believe that using this combined knowledge may eventually lead to an effective therapeutic tool to combat this highly intractable disease.

1.1. Melanoma clinical statistics

Melanoma accounts for 4% of all dermatologic cancers but remains responsible for 80% of deaths from skin cancer with the average patient diagnosed with disseminated metastases

APPENDIX B:

ST JOHN'S WORT

FOLKLORE AND MYSTICISM

Named after Saint John the Baptist this mystical plant has been used in a myriad of ways for more than 2500 years. Its multifaceted medicinal and spiritual quintessence is vividly imprinted in human culture and has been passed on from generation to generation through stories, tales and rituals. A summary of the cultural history, folklore and mysticism of St John's Wort follows (compiled from various sources¹⁻⁴).

Saint John the Baptist is said to have prepared the Path for Christ, to have seen the Light in Christ, baptizing him. "In him was Life; and the Life was the Light of men." St John's Wort is told to be named after the Baptist as it reminded of the light radiating from him.

John's Gospel 1:15,33

1. In the beginning there was the Word, and the Word was with God, and the Word was God.

4. In him was Life; and the Life was the Light of men.

14. And the Word became Flesh, and dwelt among us.

The five-petal yellow flower resembles a crown of light, a halo, treasured as a metaphor for light in the Christian tradition for 1300 years. Terms such as *Corona regia* (Monk Vitus Auslasser 1479), *Corona regis* (Albert Magnus 1193-1280) and *Chüngus chron* (Conrad von Megenberg 1309-1374) were used by several monks in these early times. The golden yellow flowers with their stamens radiate like rays from the Sun, an image so powerful in mediaeval times that the plant acquired the name *Sol Terrestris* ("Terrestrial Sun"). It is said to be under the celestial sign Leo and the dominion of the Sun. The ray-like flowers were picked on St John's birthday the 24th of June or Midsummer Day and the Summer Solstice on the 21st June. It was burnt in ceremonial midsummer fires. The plants magical powers are believed to be strongest around these marvelous cultural days. The cherry-red juice of these flowers symbolizes the spilled blood of St John, honored on the day of the Saints be-heading the 29th August.

As a symbol of light, fire and sun, St John's Wort is believed to have the ability to counteract the forces of darkness. Tales, legends and religions all use the magic of this plant to ward off evil spirits.

The Catholic Church used it in exorcisms and thus termed it *Fuga daemonum* ("Devil's flight/chaser"). During the burning times an accused witch's mouth was stuffed with St John's Wort to force her to confess as it was believed that it stripped a witch of her power and will. Ironically it is also called 'witches blood' as legend tells that its blood-red oil was used by witches as a base in sorcery from spells to love potions and poisons. Bundles and woven garlands of the plant were carried on one's body and hung on doorframes, roofs and altars of homes, sacred sites and churches for protection against evil and lightning in many traditions.

The name *Hypericum* originates from the Greek word "*Hyperikon*" which is made of "*hyper*" meaning "over" and "*eikon*" meaning "image or apparition" and is "holding power over spirits". The name *perforatum* translates to "punctured" and refers to the many tiny dots found on the flowers and leaves of the plant, giving it a perforated appearance. Tales tell that the devil was so angry that the magical plant had such power over evil spirits and himself that he pierced the plant in his rage.

These beliefs are expressed in an old English poem:

*St. John's wort doth charm all witches away
If gathered at midnight on the saint's holy day.
Any devils and witches have no power to harm
Those that gather the plant for a charm:
Rub the lintels and post with that red juicy flower
No thunder nor tempest will then have the power
To hurt or hinder your houses: and bind
Round your neck a charm of similar kind.*

St John's Wort was also used in divination for romance and longevity and is associated with fairies. Tales tell that when flowers die they become fairies and this was especially true for St John's Wort. In the Isle of Man it is believed that the plant is sacred to fairies and if you step on a St John's Wort flower at night you will be carried away on a fairy horse and won't be allowed to rest till sunrise. In Gaelic traditions however, it was believed that the plant could break fairy spells and protect against being taken away by fairies at night. Burnt in Midsummer night fires it was believed that it would bestow the ability to see fairies.

Cultures from all over the world such as Celtic, Greek, Roman, English, French, German and Native American have used St John's Wort medicinally to cure a variety of injuries and diseases and continue to do so in today's age. Its cure-all reputation gave the plant the obscure name "tutsan" originating from the French *La toute-saine*, meaning "all-heal". The flowers are picked and infused in olive oil and left out in the sun for a few days yielding cherry-red oil. This oil is also known as the "blood of Christ". Other ways of administering the herb is to make a tea which is either taken internally or applied to the skin as a spray. It is also used in cushions and baths and as a cosmetic tonic for skin, hair and scalp. The tiny tender leaves and yellow flower petals are used in salads and stir fries. Following is a list of the many medicinal uses of St John's Wort throughout the ages.

- ♥ abortions
- ♥ arthritic pain, sprains and strains
- ♥ cramps and neuralgia
- ♥ cuts, burns and wounds
- ♥ diuretic, eliminating wastes and toxins from the body, which assists the treatment of gout and arthritis
- ♥ gastrointestinal distress including Indigestion, flatulence and bloating
- ♥ headaches and migraine
- ♥ hormonal change, menstrual disorders
- ♥ jaundice reliever and detoxifier
- ♥ liver and gall bladder decongestant
- ♥ menopausal problems
- ♥ nervous problems such as illnesses of the imagination, melancholia, disturbances of understanding, nervous anxiety and depression
- ♥ normalises stomach acid and soothes and repairs stomach ulcers
- ♥ rashes, shingles, measles and chickenpox
- ♥ seasonal affected disorder (SAD)
- ♥ snake and reptile bites
- ♥ tension, sleeplessness, decreased vitality
- ♥ varicose veins

Fascinatingly St John's Wort's connection to light continues to emerge as centuries later this magical plant is investigated for its use in a light therapy for cancer. This photomedicine for cancer is called photodynamic therapy and uses one of the extracts found in this plant, hypericin, which gives the oil its cherry-red colour.

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APPENDIX C: SOLUTIONS

TISSUE CULTURE	
Dulbecco's Modified Eagle's Medium (DMEM, pH 7.4, 1 L)	
DMEM powder	27.06 g
NaHCO ₃	7.4 g
autoclaved ddH ₂ O	qs
Adjust to pH 7.4	
Sterilise through a 0.2 µm filter and store in aliquots at 4°C	
Fetal Calf Serum (FCS, 500 ml)	
FCS	500 ml
Heat inactivate by 20 min incubation in a 56°C water bath	
Allow to cool, store aliquots at -20°C	
Penicillin (100U/ml)/ Streptomycin (100µg/ml) (100x P/S, 500 ml)	
Penicillin (1662U/mg)	3 g
Streptomycin (750U/mg)	5 g
ddH ₂ O	100 ml
Sterilise through a 0.2 µm filter and store in aliquots at -20°C	
Complete culture medium (10% FCS, 1x P/S, 400 ml)	
FCS (100%)	40 ml
P/S (100x)	4 ml
DMEM	qs
Store in aliquots at 4°C	
Phosphate Buffered Saline (1x PBS, 0.14 M NaCl, 8.8 M Na₂HPO₄, 2.7 M KCl, 1.47 M KH₂PO₄, pH 7.4, 1 L)	
NaCl	8 g
Na ₂ HPO ₄	1.26 g
KCl	0.2 g
KH ₂ PO ₄	0.2 g
ddH ₂ O	qs
Autoclave and store in aliquots at 4°C	

Trypsin/EDTA (0.05 % T/ 0.02 % E, 100 ml)	
Trypsin	0.05 g
EDTA	0.02 g
ddH ₂ O	100 ml
Sterilise through a 0.2 µm filter and store in aliquots at -20°C	
Mycoplasma test- Hoechst	
grow cells on coverslips for 7 days without antibiotics	
remove culture medium	
wash cells with 1x PBS	
fix cells with 1ml glacial acetic acid:methanol (1:3) for 6 min	
remove fixative, allow to air dry	
stain cells with 5µg/ml Hoechst for 6min	
discard Hoechst and wash cells a few times with 1x PBS	
mount coverslips onto glass slides in mowiol	
view on a fluorescent microscope	
TYROSINASE ASSAY	
Sodium Phosphate Buffer (0.1 M, pH 7.2, 100 ml)	
Sodium phosphate buffer (1 M)	10 ml
ddH ₂ O	qs
Adjust to pH 7.2 and store at room temperature	
Sodium Phosphate Buffer (1 M, 200 ml)	
NaH ₂ PO ₄ ·2H ₂ O	15.6 g
Na ₂ HPO ₄ ·12H ₂ O	35.8 g
ddH ₂ O	qs
Adjust to pH 7-7.4 and store at room temperature	
DOPA (0.25 mM, 10 ml)	
L-DOPA	0.0005 g
Sodium phosphate buffer (0.1 M)	10 ml
Store in aliquots at -20°C	
HCl (0.1 N, 1 L)	
HCl (37%)	9.9 ml
ddH ₂ O	qs
Store at room temperature	

PDT	
Hypericin (2 mM, 1 ml)	
Hypericin	1 mg
DMSO	1 ml
Prepare in sterile environment under subdued light conditions	
Store in 20µl aliquots wrapped in foil at -80°C	
ORGANELLE DYES	
Hoechst (1000 µg/ml, 1 ml)	
Hoechst (10 mg/ml)	100 µl
autoclaved ddH ₂ O	900 µl
Store in aliquots at -20°C	
Hoechst (100 µg/ml, 1 ml)	
Hoechst (1000 µg/ml)	100 µl
autoclaved ddH ₂ O	900 µl
Store in aliquots at -20°C	
Lysotracker yellow (10000nM, 100µl)	
Lysotracker yellow stock 1mM	1µl
Complete tissue culture medium	99µl
Prepare fresh in tissue culture hood, use fresh	
Lysotracker yellow (75nm, 1ml)	
Lysotracker yellow 10000nM	7.5µl
Tissue culture medium	1ml
Add directly to tissue culture dish with cells	
PROTEIN EXTRACTION	
Extraction buffer (100 ml)	
Tris HCL (1 M, pH 7.2)	10 ml
Nonidet P-40 (100%)	1 ml
SDS (10%)	100 µl
ddH ₂ O	qs
Sterilise through a 0.2 µm filter and store in aliquots at 4°C for up to 6 months	

Complete extraction buffer (1 ml)	
Extraction buffer	1 ml
Aprotinin (1 mg/ml)	1 µl
PMSF (0.1 M)	1 µl
Prepare fresh on ice	
Tris HCl (1 M, 100 ml)	
Tris	12.114 g
ddH ₂ O	qs
Adjust to pH 7.2 and store at room temperature	
Aprotinin (1 mg/ml, 1 ml)	
Aprotinin	1 mg
ddH ₂ O	1 ml
Store in aliquots at -20°C	
PMSF (0.1 M, 5 ml)	
PMSF	0.087 g
Isopropanol	5 ml
Store in aliquots at -20°C	
WESTERN BLOTTING	
Resolving gel buffer (1.5 M Tris, 0.4% SDS, pH 8.8, 200 ml)	
Tris	36.342 g
SDS	0.8 g
ddH ₂ O	qs
Heat slightly to dissolve SDS, adjust to pH 8.8 and store at 4°C	
Stacking gel buffer (0.5 M Tris, 0.4% SDS, pH 6.8, 100 ml)	
Tris	6.057 g
SDS	0.4 g
ddH ₂ O	qs
Heat slightly to dissolve SDS, adjust to pH 6.8 and store at 4°C	
Running buffer (10x, 0.25 M Tris, 1.92 M Glycine, 1% SDS, 1 L)	
Tris	30.2 g
Glycine	144 g
SDS	10 g
ddH ₂ O	qs
Store at 4°C	

Running buffer (1x, 0.025 M Tris, 0.192 M Glycine, 0.01% SDS, 1 L)	
Running buffer 1x	100 ml
ddH ₂ O	900 ml
Store at 4°C	
Transfer buffer (10x, 0.31 M Tris, 1.92 M Glycine, 1 L)	
Tris	38 g
Glycine	144 g
ddH ₂ O	qs
Store at 4°C	
Transfer buffer (1x, 0.031 M Tris, 0.192 M Glycine, 20% Methanol, 1 L)	
Transfer buffer 1x	100 ml
ddH ₂ O	700 ml
Methanol	200 ml
Store at 4°C	
Tris buffered saline (10x TBS, 0.5 M Tris, 1.5 M NaCl, pH 7.5, 1 L)	
Tris	60.5 g
NaCl	87.6 g
ddH ₂ O	qs
Adjust to pH 7.5 and store at 4°C	
Tris buffered saline- Tween (1x TBS-T, 0.05 M Tris, 0.15 M NaCl, 0.1% Tween, pH 7.5, 1 L)	
TBS 10x	100 ml
ddH ₂ O	900 ml
Tween-20	1 ml
Store at 4°C	
Loading Buffer (5x, 0.29 M Tris, 0.6% glycerol, 10% SDS, pH 6.8, 50 ml)	
Tris	1.75 g
Glycerol	30 ml
ddH ₂ O	40 ml
Adjust to pH 6.8	
SDS	5 g
ddH ₂ O	qs
Store at room temperature	
Loading dye (5x)	
Loading buffer (5x)	2 parts
2-Mercaptoethanol	1 part
Bromphenol blue (0.025% w/v)	1 part
Prepare fresh	

Bromphenol blue (0.025%, 10 ml)	
Bromphenol blue	0.0025 g
ddH ₂ O	qs
Store at 4°C	
Ponceau S (0.1% Ponceau S, 1% acetic acid, 30 ml)	
ddH ₂ O	10 ml
Acetic acid (glacial)	0.3 ml
Ponceau S	0.033 g
ddH ₂ O	qs
Store at room temperature	
Acetic acid (0.5%, 500 ml)	
Acetic acid (glacial)	50 ml
ddH ₂ O	qs
Store at room temperature	
ELECTRON MICROSCOPY	
Palade's veronal acetate buffer (500 ml)	
Sodium veronal	14.7 g
Sodium acetate	9.7 g
ddH ₂ O	qs
Store at 4°C	
Sorenson's Phosphate buffer (0.1 M, pH 7.2, 400 ml)	
NaH ₂ PO ₄ 0.2 M	144 ml
Na ₂ HPO ₄ 0.2 M	56 ml
ddH ₂ O	qs
Adjust to pH 7.2, store at 4°C	
Lead citrate (50 ml)	
Rinse the container with glacial acetic acid, tap water, distilled water and autoclaved distilled water	
Lead nitrate	1.33 g
Sodium citrate	1.76 g
ddH ₂ O	30 ml
Mix, the solution will be 'milky', let stand for 30 min	
NaOH 1 N	7 ml
Shake well, the solution will be clear	
ddH ₂ O	qs
Leave overnight before use, store at room temperature	

NaOH (1 N, 100 ml)	
NaOH pellets	4 g
ddH ₂ O	qs
Store at room temperature	
Sodium di-hydrogen orthophosphate (NaH₂PO₄, 0.2 M, 500 ml)	
NaH ₂ PO ₄	15.61 g
ddH ₂ O	qs
Store at room temperature	
Di-Sodium hydrogen orthophosphate (Na₂HPO₄, 0.2 M, 1 L)	
Na ₂ HPO ₄	28.4 g
ddH ₂ O	qs
Store at room temperature	
Uranyl acetate (100 ml)	
Rinse the container with glacial acetic acid, tap water, distilled water, autoclaved distilled water	
Uranyl acetate	6.8 g
ddH ₂ O	qs
Store at room temperature	
Glutaraldehyde in 0.1 M Sorenson's phosphate buffer (2.5%, pH 7.2-7.4, 100 ml)	
Glutaraldehyde stock solution (25%)	10 ml
Sorenson's phosphate buffer (0.1 M)	90 ml
Store at 4°C	
Osmium tetroxide in Palade's veronal acetate buffer (1%, 100 ml)	
Work in a fume cabinet	
2% Osmium tetroxide stock solution	50 ml
Palade's veronal acetate buffer	20 ml
HCl (0.1 N)	20 ml
ddH ₂ O	10 ml
Store at 4°C	
Osmium tetroxide stock solution (2%)	
Work in a fume cabinet	
Make a solution of <i>Contrad soap</i> and water in a 500ml brown reagent bottle	
Place two 0.5g capsules or one 1g capsule of osmium tetroxide in the container and leave overnight	
Rinse the bottle (with capsules still inside) with running tap water until all the soap is removed	
Without touching the capsules with bare hands, place it on a piece of paper towel and dry	
Wash the empty bottle for OsO ₄ with <i>Contrad soap</i> and rinse well with distilled water	
Etch the capsule with a diamond pen and put it in the washed bottle	

Replace the lid of the bottle before breaking the capsule to prevent fumes from escaping	
Shake the bottle to break the capsule	
Add 50 ml ddH ₂ O	
Leave overnight to dissolve	
Store at room temperature	
Spurr's epoxy resin (for 1 specimen)	
Hardener: NSA	6.5 g
Plastic: ERL	2.5 g
Plastisizer: DER	1.5 g
Accelerator: S-1	0.1 g
Prior to preparation, flame the glass vial and cool completely to eliminate all damp present in the container	
Thorough mixing of the resin is essential for uniform polymerization	
Agitate the mixture by using a magnetic stirrer for at least 3 hours before use	
Cover the solution to avoid contact with oxygen and to prevent fumes from escaping	
Preferably prepare fresh just before use	
Leftover resin can be stored in a flamed glass vial in the freezer	

APPENDIX D: REAGENTS

Name	Product number	Company
2-Mercaptoethanol	8.05740.0250	Merck
Acetic acid (glacial) 100%	SAAR1021020LC	Merck
Acetone	SAAR1022020LC	Merck
Acrylamide/bis-acrylamide (30%)	A3574	SIGMA-ALDRICH
Albumin, from bovine serum	A7906	SIGMA-ALDRICH
AMPS (ammonium persulfate)	V3131	Promega
Annexin V-FITC	556420	BD Biosciences
Apoptosis inducing factor (AIF)	A7549	Sigma
Aprotinin	10 236 624 001	Roche
BCA Protein Assay Kit	23225	Thermo Scientific Pierce
Bromphenol blue	B5525	SIGMA-ALDRICH
CaCl ₂ ·2H ₂ O (calcium chloride dihydrate)	SAAR1524920EM	Merck
Caspase-3 Cleaved (Asp175) Antibody	9661	Cell Signaling Technology
Caspase-8 (1C12) Mouse mAb	9746	Cell Signaling Technology
Cell Proliferation Kit II (XTT)	11 465 015 001	Roche
Chromatography Paper Sheets FN100	2.527.460570N	Munktell
Compensation Particles Set, Anti-Rat Ig, κ/Negative Control (FBS)	552844	BD Biosciences
Developer/replenisher	G150	AGFA
DMSO (dimethyl sulfoxide)	8.02912.1000	Merck
DOPA (3,4-Dihydroxy-L-phenylalanine)	D9628	SIGMA-ALDRICH
Dulbecco's Modified Eagle's MEM	No.P02	Highveld Biological
EDTA (ethylenediaminetetraaceticacid)	E9884	SIGMA-ALDRICH
Ethanol absolute	1.00983.2500	Merck
Fat free Instant Milk Powder		Elite
Fetal Calf Serum	No.306	Highveld Biological
FITC Anti-Human IL-2	340448	BD Biosciences
Fixing bath manual	G354	AGFA
GeneCellin	GC500	BioCellChallenge
Glass microfibre filters GF/C 25mm	1822-025	Whatman
Glycerol	2676500LC	Merck
Glycine	G7126	SIGMA-ALDRICH
Goat Anti-Mouse IgG (H + L) Horseradish Peroxidase Conjugate	170-6516	BIO-RAD
Goat Anti-Rabbit IgG (H + L) Horseradish Peroxidase Conjugate	170-6515	BIO-RAD
HCl (hydrochloric acid, 37%)	SAAR3063054LCA	Merck
HEPES	H3375	SIGMA-ALDRICH
Hoechst 33342	H1399	Invitrogen Molecular Probes

Hybond ECL Amersham	RPN203D	GE Healthcare
Hypericin from <i>Hypericum perforatum</i>	56690	SIGMA-ALDRICH
Isopropanol (propan-2-ol 2 propanol)	SAAR5075040LC	Merck
KCl (potassium chloride)	SAAR5042000EM	Merck
KH ₂ PO ₄ (potassium dihydrogen orthophosphate)	10203	BDH AnalaR
L-[U- ¹⁴ C]Tyrosine (50µCi/ml, 1.85MBq/ml)	CFB74	Amersham
LysoTracker® Yellow-HCK-123	L12491	Invitrogen Molecular Probes
Methanol	SAAR4164060LP	Merck
Microscope cover glasses 22x22mm	101050	MARIENFELD
Microscope slides	1001412	MARIENFELD
MycoAlert™ mycoplasma detection kit	LT07-318	Lonza
Na ₂ HPO ₄ (di-sodium hydrogen phosphate)	1.06586.0500	Merck
Na ₂ HPO ₄ · 12H ₂ O (di-sodium hydrogen phosphate dodecahydrate)	1.06579	Merck
NaCl (sodium chloride)	SAAR5822320EM	Merck
NaH ₂ PO ₄ · 2H ₂ O (sodium dihydrogen orthophosphate)	10245	BDH AnalaR
NaHCO ₃ (sodium hydrogen carbonate)	SAAR5822820EM	Merck
NaHPO ₄ · 12H ₂ O (di-sodium hydrogen phosphate dodecahydrate)	1.06579	Merck
Nonidet P40 Substitute	74385	SIGMA-ALDRICH
Pacific Blue® Mouse Anti-human CD3	558117	BD Biosciences
PARP-1 (H-250): sc-7150	sc-7150	Santa Cruz Biotechnology
PE Mouse Anti-Human Ki-67 Set	556027	BD Biosciences
pEGFP-pOTC		Dr LM Davids, University of Cape Town
pEGFP-rab7WT	12605	Addgene
Penicillin G sodium salt	P3032	SIGMA-ALDRICH
pEYFP-ER	6906, Clontech	Dr G Schafer, Dr C Kaschula, ICGEB, University of Cape Town
pGFP-Bcl2CB5	18000	Addgene
pGFP-MyosinVa		Prof J Lambert, University of Ghent
pGFP-Rab27a		Prof J Lambert, University of Ghent
PMSF (phenylmethylsulfonylfluoride)	P7626	SIGMA-ALDRICH
Ponceau S	P3504	SIGMA-ALDRICH
Prestained Protein Ladder	26616	Thermo Scientific Pierce
PureYield™ Plasmid Maxiprep System	A2393	Promega
Quicksafe A scintillation cocktail	1008000	Zinsser Analytic
SDS (sodium dodecyl sulfate)	L4390	SIGMA-ALDRICH
Streptomycin sulfate salt	S9137	SIGMA-ALDRICH
SuperSignal West Pico		Thermo Scientific Pierce
TEMED (N,N,N',N'-Tetramethylethylenediamine)	T9281	SIGMA-ALDRICH
TransFectin	170-3351	BIO-RAD
Tris (trizma base)	T6066	SIGMA-ALDRICH

Trypsin, from porcine pancreas	T4799	SIGMA-ALDRICH
Tween-20	P9416	SIGMA-ALDRICH
VIVID LIVE/DEAD® Fixable Violet Dead Cell Stain Kits	L34955	Invitrogen Molecular Probes
X-ray film medical CP-BU	LEEFV	AGFA

APPENDIX E: FACS GATING

Gating of fluorescent activated cell sorting (FACS) data can be done in several ways. After consulting various FACS experts the following approach by Ryan Duggan was chosen.

This 3-point gating approach can be found at these sources and the information is listed below:

<http://youtu.be/b6dKCJ0rpG4>

<http://ucflow.blogspot.com/2012/07/my-3-step-approach-to-gating-annexin-v.html>

Using flow cytometry to assess cell death, and more specifically, apoptosis, is an everyday occurrence in a flow core. Technically speaking, performing the assay couldn't be easier when utilizing numerous kits available from any number of vendors. It's typically set up in the format, add 'x' ul of reagent A to your cells, then 'x' ul of reagent B, and analyze. So, with such a simple assay, what could possibly go wrong?... Lots!

Let's assume there are no faults with setting up the instrument or parameter voltages and the like. Let's also assume the assay worked; that is, control cells looked relatively live, and treated cells looked nicely dead. The possible problem I've been coyly hinting at therefore, is in the analysis.

What's typically the first thing someone does when they analyze their flow data? They make a forward scatter (FS) versus side scatter (SS) plot and gate on the 'live' cells. Obviously, if you're trying to determine frequencies of live and dead cells, you wouldn't use a 'live' gate, you'd extend the gate lower on the FS parameter to include the dead cells. However, when you do so, you could be including small debris and bits of cells, and it's not exactly fair to count fragments of cells as events. So, herein lies the dilemma: You'd like to count whole cells, while drawing a light scatter gate that encompasses both live and dead/dying cells.

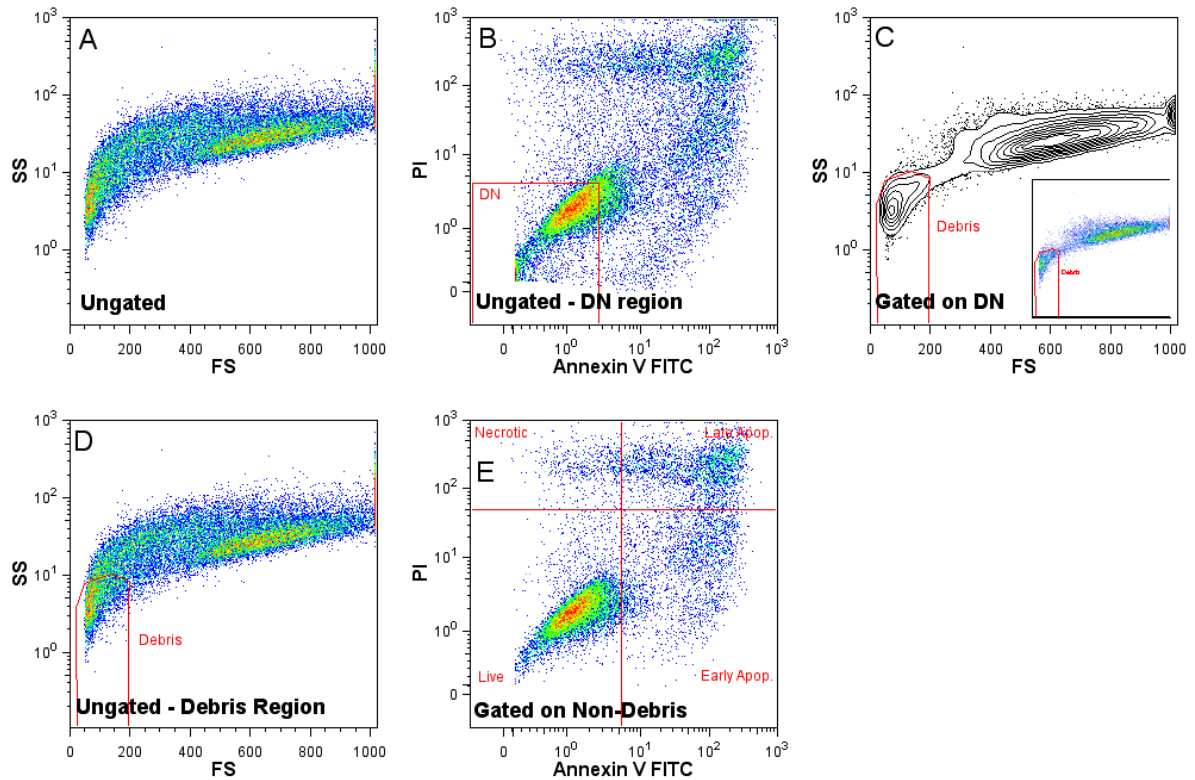
Hence, **My 3-step approach to gating Annexin V data appropriately.**

Step 1: Display your Annexin V channel versus your Membrane Permeability Channel (for ease of typing, I'll assume we're using Annexin V FITC and PI) on the entire, ungated data set, and draw a region around the double negative (DN) fraction.

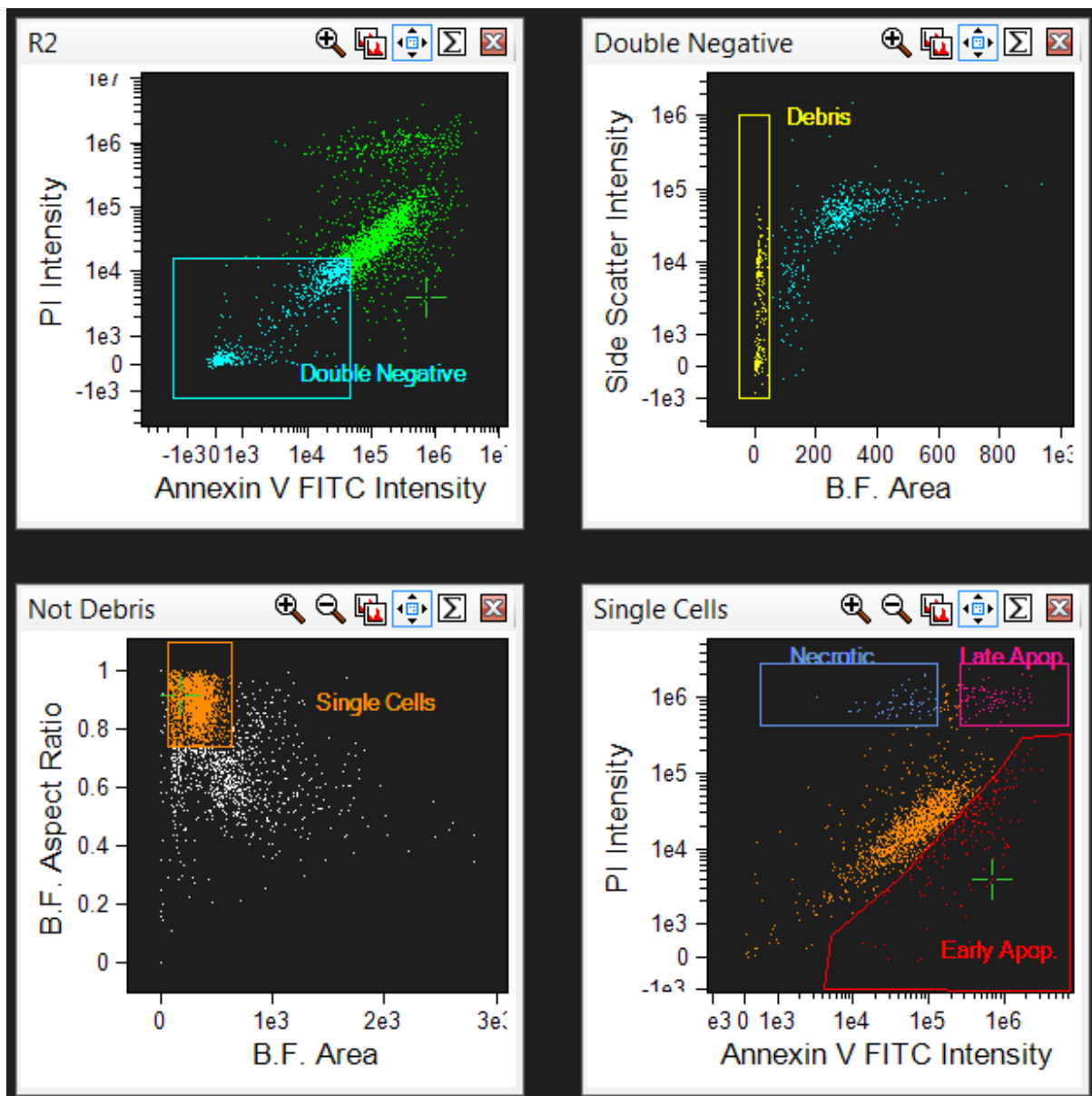
Step 2: Using the DN region from Step 1, gate a FS versus SS plot on these DN events, and draw a tight region around the subset of cells with low FS, now called Debris.

Step 3: Invert this Debris gate, creating a Not-Debris gate, and use this Not-Debris gate on the total population for your downstream Annexin V vs. PI display.

Let me break this down for you using an example. The entire point of the 3-step exercise is to properly define an area of the FS vs. SS plot that you'll call Debris, which will not be counted when generating your downstream frequencies. Depending on how much death you induce, and how toxic your treatment is, you could have a significant amount of Debris in your sample. It's important to note that you're not simply throwing away these events, you're just removing them from the downstream classification as Live (Annexin V neg, PI neg) Early Apoptotic (Annexin V pos, PI neg), Late Apoptotic (Annexin V pos, PI pos), or 'Necrotic' (Annexin V neg, PI pos). These debris events belong to none of these categories and therefore should not be included. The harm in including these debris events is that they usually end up in the Live fraction since they neither have a nucleus nor an intact membrane. Therefore, leaving them in inflates the Live fraction of cells, yielding potentially misleading results. The figure below shows the respective plots and regions.

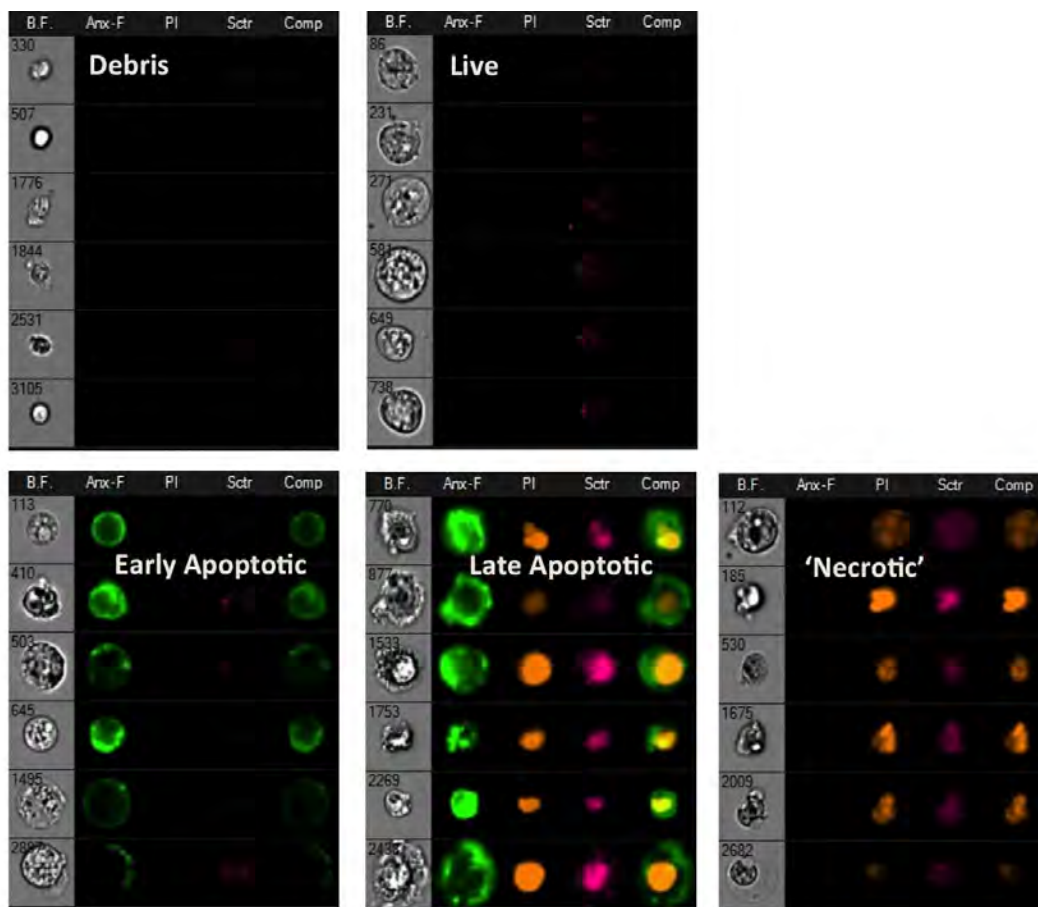


Plot A shows a FS vs. SS plot of the full data set. Plot B shows the Annexin V and PI fluorescence on the entire data set with the double negative region drawn. In C, we are showing a plot of FS vs. SS gated on the DN cells. Here we can see we're left with two distinct populations. The larger population is the true Live cells (Annexin V neg, PI neg, and large), and the other population is the debris. I use a contour plot to show a nice outline of the region that should be drawn. This region is then applied to the top level FS vs. SS plot (as in D), and is inverted. This inverted Debris gate (i.e. Not-Debris) is then applied to the Annexin V vs. PI plot and quadrants are drawn to define the 4 populations present.



Not quite convinced yet? Well, I ran a similar sample on the ImageStreamX, gated in a similar way, but now, using the imaging power of the ISX, we're able to visually confirm what each of these populations is. To the left, we see the analogous plots and regions as prescribed in the 3-step method, and below we can see a representative pool of images from each of the regions. The panels labeled Debris and Live came from the DN gate drawn, and as in the flow cytometry data, the FS vs. SS plot (here presented as Bright Field Area and Side Scatter) separates into 2 distinct populations. A larger Live cell population and a smaller Debris population. Once the Debris is removed from the downstream classification, we are left with the Live, Early/Late Apoptotic, and Necrotic fractions. Of special note is the fact that we are not simply gating out small events, as there are small events that do in fact belong to the Necrotic or Apoptotic groups (see especially event #2269 in the Late

Apoptotic panel). We're specifically gating out events that are small and have no fluorescence, which in this case, is a pretty good definition of debris.



Now obviously there are a whole bunch of caveats. This works really well for a homogeneously sized cell line, and perhaps less well for a mixed population. Also, as was briefly mentioned above, you'll need to still factor in that % Debris metric somehow. It could be your treatment makes either live cells or debris, and excluding the Debris from the Annexin V plot may show you have a healthy profile. However, it's important to track % Debris in addition to the other populations when comparing your conditions. If nothing else, though, you now have a reproducible method for gating out debris from your apoptosis analysis that could actually be explained properly in the methods section of your manuscript.

APPENDIX F: SUPPLEMENTARY DATA

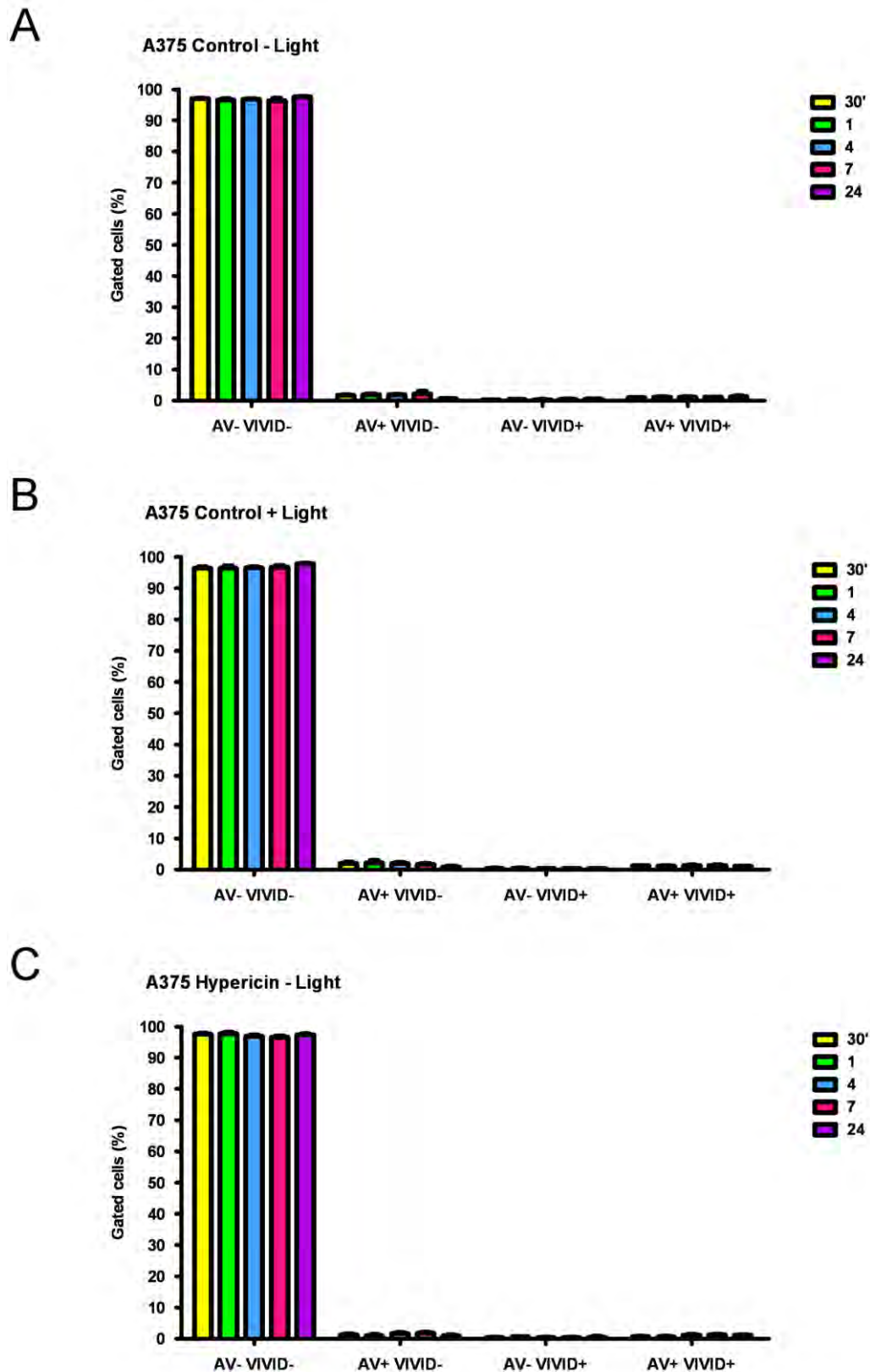


Figure A.F.1: PDT induced A375 cell death control treatments. (A) Untreated non-irradiated (B) untreated irradiated and (C) hypericin treated sham-irradiated controls at 30', 1, 4, 7 and 24h after treatment. Data is shown as percentage gated cells of 4 different populations labeled with Annexin V (phosphatidyl serine exposure) and VIVID (loss of cell membrane integrity): AV- VIVID-, AV+ VIVID-, AV- VIVID+ and AV+ VIVID+. Data is shown as mean $\pm$ SEM of gated cells ($n \geq 3$).

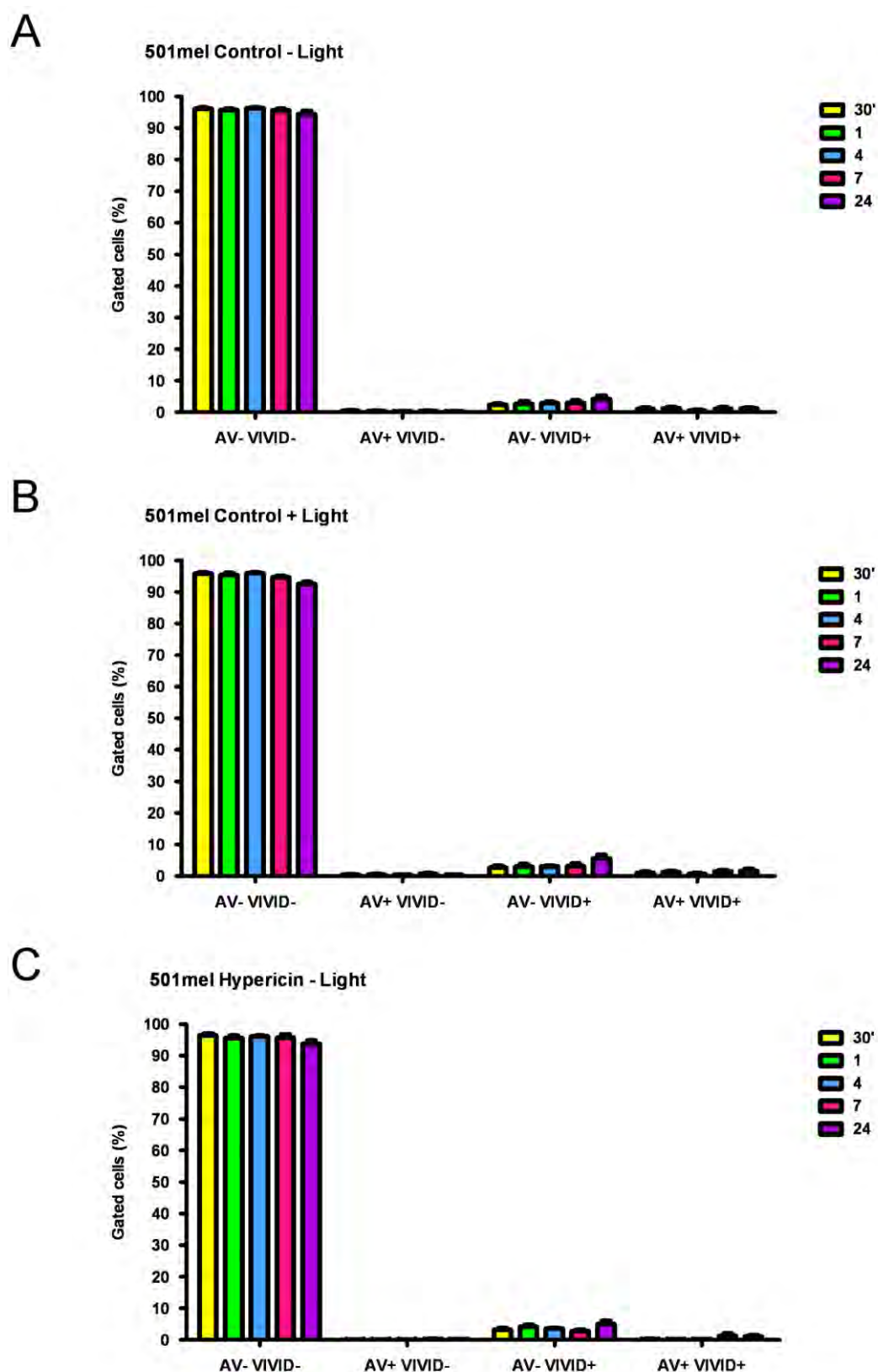


Figure A.F.2: PDT induced 501mel cell death control treatments. (A) Untreated non-irradiated (B) untreated irradiated and (C) hypericin treated sham-irradiated controls at 30', 1, 4, 7 and 24h after treatment. Data is shown as percentage gated cells of 4 different populations labeled with Annexin V (phosphatidyl serine exposure) and VIVID (loss of cell membrane integrity): AV- VIVID-, AV+ VIVID-, AV- VIVID+ and AV+ VIVID+. Data is shown as mean $\pm$ SEM of gated cells ($n \geq 3$).

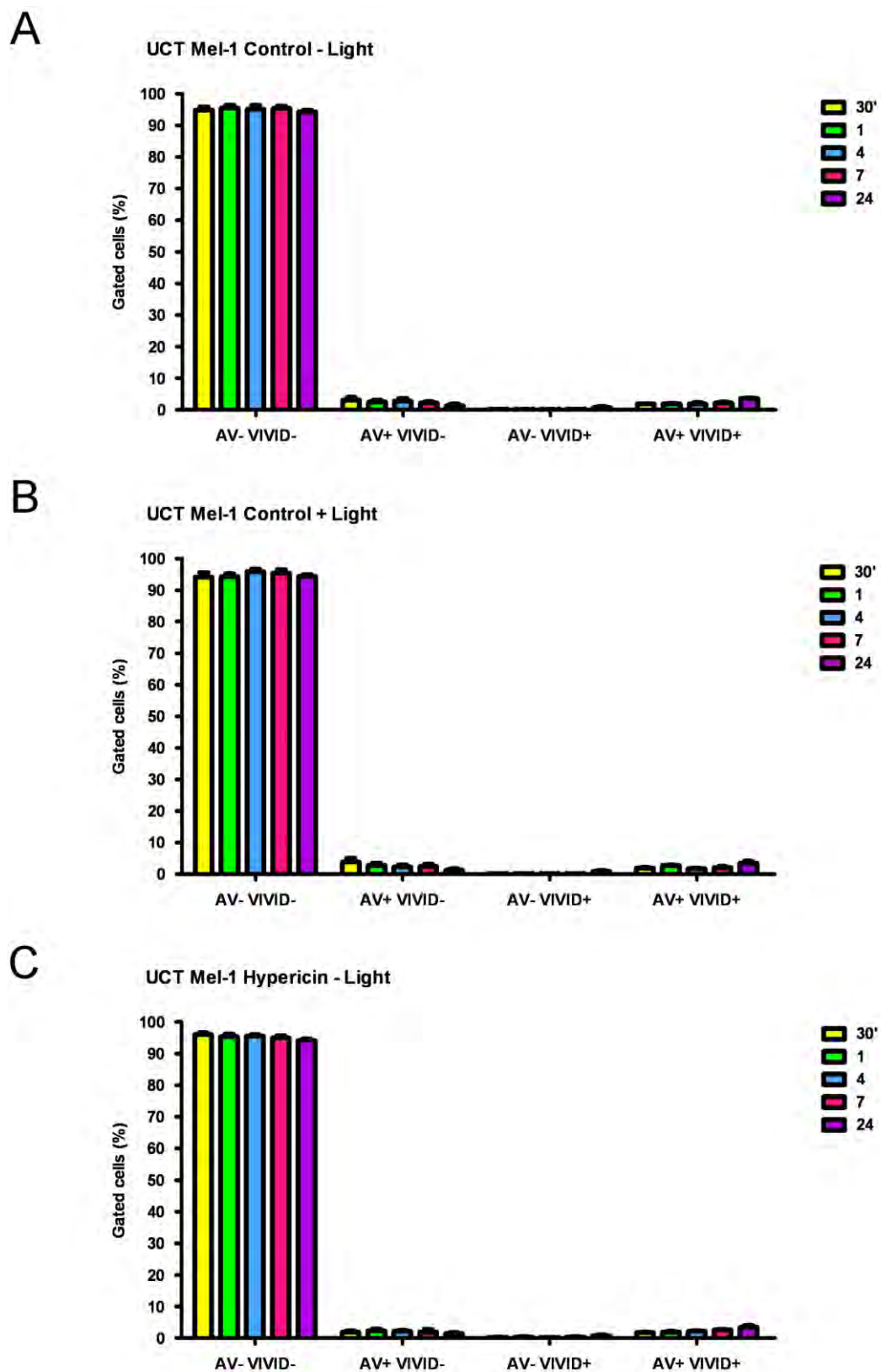
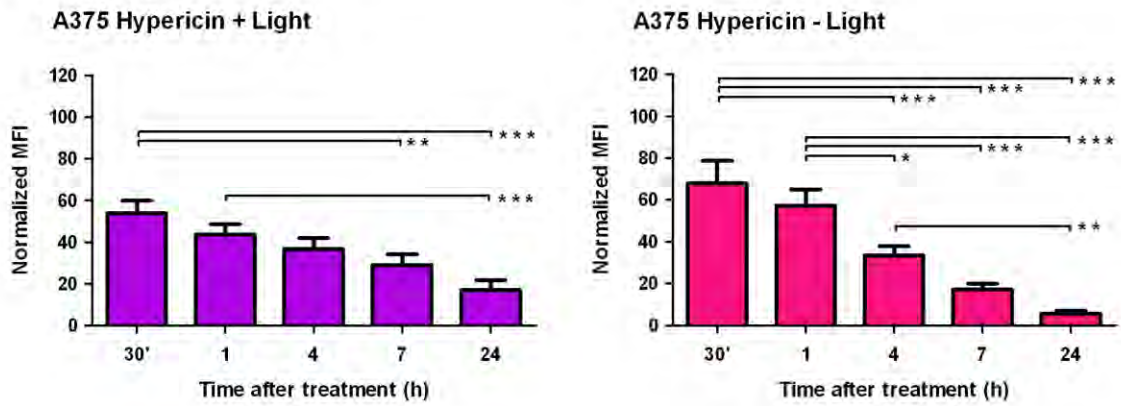
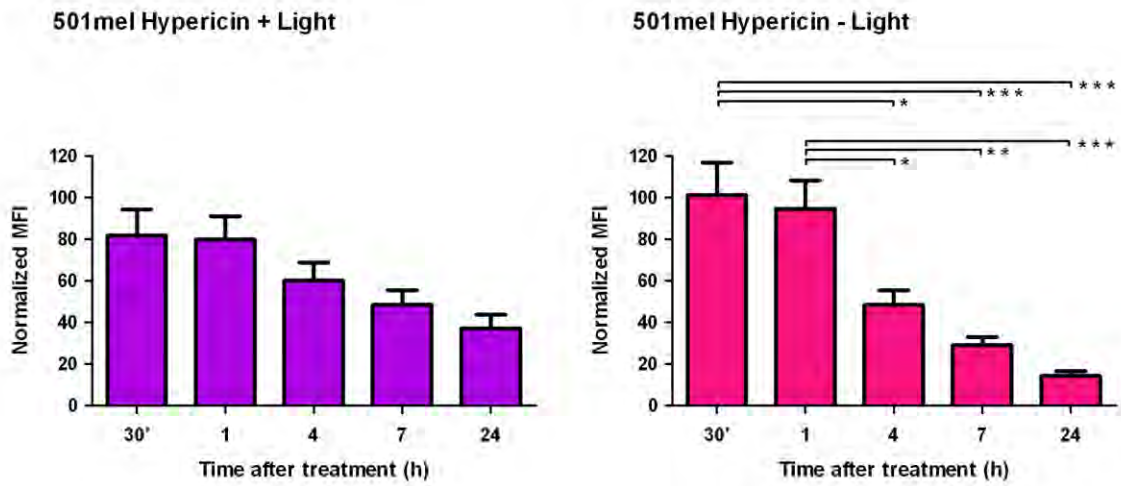


Figure A.F.3: PDT induced UCT Mel-1 cell death control treatments. (A) Untreated non-irradiated (B) untreated irradiated and (C) hypericin treated sham-irradiated controls at 30', 1, 4, 7 and 24h after treatment. Data is shown as percentage gated cells of 4 different populations labeled with Annexin V (phosphatidyl serine exposure) and VIVID (loss of cell membrane integrity): AV- VIVID-, AV+ VIVID-, AV- VIVID+ and AV+ VIVID+. Data is shown as mean $\pm$ SEM of gated cells ($n \geq 3$).

A



B



C

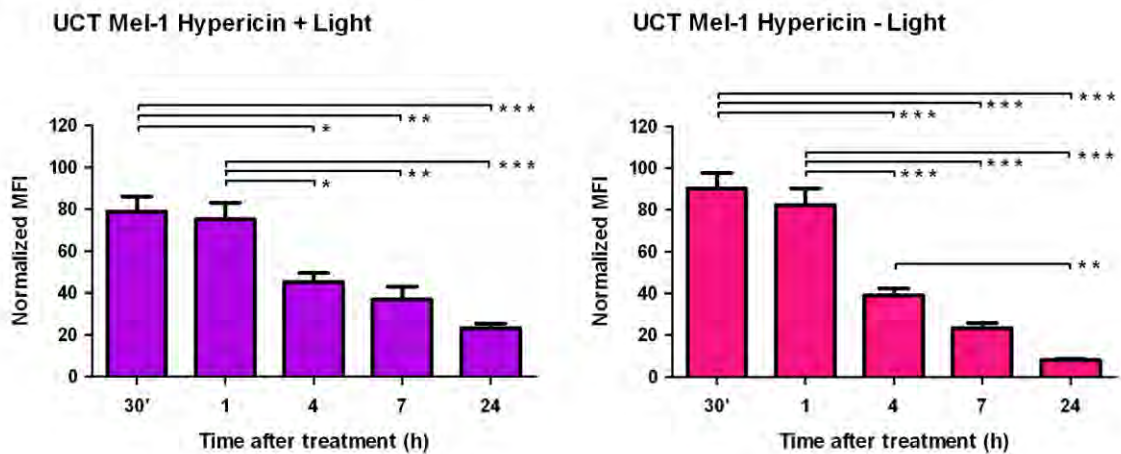


Figure A.F.4: Intracellular hypericin reduction post PDT. (A) A375, (B) 501mel and (C) UCT Mel-1 hypericin median fluorescent intensities (MFI) normalized to the untreated, sham-irradiated control at 30', 1, 4, 7 and 24h after treatment (median±SEM, n≥3, ***p<0.0001, **p<0.01, *p<0.05, L: light).

(((♥)))

May you sparkle your magick

With every step you take

May you be grateful

For

The Gift of Life

The Light of Love

The Wisdom of the Moment

The sheer Beauty of Being Alive

.

.

.

Such DeLight

Oh so Bright

Dance Dance Dance

Love Love Love

Be Be Be

YOU

.

... The End of one...

... The beginning of another...

Merry meet

And Merry part

And so we meet again

(((♥)))