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The Multifaceted Photocytotoxic Profile of Hypericin

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Abstract: Photodynamic therapy (PDT) is an established anticancer treatment employing a phototoxin (photosensitizer), visible light and oxygen. The latter is photochemically converted into reactive oxygen species, which are highly toxic to the cells. Hypericin, a natural pigment of hypericum plants, is prominent among photosensitizers. The unique perylenequinone structure of hypericin is responsible for its intriguing multifaceted photochemical cytotoxicity. The diverse photodynamic action of hypericin targets a range of subcellular organelles most importantly the mitochondria and the endoplasmic reticulum (ER)−Golgi complex. Hypericin exerts its phototoxicity through intricate mechanisms, implicating key proteins, vital enzymes, organelle membranes and changes in cellular homeostasis. This, depending on drug and light administration conditions, leads to cell death, which occurs mainly by the induction of apoptosis and/or necrosis. Cell photosensitization with hypericin is also associated with the stimulation of macroautophagy, which may promote cell demise when the apoptotic machinery is defective. Herein, we aim to integrate the most important findings with regard to hypericin photocytotoxicity, into a unified scenario, detailing its potential in cancer photomedicine.

Keywords: Hypericin; photodynamic therapy of cancer; photocytotoxicity; mitochondria; endoplasmic reticulum

Introduction

Hypericin (Figure 1) is a phenanthroperylenequinone, naturally occurring in plants of the genus *Hypericum*, especially *Hypericum perforatum*. In organic solvents and in the pH range 4−11, hypericin is only present as a monoanion thus tending to form monobasic salts;[1] in fact, in most biological assays hypericin is present as its monosodium salt. Hypericin salts produce wine-red solutions in organic solvents (absorbance λ_{max}: 548 and 591 nm in ethanol), with a characteristic red fluorescence (λ_{max}: 594 and 642 nm in ethanol), whereas they form nonfluorescent high molecular weight aggregates in aqueous solutions.[2,3] Most importantly, hypericin can strongly associate with a large variety of proteins (e.g., albumin, LDL, amyloid fibrils) and macromolecules like PVP (poly vinyl pyrrolidone), thereby forming red fluorescent monomers.[4,5]

Hypericin can be chemically synthesized by base catalyzed oxidative dimerization of emodin anthrone.[6] Recently a *H.*

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(1) Freeman, D.; Frolow, F.; Kapinus, E.; Lavie, D.; Lavie, G.; Meruelo, D.; Mazur, Y. Acidic properties of hypericin and its octahydroxy analogue in the ground and excited states. *J. Chem. Soc., Chem. Commun.* **1994**, 891–2.

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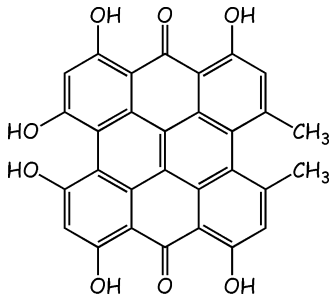

Figure 1. Chemical structure of hypericin.

perforatum gene, cloned and subsequently expressed in *Escherichia coli*, encoded for HYP-1, the enzyme catalyzing the conversion of emodin to hypericin with 84.6% efficiency.[7]

The photosensitizing properties of *Hypericum* were first recognized through studying the causes of hypericism[8] in animals. Hypericism is a state of cutaneous photosensitivity following the ingestion of very large quantities of *Hypericum* plants and exposure to sunlight. Using laboratory animals, hypericin was shown to be responsible for this photosensitization and it was further shown that oxygen was a necessary component since ischemic tissues were substantially unaffected, thus classifying it as a photodynamic process. Hypericin has also been shown to possess a profound antiviral activity[9−11] which was markedly enhanced by photoactivation, and involved protein kinase C inhibition.[12,13] This antiviral activity seemed to be specific to enveloped viruses such as herpes simplex virus, cytomegalovirus and human immunodeficiency virus (HIV).[2,11] Despite the initially promising results, a phase I clinical trial of hypericin on HIV-infected adults revealed that even high doses of hypericin (0.25 mg/kg) induced significant cutaneous phototoxicity without showing any significant antiretroviral activity.[14]

The main focus of hypericin research has been and currently is into its photocytotoxic action. Both experimental and clinical evidence concur that hypericin is a potent natural photosensitizer with great potential in photodynamic therapy[15] (PDT) especially because of its high triplet quantum yield and its efficient singlet oxygen [$^1O_2$ ($^1\Delta_g$)] and superoxide anion[16,17] generation.

In brief, PDT requires the synergy of a photoactive drug, light at the appropriate wavelength and molecular oxygen for the production of either $^1O_2$[18] by direct energy transfer from the photosensitizer triplet state or free radicals[19] following charge exchange between the photosensitizer and the substrate. Excessive production of reactive oxygen species (ROS) during PDT leads to oxidative stress and may trigger the induction of apoptosis, necrosis or autophagy-associated cell death.[20] In addition, some photosensitizers, including hypericin, exert a vascular effect, practically

(2) Lavie, G.; Mazur, Y.; Lavie, D.; Meruelo, D. The chemical and biological properties of hypericin—a compound with a broad spectrum of biological activities. *Med. Res. Rev.* **1995**, *15*, 111–9.
(3) Yamazaki, T.; Ohta, N.; Yamazaki, I.; Song, P.-S. Excited-state properties of hypericin: Electronic spectra and fluorescence decay kinetics. *J. Phys. Chem.* **1993**, *97*, 7870–5.
(4) Kubin, A.; Loew, H. G.; Burner, U.; Jessner, G.; Kolbabek, H.; Wierrani, F. How to make hypericin water-soluble. *Pharmazie* **2008**, *63*, 263–9.
(5) Sgarbossa, A.; Buselli, D.; Lenci, F. In vitro perturbation of aggregation processes in beta-amyloid peptides: a spectroscopic study. *FEBS Lett.* **2008**, *582*, 3288–92.
(6) Falk, H.; Schoppel, G. A synthesis of emodin anthrone. *Monatsh. Chem.* **1991**, *122*, 739–44.
(7) Bais, H. P.; Vepachedu, R.; Lawrence, C. B.; Stermitz, F. R.; Vivanco, J. M. Molecular and biochemical characterization of an enzyme responsible for the formation of hypericin in St. John's wort (Hypericum perforatum L. *J. Biol. Chem.* **2003**, *278*, 32413–22.
(8) Giese, A. C. Hypericism. *Photochem. Photobiol. Rev.* **1980**, *5*, 229–55.
(9) Meruelo, D.; Lavie, G.; Lavie, D. Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. *Proc. Natl. Acad. Sci U.S.A.* **1988**, *85*, 5230–4.
(10) Lavie, G.; Valentine, F.; Levin, B.; Mazur, Y.; Gallo, G.; Lavie, D.; Weiner, D.; Meruelo, D. Studies of the mechanisms of action of the antiretroviral agents hypericin and pseudohypericin. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5963–7.
(11) Lavie, G.; Mazur, Y.; Lavie, D.; Prince, A. M.; Pascual, D.; Liebes, L.; Levin, B.; Meruelo, D. Hypericin as an inactivator of infectious viruses in blood components. *Transfusion* **1995**, *35*, 392–400.
(12) Takahashi, I.; Nakanishi, S.; Kobayashi, E.; Nakano, H.; Suzuki, K.; Tamaoki, T. Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antiretroviral activity. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 1207–12.
(13) Utsumi, T.; Okuma, M.; Utsumi, T.; Kanno, T.; Yasuda, T.; Kobuchi, H.; Horton, A. A.; Utsumi, K. Light-dependent inhibition of protein kinase C and superoxide generation of neutrophils by hypericin, an antiretroviral agent. *Arch. Biochem. Biophys.* **1995**, *316*, 493–7.
(14) Gulick, R. M.; McAuliffe, V.; Holden-Wiltse, J.; Crumpacker, C.; Liebes, L.; Stein, D. S.; Meehan, P.; Hussey, S.; Forcht, J.; Valentine, F. T. Phase I studies of hypericin, the active compound in St. John's Wort, as an antiretroviral agent in HIV-infected adults. AIDS Clinical Trials Group Protocols 150 and 258. *Ann. Intern. Med.* **1999**, *130*, 510–4.
(15) Macrobert, A. J.; Theodossiou, T. Photodynamic Therapy of Cancer. In *Encyclopedia of Modern Optics*; Guenther, R. D., Ed.; Elsevier: Amsterdam, 2005; Vol. 1, pp 53−62.
(16) Diwu, Z.; Lown, J. W. Photosensitization with anticancer agents. 17. EPR studies of photodynamic action of hypericin: formation of semiquinone radical and activated oxygen species on illumination. *Free Radical Biol. Med.* **1993**, *14*, 209–15.
(17) Hadjur, C.; Richard, M. J.; Parat, M. O.; Jardon, P.; Favier, A. Photodynamic effects of hypericin on lipid peroxidation and antioxidant status in melanoma cells. *Photochem. Photobiol.* **1996**, *64*, 375–81.
(18) Fuchs, J.; Thiele, J. The role of oxygen in cutaneous photodynamic therapy. *Free Radical Biol. Med.* **1998**, *24*, 835–47.
(19) Ochsner, M. Photophysical and photobiological processes in the photodynamic therapy of tumours. *J. Photochem. Photobiol. B* **1997**, *39*, 1–18.
(20) Buytaert, E.; Dewaele, M.; Agostinis, P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim. Biophys. Acta* **2007**, *1776*, 86–107.

disrupting tumor microcirculation and resulting in both hypoxia and energy dysfunction.

Although hypericin is an attractive, natural alternative to chemically synthesized photosensitizers (mainly porphyrin-based) entering or undergoing clinical trials,[21−24] it absorbs in the green-orange spectral region where light penetration into biological tissue is limited, mainly due to hemoglobin absorption. More specifically, hypericin has an action spectrum that peaks around 595 nm, and unlike the second-generation photosensitizers (e.g., chlorins and phtalocyanines) the compound has virtually no absorption above 630 nm.[25] Attempts to shift the absorption spectrum of hypericin by chemical modification have so far met with limited success.[26]

In this context, the potential of hypericin in clinical PDT mainly lies in the treatment of superficial lesions. Ongoing clinical work is addressing the potential of hypericin as the photosensitizer of choice in bladder cancer PDT. This is because of its attested, specific accumulation in urothelial carcinoma lesions and its proven safety and efficacy as a diagnostic tool when administered intravesically.[27−30]

Based on the current literature, hypericin is a photosensitizer endowed with a multifaceted photocytotoxic profile. While several reviews have recently been published on the general molecular and cellular mechanisms instigated by PDT,[20,31−33] the aim of the present review is to assemble the existing evidence on hypericin into a unified scenario, elucidating the mechanism of action of this potent, natural phototoxin.

The Subcellular Distribution of Hypericin

Crucial parameters in determining the photocytotoxic activity of hypericin are its cell permeability and subcellular localization. It is clear that the final destination of hypericin within the cell will determine its apical molecular targets and will influence its photocytotoxic profile. This is because, within the cellular milieu, $^1O_2$ and the majority of other ROS have extremely short lives and small radii of diffusion due to their rapid interaction with biological targets; this interaction ultimately leads to modification of cellular functionality and potentially cell death.

Reports on the localization of hypericin following cellular uptake indicate a general association with lipid membranes.[34] Cell colabeling of hypericin with fluorescent dyes specific for subcellular organelles, followed by conventional or confocal fluorescence microscopy imaging and analysis, revealed that hypericin redistributes and accumulates in the membranes of various organelles, including mitochondria,[35−37] ER,[36,38−41] Golgi apparatus[36,38,39,41] and lysosomes.[35,38,40,42]

The preferential distribution of the dye among subcellular organelles in cultured cells is complex and appears to be dictated by a number of factors making a direct comparison of data from different studies difficult. For example, in DU145 cells at higher incubation concentrations of hypericin (1−5 μM) after a rapid, initial (1 h) accumulation in the mitochondria (Figure 2I), a subsequent

(21) Ethirajan, M.; Saenz, C.; Gupta, A.; Dobhal, M. P.; Pandey, R. K. Photosensitisers for photodynamic therapy imaging. In *Advances In Photodynamic Therapy: Basic, Translational And Clinical*; Hamblin, M. R., Mroz, P., Eds.; Artech House Publishers: 2008; pp 13−39.

(22) Berg, K.; Selbo, P. K.; Weyergang, A.; Dietze, A.; Prasmickaite, L.; Bonsted, A.; Engesaeter, B. O.; Angell-Petersen, E.; Warloe, T.; Frandsen, N.; Hogset, A. Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J. Microsc.* **2005**, *218*, 133–47.

(23) Peng, Q.; Berg, K.; Moan, J.; Kongshaug, M.; Nesland, J. M. 5-Aminolevulinic acid-based photodynamic therapy: principles and experimental research. *Photochem. Photobiol.* **1997**, *65*, 235–51.

(24) Wainwright, M. Photodynamic therapy: the development of new photosensitisers. *Anticancer Agents Med. Chem.* **2008**, *8*, 280–91.

(25) Chen, B.; Xu, Y.; Roskams, T.; Delaey, E.; Agostinis, P.; Vandenheede, J. R.; de Witte, P. Efficacy of antitumoral photodynamic therapy with hypericin: relationship between biodistribution and photodynamic effects in the RIF-1 mouse tumor model. *Int. J. Cancer* **2001**, *93*, 275–82.

(26) Roelants, M.; Lackner, B.; Waser, M.; Falk, H.; Agostinis, P.; Van Poppel, H.; De Witte, P. In vitro study of the photocytotoxicity of bathochromically-shifted hypericin derivatives. *Photochem. Photobiol. Sci.* **2009**, DOI: 10.1039/b820817d.

(27) D'Hallewin, M. A.; Kamuhabwa, A. R.; Roskams, T.; De Witte, P. A.; Baert, L. Hypericin-based fluorescence diagnosis of bladder carcinoma. *BJU Int.* **2002**, *89*, 760–3.

(28) D'Hallewin, M. A.; De Witte, P. A.; Waelkens, E.; Merlevede, W.; Baert, L. Fluorescence detection of flat bladder carcinoma in situ after intravesical instillation of hypericin. *J. Urol.* **2000**, *164*, 349–51.

(29) Sim, H. G.; Lau, W. K.; Olivo, M.; Tan, P. H.; Cheng, C. W. Is photodynamic diagnosis using hypericin better than white-light cystoscopy for detecting superficial bladder carcinoma. *BJU Int.* **2005**, *95*, 1215–8.

(30) Kubin, A.; Meissner, P.; Wierrani, F.; Burner, U.; Bodenteich, A.; Pytel, A.; Schmeller, N. Fluorescence diagnosis of bladder cancer with new water soluble hypericin bound to polyvinylpyrrolidone: PVP-hypericin. *Photochem. Photobiol.* **2008**, *84*, 1560–3.

(31) Oleinick, N. L.; Morris, R. L.; Belichenko, I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem. Photobiol. Sci.* **2002**, *1*, 1–21.

(32) Almeida, R. D.; Manadas, B. J.; Carvalho, A. P.; Duarte, C. B. Intracellular signaling mechanisms in photodynamic therapy. *Biochim. Biophys. Acta* **2004**, *1704*, 59–86.

(33) Moor, A. C. Signaling pathways in cell death and survival after photodynamic therapy. *J Photochem. Photobiol. B* **2000**, *57*, 1–13.

(34) English, D. S.; Doyle, R. T.; Petrich, J. W.; Haydon, P. G. Subcellular distributions and excited-state processes of hypericin in neurons. *Photochem. Photobiol.* **1999**, *69*, 301–5.

(35) Ali, S. M.; Olivo, M. Bio-distribution and subcellular localization of Hypericin and its role in PDT induced apoptosis in cancer cells. *Int. J. Oncol.* **2002**, *21*, 531–40.

(36) Galanou, M. C.; Theodossiou, T. A.; Tsiourvas, D.; Sideratou, Z.; Paleos, C. M. Interactive transport, subcellular relocation and enhanced phototoxicity of hypericin encapsulated in guanidinylated liposomes via molecular recognition. *Photochem. Photobiol.* **2008**, *84*, 1073–83.

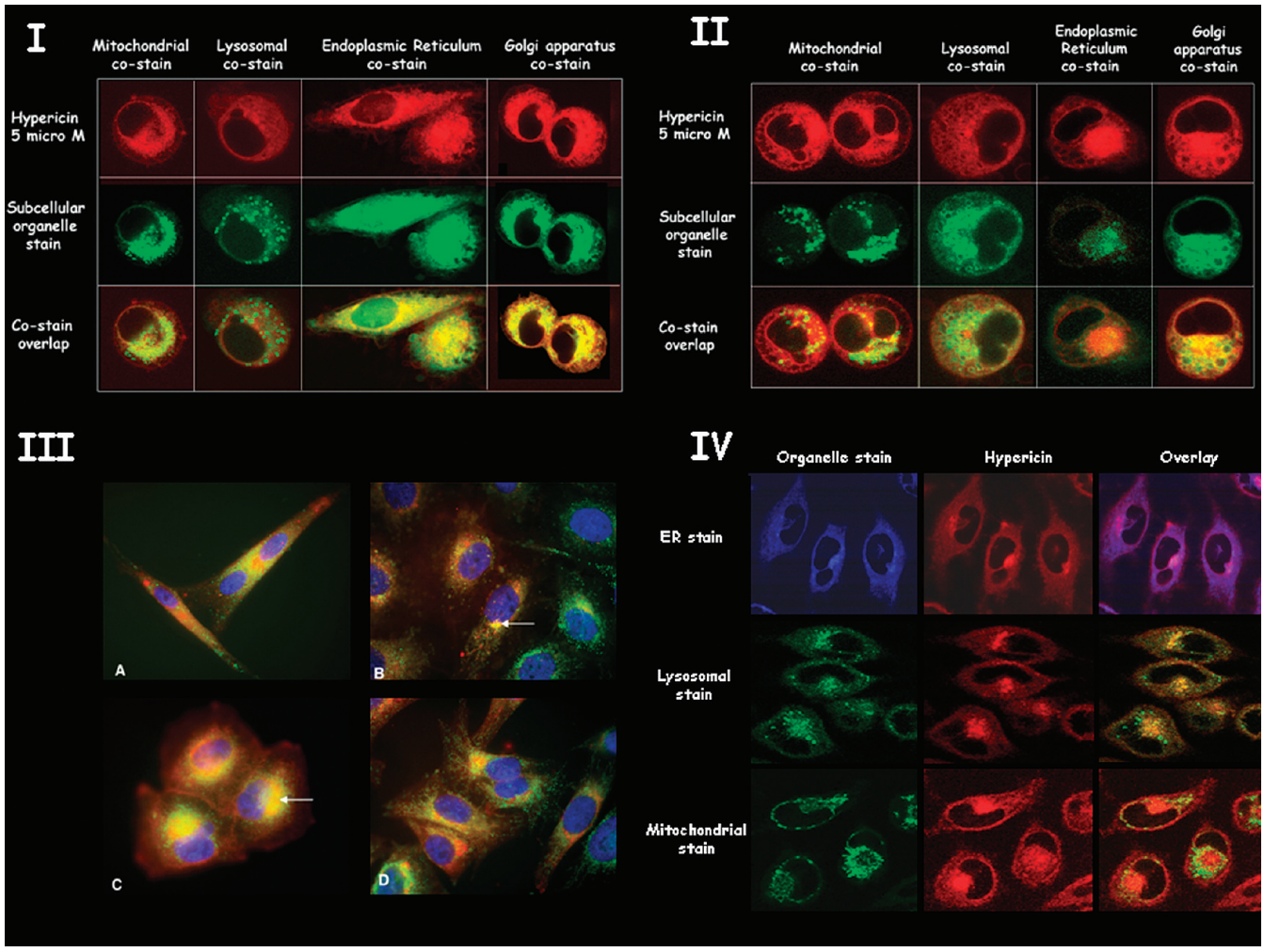

Figure 2. Subcellular localization studies on hypericin. Confocal fluorescence microscopy on DU145 human prostate carcinoma cells incubated with 5 μM hypericin for 1 h (I) and 5 h (II).The cells in I and II were costained with a mitochondria specific fluorescence probe (MitoTracker Green FM), a lysosome specific probe (LysoTracker Green DND-26), an ER specific probe (ER-Tracker Green) and a Golgi specific probe (Bodipy FL C5-ceramide). All subcellular organelle probes were added 1 h prior to imaging. The images in I and II were originally published in Galanou et al. Interactive Transport, Subcellular Relocation and Enhanced Phototoxicity of Hypericin Encapsulated in Guanidinylated Liposomes via Molecular Recognition. *Photochem. Photobiol.* **2008**, *84*, 1073–83. (III) Hypericin (red) was colocalized with MitoTracker fluorescent dye (green) in (A) melanocytes; (B) keratinocytes; (C) UCT Mel-3; (D) UCT Mel-1. White arrows indicate the perinuclear colocalization pattern. The images in III were reprinted from *J. Photochem. Photobiol., B* **2008**, *91*, 67–76 with permission from Elsevier. (IV) Hypericin localizes to ER and lysosomes in HeLa cells. Confocal microscopic analysis of HeLa cells incubated for 16 h with hypericin (500 nM, red). The panels show staining with the ER-specific probe ER-Tracker Blue-White DPX (500 nM, blue), the lysosomal-specific probe LysoTracker Green DND-26 (1 μM, green), and the mitochondrial dye Rhodamine 123 (500 nM, green). Merged fluorescence (purple for blue and red and yellow for green and red) indicating colocalization is shown on the right. The images in IV were reprinted from *FASEB J.* **2006**, *20*, 756–8 (full text version) with permission from FASEB J.

(5 h) redistribution toward the ER and Golgi membranes was observed (Figure 2II).

Elsewhere,[37] hypericin (3 μM) accumulated in mitochondria of human cultured keratinocytes, whereas at the same incubation concentration and incubation time, a predominant ER and Golgi localization was observed in melanocytes (Figure 2III).

(37) Davids, L. M.; Kleemann, B.; Kacerovska, D.; Pizinger, K.; Kidson, S. H. Hypericin phototoxicity induces different modes of cell death in melanoma and human skin cells. *J. Photochem. Photobiol B* **2008**, *91*, 67–76.

(38) Buytaert, E.; Callewaert, G.; Hendrickx, N.; Scorrano, L.; Hartmann, D.; Missiaen, L.; Vandenheede, J. R.; Heirman, I.; Grooten, J.; Agostinis, P. Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. *FASEB J.* **2006**, *20*, 756–8.

At lower incubation concentrations (0.1−0.2 μM) hypericin initially flooded into the plasma membrane (1−2 h) and subsequently (5−16 h) redistributed into ER and Golgi apparatus membranes, and to a lesser extent to lysosomes[38] (Figure 2IV).

Incubation of cells with hypericin in the presence of serum proteins has been found to favor preferential binding of hypericin to either LDL or HDL lipoproteins, depending on their relative abundance.[25] Using cultured murine colon carcinoma CT26 or human glioma U87 MG cells, LDL was shown to be not only an important hypericin carrier but also a mediator of receptor-dependent internalization and subsequent lysosomal accumulation of the dye.[40,43] However, acetylated LDL (preserving LDL structure but not recognized by LDL receptors) when used as hypericin carrier in TCC RT-112 cells resulted in an unchanged uptake in comparison to LDL.[44] This observation combined with the fact that hypericin preferentially accumulates in organelles other than lysosomes suggested that LDL receptor-mediated endocytosis is not the only internalization mechanism. This endocytotic route arguably prevails in cancer cells expressing high levels of LDL surface receptors.

In a very recent report, cholesterol was found to serve as a molecular determinant for the uptake of hypericin into cellular membranes.[45] Cholesterol and hypericin feature a common rigid planar configuration and are believed to form a molecular complex promoting initial hypericin buildup in cholesterol-rich domains. Hence, hypericin may be taken up by partitioning between LDL (or HDL) particles and cholesterol-rich lipid domains i.e. cell membranes. Preferential distribution of the dye among subcellular organelles is thus determined by vesicular or nonvesicular transport known to regulate endosomal cholesterol traffic.[46] Vesicular intracellular trafficking of hypericin is supported by a study showing that brefeldin A, an inhibitor of membrane cycling between the ER and Golgi, counteracted the intracellular accumulation of hypericin in cultured cells.[47]

Moreover, partial diffusion and redistribution of hypericin from mitochondrial to the neighboring ER membranes could be facilitated through intermembrane cholesterol−hypericin complex trafficking. The varying size, shape and layout of the mitochondrial network, as well as its proximity and extent of contact with the ER, could account for different times of retention of hypericin in the mitochondria before redistribution to the ER.

Irrespective of the exact mechanism involved, which requires further clarification, the overall consensus emerging from the currently available studies is that three important intracellular target sites are prevalent for hypericin, namely, the mitochondria, the ER (and ER−Golgi network) and to a lesser extent the lysosomes.

ROS production, perturbation of organellar functions, and crucial cytotoxic mechanisms resulting from light activation of hypericin localized at the above-designated intracellular loci will be comprehensively discussed below.

Hypericin and ROS Production

In principle a type II oxygen-dependent photosensitization mechanism generating singlet oxygen ($^1O_2$) was postulated to explain the hypericin photodynamic efficacy, via events resulting in lipid peroxidation, amino acid and protein photomodification, disruption of membrane function and inhibition of viral activity. The generation of $^1O_2$ ($^1\Delta_g$) from the lowest excited triplet state of hypericin was verified quite early by laser spectroscopy.[48,49]

It is clear from all published work on hypericin that $^1O_2$ plays a central role in the associated phototoxicity. Indeed, the $^1O_2$ quantum yield of hypericin in methanol or hypericin

(39) Ritz, R.; Roser, F.; Radomski, N.; Strauss, W. S.; Tatagiba, M.; Gharabaghi, A. Subcellular colocalization of hypericin with respect to endoplasmic reticulum and Golgi apparatus in glioblastoma cells. *Anticancer Res.* **2008**, *28*, 2033–8.

(40) Siboni, G.; Weitman, H.; Freeman, D.; Mazur, Y.; Malik, Z.; Ehrenberg, B. The correlation between hydrophilicity of hypericins and helianthrone: internalization mechanisms, subcellular distribution and photodynamic action in colon carcinoma cells. *Photochem. Photobiol. Sci.* **2002**, *1*, 483–91.

(41) Uzdensky, A. B.; Ma, L. W.; Iani, V.; Hjortland, G. O.; Steen, H. B.; Moan, J. Intracellular localisation of hypericin in human glioblastoma and carcinoma cell lines. *Lasers Med. Sci.* **2001**, *16*, 276–83.

(42) Theodossiou, T.; Spiro, M. D.; Jacobson, J.; Hothersall, J. S.; Macrobert, A. J. Evidence for intracellular aggregation of hypericin and the impact on its photocytotoxicity in PAM 212 murine keratinocytes. *Photochem. Photobiol.* **2004**, *80*, 438–43.

(43) Kascakova, S.; Nadova, Z.; Mateasik, A.; Mikes, J.; Huntosova, V.; Refregiers, M.; Sureau, F.; Maurizot, J. C.; Miskovsky, P.; Jancura, D. High level of low-density lipoprotein receptors enhance hypericin uptake by U-87 MG cells in the presence of LDL. *Photochem. Photobiol.* **2008**, *84*, 120–7.

(44) Crnolatac, I.; Huygens, A.; Agostinis, P.; Kamuhabwa, A. R.; Maes, J.; van Aerschot, A.; De Witte, P. A. In vitro accumulation and permeation of hypericin and lipophilic analogues in 2-D and 3-D cellular systems. *Int. J. Oncol.* **2007**, *30*, 319–24.

(45) Ho, Y. F.; Wu, M. H.; Cheng, B. H.; Chen, Y. W.; Shih, M. C. Lipid-mediated Preferential Localization of Hypericin in Lipid Membranes. *Biochim. Biophys. Acta* **2009**, *1788* (6), 1287–95.

(46) Holtta-Vuori, M.; Ikonen, E. Endosomal cholesterol traffic: vesicular and non-vesicular mechanisms meet. *Biochem. Soc. Trans.* **2006**, *34*, 392–4.

(47) Weber, N. D.; Murray, B. K.; North, J. A.; Wood, S. G. The antiviral agent hypericin has in vitro activity against HSV-1 through non-specific association with viral and cellular membranes. *Antiviral Chem. Chemother.* **1994**, *5*, 83–90.

(48) Jardon, P.; Lazortchak, N.; Gautron, R. Formation d'oxygene singulet $1\Delta_g$ photosensibilisee par l'hypericine. Characterisation et etude du mechanisme par spectroscopie laser. *J. Chim. Phys.* **1987**, *84*, 1143–1145.

(49) Racinet, H.; Jardon, P.; R., G. Formation d'oxygene singulet $^1\Delta_g$ photosensibilisee par l'hypericine. Etude cinetique en millieu micellaire non ionique. *J. Chim. Phys.* **1988**, *85*, 971–977.

bound to the bilayer of L-α-lecithin liposomes was found to be substantial ($\Phi\Delta$ ~0.35 and 0.39, respectively).[50]

In this context, hypericin derived $^1O_2$ has so far been implicated in

1. the photodestruction of the sarco/endoplasmic reticulum Ca^{2+}-ATPase-2 (SERCA2) pumps[38] leading to disruption of Ca^{2+} homeostasis in the cell and most probably also triggering the unfolded protein response (UPR, *vide infra*);
2. inhibition or depletion of mitochondrial enzymes such as succinoxidase[51] and aconitase,[52] as well as detachment of mitochondria-bound hexokinase[53] (*vide infra*);
3. the downregulation of metalloproteinase-9 expression in nasopharyngeal cancer cells,[54] quite possibly by inhibition of the oxidative stress responsive transcriptional activity of NF-κB; and
4. lipid peroxidation of cell membranes as demonstrated in melanoma cells[17] followed by catalase, glutathione peroxidase and SOD increase and glutathione (GSH) depletion.

In addition to $^1O_2$, other ROS have been reported to form downstream of hypericin photoactivation. More specifically H_2O_2 generation has been reported within an hour of hypericin photosensitization followed by GSH depletion.[55]

Although some of the above reports[17,53,55] present data on GSH depletion following hypericin photoactivation, this seems to be a rather late event secondary to the first burst of $^1O_2$ production. These are mostly related to free radical formation (hydrogen and other peroxides e.g. following lipid peroxidation), as measurement of total GSH immediately following irradiation in DU145 human prostate carcinoma cells did not show any significant decrease.[52] Moreover, in the same work,[52] cells depleted of GSH [using buthionine sulfoxide (BSO)] exhibited phototoxicity responses comparable to control cells with physiological GSH levels, somewhat downgrading the role of peroxides in hypericin mediated photocytotoxicity.

One important reactive intermediate related to hypericin phototoxicity, however, seems to be the photogenerated semiquinone radical. The formation of hypericin semiquinone radical anion following photoactivation has been shown using electron paramagnetic resonance (EPR),[16,56,57] while the presence of electron donors significantly enhances this photogeneration.[56] In corroboration of these results, we recently demonstrated, using specific mitochondrial inhibitors, that the target site of hypericin photodamage in the mitochondria was the electron transport chain (ETC) at complex III.[58] More precisely the focus of damage was identified at the quinone reducing center (Q_i) of complex III, since antimycin A (inhibitor of complex III at Q_i) protected cells from hypericin phototoxicity while myxothiazol [complex III inhibitor at the quinol oxidizing center (Q_o)] conferred no measurable protection.[58] This suggests involvement of the excited state hypericin semiquinone radical, since the latter would preferentially be generated at Q_i, due to the enhanced stability of the semiquinone, resulting from efficient electron donation at that site.[59] If $^1O_2$ were implicated in complex III impairment, photodamage at Q_o would be reversed by myxothiazol, which, as stated above, was not the case. It should be noted that, through enzyme activity measurements in submitochondrial particles, hypericin was shown to be an efficient substrate of complex III efficiently reducing cytochrome *c*[58] for which hypericin docking at Q_o is a prerequisite. The possible interactions of hypericin at ETC complex III leading to generation of free radicals and reactive intermediates are illustrated in the schematic diagram of Figure 3.

Formation of the superoxide anion has been reported following hypericin photoactivation, as demonstrated by EPR studies.[16] Superoxide anion radical is generated (to a lesser extent than $^1O_2$) from oxygen reduction by the semiquinone radical. Although some studies[17] have presented elevated SOD levels consistent with increased superoxide anion dismutation demands, our own measurements, performed immediately following irradiation, indicate comparable SOD

(50) Roslaniec, M.; Weitman, H.; Freeman, D.; Mazur, Y.; Ehrenberg, B. Liposome binding constants and singlet oxygen quantum yields of hypericin, tetrahydroxy helianthrone and their derivatives: studies in organic solutions and in liposomes. *J. Photochem. Photobiol. B* **2000**, *57*, 149–58.

(51) Thomas, C.; MacGill, R. S.; Miller, G. C.; Pardini, R. S. Photoactivation of hypericin generates singlet oxygen in mitochondria and inhibits succinoxidase. *Photochem. Photobiol.* **1992**, *55*, 47–53.

(52) Theodossiou, T. A.; Noronha-Dutra, A.; Hothersall, J. S. Mitochondria are a primary target of hypericin phototoxicity: synergy of intracellular calcium mobilisation in cell killing. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 1946–56.

(53) Miccoli, L.; Beurdeley-Thomas, A.; De Pinieux, G.; Sureau, F.; Oudard, S.; Dutrillaux, B.; Poupon, M. F. Light-induced photoactivation of hypericin affects the energy metabolism of human glioma cells by inhibiting hexokinase bound to mitochondria. *Cancer Res.* **1998**, *58*, 5777–86.

(54) Du, H. Y.; Olivo, M.; Mahendran, R.; Huang, Q.; Shen, H. M.; Ong, C. N.; Bay, B. H. Hypericin photoactivation triggers downregulation of matrix metalloproteinase-9 expression in well-differentiated human nasopharyngeal cancer cells. *Cell. Mol. Life Sci.* **2007**, *64*, 979–88.

(55) Ali, S. M.; Chee, S. K.; Yuen, G. Y.; Olivo, M. Hypocrellins and Hypericin induced apoptosis in human tumor cells: a possible role of hydrogen peroxide. *Int. J. Mol. Med.* **2002**, *9*, 461–72.

(56) Rahimipour, S.; Palivan, C.; Barbosa, F.; Bilkis, I.; Koch, Y.; Weiner, L.; Fridkin, M.; Mazur, Y.; Gescheidt, G. Chemical and photochemical electron transfer of new helianthrone derivatives: aspects of their photodynamic activity. *J. Am. Chem. Soc.* **2003**, *125*, 1376–84.

(57) Rahimipour, S.; Palivan, C.; Freeman, D.; Barbosa, F.; Fridkin, M.; Weiner, L.; Mazur, Y.; Gescheidt, G. Hypericin derivatives: substituent effects on radical-anion formation. *Photochem. Photobiol.* **2001**, *74*, 149–56.

(58) Theodossiou, T. A.; Papakyriakou, A.; Hothersall, J. S. Molecular modeling and experimental evidence for hypericin as a substrate for mitochondrial complex III; mitochondrial photodamage as demonstrated using specific inhibitors. *Free Radical Biol. Med.* **2008**, *45*, 1581–90.

(59) Osyczka, A.; Moser, C. C.; Dutton, P. L. Fixing the Q cycle. *Trends Biochem. Sci.* **2005**, *30*, 176–82.

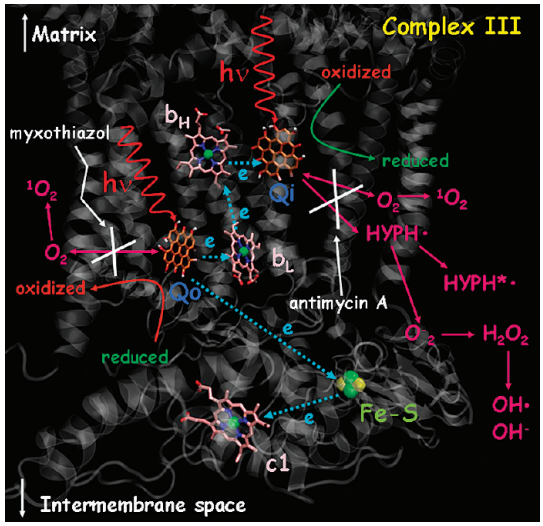

Figure 3. Diagram based on computer modeling showing the possible interactions of hypericin (HYP) as a substrate of complex III leading to formation of free radicals and reactive intermediates. Qo and Qi are the quinol oxidizing and quinone reducing centers of complex III. Hemes b_{566} (b_H), b_{562} (b_L), and c1 are shown in pink. HYP molecules occupying the Qi and Qo sites are shown in orange. Electron transport in the complex is represented with cyan dotted lines. The Fe_2-S_2 mobile Rieske center is shown in yellow-green. On light activation HYP can (a) generate singlet oxygen and (b) form the semiquinone radical which can (i) inflict direct damage as an excited state species or (ii) lead to the generation of oxidative species such as superoxide anion, H_2O_2, and subsequently hydroxyl radicals. It is very likely that the latter processes [(b)] are favored at the Qi site due to the stability of the semiquinone at that site and are inhibited by antimycin through displacement of HYP from the Qi site.

levels between hypericin photosensitized and control cells.[52] This of course could be due to the competition between $^1O_2$ and superoxide anion (semiquinone radical) formation which greatly depends on the microenvironment/experimental conditions.[16] Alternatively, it could be due to semiquinone radical interaction with target substrates (causing photodamage) rather than with oxygen (leading to superoxide anion formation). There is of course also the well documented possibility of superoxide anion generation following electron leakage in the ETC of mitochondria damaged from hypericin photosensitization. However, this generation is expected to be a delayed effect.

Mitochondrial Targets of Hypericin Phototoxicity

Historically, mitochondria have been considered critical organelles of eukaryotic cells where the energy derived from the oxidation of reducing substrates is converted to ATP via oxidative phosphorylation. In noninflammatory cells, mitochondria are also the main source of intracellular ROS and therefore are major mediators of ROS signaling in the cells. More recently their importance in controlling the response to cell death signals has been established.[60] Both oxidative phosphorylation and ROS signaling involve the shuttling of electrons along inner membrane complexes via multiple transfer sites to provide and sustain a proton (H^+) gradient between the mitochondrial intermembrane space and the matrix. This proton gradient, expressed as mitochondrial membrane potential ($\Delta\Psi_m$), is dissipated in a controlled fashion by F_1F_0-ATP synthase, using oxygen as a terminal electron acceptor to drive ADP oxidative phosphorylation to ATP.

Photoactivation of hypericin in the glioblastoma cell line SNB-19[53] was found to mediate the dissociation of hexokinase (HK) from the mitochondria. This concentration and light-dependent release of mitochondrial HK, downstream of an intracellular pH decrease (0.6−0.8 pH unit), was accompanied by an increase in cytoplasmic HK activity. Parallel measurement of the intracellular ATP, following treatment with 5 μM hypericin and light, showed a marked decrease in ATP content. Binding of HK to the mitochondria occurs through the association with porin or the voltage-dependent anion channel (VDAC), located in the outer mitochondrial membrane. Irrespective of the yet unclear photoinduced mechanism, VDAC−HK dissociation following hypericin photosensitization is expected to have a severe impact on the energy metabolism of cancer cells. HK catalyzes the phosphorylation of glucose to glucose-6-phosphate in the glycolytic pathway, and its increased expression in many cancers is thought to support the glycolytic phenotype (Warburg effect), a typical hallmark of cancer cells.[61] Furthermore, HK bound to mitochondria is thought to have an important role in the control of apoptosis (*vide infra*). Thus, VDAC−HK interaction is emerging as a crucial target for anticancer drugs, which aim to block cancer progression through interfering with the glycolytic phenotype.[62]

Hypericin-mediated photoactivation is not restricted to outer mitochondrial targets only, but involves photo-oxidation mechanisms occurring in the mitochondrial matrix.

In an early report,[51] oxygen consumption was measured before and after hypericin photoactivation in isolated bovine heart mitochondria. A drug, light dose and wavelength dependent photosensitized inhibition of mitochondrial succinoxidase was recorded, while simultaneous singlet oxygen generation was confirmed by the use of the specific $^1O_2$ trap tetramethylethylene.

(60) Oberst, A.; Bender, C.; Green, D. R. Living with death: the evolution of the mitochondrial pathway of apoptosis in animals. *Cell Death Differ.* **2008**, *15*, 1139–46.
(61) Deberardinis, R. J.; Sayed, N.; Ditsworth, D.; Thompson, C. B. Brick by brick: metabolism and tumor cell growth. *Curr. Opin. Genet. Dev.* **2008**, *18*, 54–61.
(62) Mathupala, S. P.; Ko, Y. H.; Pedersen, P. L. Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. *Oncogene* **2006**, *25*, 4777–86.

In a subsequent work,[63] studying the photosensitizing effect of hypericin on isolated rat liver mitochondria, inhibition of state 3 respiration (ADP dependent) and enhancement of state 4 respiration (ADP independent) were observed following hypericin photoactivation. The authors thus postulated that photosensitization of hypericin might increase the permeability of the mitochondrial membrane to H^+. Furthermore, they proposed that the transmembrane potential generated by succinate oxidation was decreased by hypericin photo-oxidation and that the concomitant formation of thiobarbituric acid reactive substances (TBARS) implicated $^1O_2$-mediated lipid peroxidation of mitochondrial membranes as a contributory factor. In another study, light activation of hypericin was found to deplete mitochondrial aconitase in DU145 human prostate carcinoma cells.[52] Aconitase is an enzyme found both in the mitochondria and cytosol and is particularly susceptible to oxidative insult, due to its cubane cluster $[4Fe\text{-}4S]^{2+}$. This hypericin-induced differential photoinactivation of mitochondrial aconitase may, on one hand, increase the generation of highly cytotoxic hydroxyl radicals, following the release of Fe^{2+} and H_2O_2,[64] while secondarily affect the TCA cycle by inhibiting the aconitase-catalyzed conversion of citrate to isocitrate.

The recent finding that hypericin photoactivation damages complex III of the mitochondria, an effect reversed by antimycin A, but not by myxothiazol,[58] probably constitutes the most conclusive evidence for direct mitochondrial impairment. This differential observation pinpoints, as stated earlier, the damage to the quinone reducing center of complex III (Q_i) making several enzymes of the TCA cycle (e.g., aconitase and succinoxidase), also located on the matrix side, possible targets. In addition inhibition of the F_1F_0-ATP synthase by oligomycin during hypericin photosensitization enhances mitochondrial injury, while inhibition of ATP transport outside the mitochondrial matrix by atractyloside is substantially cytoprotective. These results suggest the activation of reverse proton pumping (i.e., out of the mitochondrial matrix) by F_1F_0-ATP synthase. This is an attempt to maintain $\Delta\Psi_m$ at the expense of ATP, in order to sustain cell viability.

Consequently the pH_i elevation observed in previous studies[53,65] could be a result of (a) hypericin excited state proton transfer properties,[34] (b) F_1F_0-ATPase pumping protons out of the mitochondrial matrix in an attempt to preserve mitochondrial functionality or (c) inner mitochondrial membrane damage as a result of the photoactivated hypericin assault.

Similarly, the observed ATP depletion[53] could be attributed to ATP consumption during the reverse function of F_1F_0-ATPase as discussed above and in our recent work.[58]

The primary effects of hypericin on mitochondria are summarized in Figure 4. Depending on the strength of the photodynamic process (e.g., light dose and dye concentration) hypericin may inflict severe damage to the mitochondria leading to a bioenergetic collapse which favors necrotic cell death, or diminish their defenses against cell death pathways, perturbing mitochondrial membrane integrity.

Endoplasmic Reticulum Targets of Hypericin Phototoxicity

Light-activation of hypericin accumulated in the ER (e.g., prolonged incubation time) has been shown to induce an immediate increase in $[Ca^{2+}]_{cyt}$ followed by striking changes in ER Ca^{2+} homeostasis.[38] This event eventually results in mitochondria mediated apoptosis. These perturbations shown in HeLa cells using bespoke aequorin chimeras targeted to the ER, cytosol or mitochondria, are functionally linked to the incapability of photosensitized cells to refill ER Ca^{2+} pools, following the abrupt $^1O_2$-mediated damage of the SERCA2 pumps.[38] SERCA2 couple ATP hydrolysis to Ca^{2+} transport from the cytosol into the ER, thus maintaining the physiological levels of $[Ca^{2+}]_{ER}$, i.e. 3 to 4 orders of magnitude higher than $[Ca^{2+}]_{cyt}$. As discussed in the following sections dealing with the signaling pathways in cell death, there is evidence supporting the notion that hypericin-mediated photokilling is due to the incapability of photosensitized cells to refill ER Ca^{2+} pools, a direct consequence of ROS-attack to the SERCA2 pump.

Another crucial function of the ER is to fold, modify and sort newly synthesized cell surface or secreted proteins. Disruption of these processes can lead to "ER stress" and subsequent activation of the UPR. The UPR is primarily a prosurvival mechanism activated to reduce the accumulation and aggregation of unfolded or misfolded proteins in order to restore normal ER operation.[66] However, if ER stress becomes irreversible, the UPR can ultimately result in apoptosis, usually through the mitochondrial caspase-cascade pathway. Depletion of the ER Ca^{2+} pool is a well-known stimulus of ER stress and UPR.[66] A recent genome-wide analysis in hypericin-photosensitized human bladder cancer cells (T24 cells) revealed that proximal molecular sensors and effectors of the UPR are induced in a coordinated manner.[67] Thus, it is possible that ER perturbations following SERCA2 photodamage promote activation of the UPR

(63) Utsumi, T.; Okuma, M.; Kanno, T.; Takehara, Y.; Yoshioka, T.; Fujita, Y.; Horton, A. A.; Utsumi, K. Effect of the antiretroviral agent hypericin on rat liver mitochondria. *Biochem. Pharmacol.* **1995**, *50*, 655–62.

(64) Orrenius, S.; Gogvadze, V.; Zhivotovsky, B. Mitochondrial oxidative stress: implications for cell death. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 143–83.

(65) Sureau, F.; Miskovsky, P.; Chinsky, L.; Turpin, P. Hypericin induced cell photosensitization involves an intracellular pH decrease. *J. Am. Chem. Soc.* **1996**, *118*, 9484–7.

(66) Ron, D.; Walter, P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 519–29.

(67) Buytaert, E.; Matroule, J. Y.; Durinck, S.; Close, P.; Kocanova, S.; Vandenheede, J. R.; de Witte, P. A.; Piette, J.; Agostinis, P. Molecular effectors and modulators of hypericin-mediated cell death in bladder cancer cells. *Oncogene* **2008**, *27*, 1916–29.

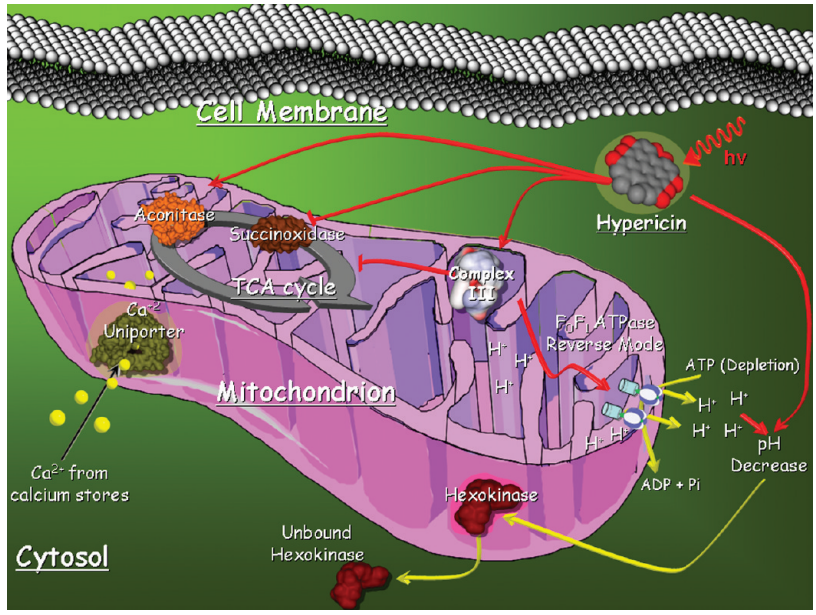

Figure 4. The effect of hypericin photosensitization on cell mitochondria. Hypericin photoactivation has been found to inhibit several enzymes of the TCA cycle like succinoxidase and aconitase. Furthermore, hypericin photosensitization seems to confer an intracellular pH drop which results in hexokinase unbinding from the mitochondrial outer membrane. Recently a direct, light activated assault of hypericin on ETC complex III was reported, and more specifically in the vicinity of the quinone reducing center. The oxidative stress caused by hypericin photoactivation seems to result in the F_0-F_1 ATP synthase running in reverse mode, in an attempt to maintain $\Delta\Psi_m$ resulting in ATP depletion and pH decrease.

pathway through photo-oxidized/misfolded protein accumulation which can eventually lead to cell death (*vide infra*). Although this assumption still needs to be thoroughly investigated, these molecular and functional data identify the ER as a main target of the photoactivity of hypericin. Nothing is known on the effects of this phototoxin on the Golgi apparatus; however, due to the colocalization of hypericin in this dynamic membrane system, it is expected to be a prominent site of photodamage.

Lysosomes as Targets of Hypericin Phototoxicity

Another reported site of hypericin intracellular accumulation is the lysosomes. Lysosomes function as cell recycling centers breaking down complex molecules, damaged or unnecessary organelles and cellular components including foreign pathogens. All these are degraded by the lysosomal hydrolases, and the products of this breakdown are subsequently released into the cytosol to be either further catabolized or recycled into new cellular components.

Lysosomes are sensitive to oxidative stress, and in several paradigms light irradiation of lysosomal-associated photosensitizers has been shown to cause rupture of the lysosomal membrane, which then released its contents along with the lysosomal hydrolases into the cytosol. Recently, a clear link has been established between lysosomal rupture and apoptosis initiation, although the exact molecular mechanism underlying lysosomal membrane permeability (LMP) remains elusive.[68] The lysosomal integrity of DU145 cells following hypericin sensitization was assessed by monitoring the activity of hexosaminidase in several cellular compartments.[52] Hexosaminidase release into the cytosol, a reflection of lysosomal rupture, was found to be constant over a period of 24 h and accounted for 25% of total hexosaminidase activity. Moreover total hexosaminidase loss following hypericin PDT did not correlate with the very rapid and complete loss of mitochondrial function. Likewise light exposure of HeLa and mouse embryonic fibroblast (MEF) cells incubated with hypericin did not significantly affect lysosomal stability and the use of various lysosomal hydrolase inhibitors did not notably affect mitochondrial cytochrome *c* release or overall apoptosis.[38]

We conclude from this that lysosomal damage does not appear to be a primary modus operandi of the multifaceted hypericin phototoxicity. Cells survive partial lysosomal disruption probably because lysosomal enzymes are inactivated either from the lysosome lumen − cytoplasm pH difference, by the photodynamic treatment itself, or by cytosolic inhibitors.[69]

(68) Boya, P.; Kroemer, G. Lysosomal membrane permeabilization in cell death. *Oncogene* **2008**, *27*, 6434–51.

(69) Berg, K.; Moan, J. Lysosomes as photochemical targets. *Int. J. Cancer* **1994**, *59*, 814–22.

Apoptotic Pathways in Hypericin-Photosensitized Cells

A. Mitochondrial Apoptosis. The *intrinsic* or mitochondrial pathway of apoptosis, which is tightly regulated by the Bcl-2 protein family, can be triggered by numerous intra- and extracellular stresses that cause the permeabilization of the mitochondrial outer membrane (MOM). This is a lethal event resulting in the release of apoptogenic molecules, such as cytochrome *c*, from the mitochondria into the cytosol. Cytosolic cytochrome *c* binds to Apaf-1 (apoptotic protease-activating factor 1) and, in the presence of ATP or dATP, recruits and activates procaspase-9 through the formation of an oligomeric complex called the apoptosome. Caspase-9 activates effector caspases leading to apotosis.[70]

Increasing evidence places the mitochondria either as initiators or as critical checkpoints of death pathways triggered by light-activation of hypericin and other photosensitizers.[20] Indeed, the crucial role of the mitochondrial pathway of caspase activation in PDT-treated cells has been extensively documented and reviewed.[20,31,32] While this is especially true for agents with a prevalent mitochondrial localization, able to directly affect mitochondrial targets or vital apoptotic modulators, mitochondria also serve as critical executers of lethal pathways stemming from photodamage to other subcellular sites or organelles; in the latter case, however, apoptogenic protein release from the mitochondria is usually delayed.

As detailed earlier, direct mitochondria photosensitization by hypericin could result in a rapid imbalance of mitochondria bioenergetics, ROS overproduction and possibly direct compromise of the mitochondrial membranes, depending on the intensity of the photodynamic assault. These conditions have been linked to primary necrotic cell death.[20]

Photosensitization of hypericin accumulated in the mitochondria results, as discussed above, in HK-inhibition in a concentration and light-dependent fashion. HK moreover is thought to be a molecular component of the mitochondrial permeability transition pore (MPTP), whose persistent opening is suggested to have a major impact in cell death.[71] The swollen morphology of the mitochondria and the assessment of the MPTP involvement through the calcein-quenching methods[72] in cells photosensitized with hypericin suggest that MPTP may be a consequence of a hypericin-associated, direct mitochondrial photodamage.

Recent studies indicated that HK bound to VDAC prevents cytochrome *c* release in tumors thus inhibiting apoptosis.[73−75] Conversely, disruption of HK−VDAC binding, via modification of key VDAC amino acids, was found to enhance apoptosis in cancer cells.[76] Although the exact correlation between hypericin-photoinduced HK−VDAC dissociation and apoptosis onset has not been fully determined, it is possible that, following HK release, VDAC becomes available for proapoptotic proteins (e.g., Bax) to bind to, thus promoting mitochondrial apoptosis.[23]

Bax has been recently shown to be a central mediator of hypericin-PDT apoptosis. All biochemical hallmarks of apoptotic cell death following the light activation of ER-localized hypericin are absent in $Bax^{-/-}Bak^{-/-}$ double knockout (DKO) cells, which die in a caspase-independent fashion. Conversely, re-expression of Bax in DKO cells proved both essential and sufficient to switch the cytocidal mode from caspase-independent back to mitochondrial apoptosis.[38,77] This pathway is mainly, yet not entirely, dependent on the mitochondria-driven caspase cascade activation; indeed apoptosis is only partially inhibited by caspase antagonists.[38,77] This suggests that subsequent to Bax/Bak permeabilization of the outer mitochondrial membrane, other apoptogenic factors are released along with cytochrome *c*, contributing to apoptotic-like cell demise in a caspase-independent fashion. The apoptosis inducing factor (AIF), for instance, is a prime suspect.

The exact mechanism underlying Bax activation subsequent to hypericin-photosensitization at the ER is not clear. The question arises whether the signal propagating mitochondrial apoptosis in hypericin-photosensitized cells is provided by the increase in $[Ca^{2+}]_{cyt}$ and its subsequent sequestration into mitochondria, or rather by the overwhelming depletion of the ER-Ca^{2+} stores.

Recent studies seem to favor the latter hypothesis. Overexpression of SERCA2 in DKO cells restored ER-

(70) Hengartner, M. O. The biochemistry of apoptosis. *Nature* **2000**, *407*, 770–6.
(71) Rasola, A.; Bernardi, P. The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis* **2007**, *12*, 815–33.
(72) Chaloupka, R.; Petit, P. X.; Israel, N.; Sureau, F. Over-expression of Bcl-2 does not protect cells from hypericin photo-induced mitochondrial membrane depolarization, but delays subsequent events in the apoptotic pathway. *FEBS Lett.* **1999**, *462*, 295–301.
(73) Pastorino, J. G.; Hoek, J. B. Hexokinase II: the integration of energy metabolism and control of apoptosis. *Curr. Med. Chem.* **2003**, *10*, 1535–51.
(74) Vyssokikh, M.; Brdiczka, D. VDAC and peripheral channelling complexes in health and disease. *Mol. Cell. Biochem.* **2004**, *256−257*, 117–26.
(75) Vyssokikh, M.; Zorova, L.; Zorov, D.; Heimlich, G.; Jurgensmeier, J.; Schreiner, D.; Brdiczka, D. The intra-mitochondrial cytochrome c distribution varies correlated to the formation of a complex between VDAC and the adenine nucleotide translocase: this affects Bax-dependent cytochrome c release. *Biochim. Biophys. Acta* **2004**, *1644*, 27–36.
(76) Shoshan-Barmatz, V.; Zakar, M.; Rosenthal, K.; Abu-Hamad, S. Key regions of VDAC1 functioning in apoptosis induction and regulation by hexokinase. *Biochim. Biophys. Acta* **2009**, *1787* (5), 421–30.
(77) Buytaert, E.; Callewaert, G.; Vandenheede, J. R.; Agostinis, P. Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy* **2006**, *2*, 238–40.

Ca^{2+} steady state levels[78] but did not reinstate apoptosis as the principal mode of cell death following hypericin PDT. Since SERCA2 photodamage is analogous in wild type, $Bax^{-/-}Bak^{-/-}$ DKO and SERCA2 overexpressing cells, the cell death commitment event occurs independently of the amount of Ca^{2+} released from the ER and is upstream to Bax activation.

Additionally, inhibition of mitochondrial Ca^{2+} uptake by ruthenium red or use of intracellular Ca^{2+} chelator BAPTA-AM conferred no cytoprotective effects against hypericin-mediated phototoxicity.[38,58] This further suggests that Ca^{2+} release into the cytoplasm following intracellular Ca^{2+} store damage is not on its own merit sufficient to trigger Bax-mediated mitochondrial membrane permeabilization. Nevertheless, cytosolic Ca^{2+} overload involving influx of extracellular Ca^{2+} selectively directed to the mitochondria by Ca^{2+} ionophore ionomycin was shown to enhance hypericin photocytotoxicity, and this synergy was reversed by EGTA, a specific calcium chelator.[52] The above observations suggest that Ca^{2+} signaling has a regulatory role and may augment hypericin photocytotoxicity, while irreparable photodamage to the ER is the commitment event in cell death.

Fatty acids derived from Ca^{2+}-dependent cytosolic phospholipase A2 ($cPLA_2$) activation, such as arachidonic acid and lysophosphatidylcholine, are suspect Bax proapoptotic triggers. These fatty acids have been shown to promote positive membrane curvature[79] and thus favor Bax pore forming activity leading to mitochondrial membrane permeabilization and concomitant release of apoptogenic factors. In support of this hypothesis, pharmacological inhibition of $cPLA_2$ proved to be cytoprotective, delaying the kinetics of cytochrome *c* release and consequently of procaspase-3 activation in HeLa cells after hypericin photoactivation.[80]

In addition it is also possible that oligomerization of Bax and/or Bak involves the post-transcriptional activation and/or transcriptional expression of BH3-only proteins, which may directly activate multidomain Bax/Bak proteins to form transmembrane channels on mitochondria allowing cytochrome *c* into the cytosol.[81,82] A plausible candidate in this context appears to be the BH3-only protein Bim, as it was recently found to be implicated in ER stress induced apoptosis.[83]

As earlier discussed, partial LMP could have an auxiliary role in the apoptotic mechanisms already set into action by hypericin PDT. For instance, Bax mediated permeabilization of the mitochondrial outer membrane could be enhanced following Bid cleavage by lysosomal hydrolases, thereby amplifying the initial damage and apical proapoptotic pathways evoked by ER stress.

Interestingly, HSP70, a molecular chaperone whose expression is rapidly upregulated at the mRNA and protein levels in cells photosensitized with hypericin,[67] has been found to exert its intracellular antiapoptotic activity by stabilizing lysosomal membranes.[84] Although this connection has yet to be proven experimentally, it could provide a cytoprotective mechanism for the extensive HSP70 upregulation generally observed in several PDT paradigms.

B. Death Receptor Mediated Apoptosis. Apoptosis, as a programmed cell death routine, can also be stimulated by positively acting, extrinsic, "death" signals. For instance, tumor necrosis factor (TNF), TNF related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) can propagate cell death by binding to their complementary cell-surface receptors, thus bringing three receptor molecules in close proximity. Receptor trimerization triggers the recruitment of death domain (DD)-containing proteins (FADD, TRADD etc.) and initiator caspase-8 (via DED domains). This leads to the formation of the death-inducing signaling complex (DISC), the molecular platform required for caspase-8 activation.[85] An increased expression of Fas and FasL following PDT with different photosensitizers has been shown *in vitro* as well as in tumor xenografts and is extensively reviewed elsewhere.[20,31,32] This suggests that the extrinsic caspase activation pathway contributes either directly or indirectly (i.e., through autocrine/paracrine signals resulting from the increased expression of DRs and their ligands in photodamaged cells) to an efficient apoptotic response engaged by PDT.

(78) Scorrano, L. Proteins that fuse and fragment mitochondria in apoptosis: con-fissing a deadly con-fusion. *J. Bioenerg. Biomembr.* **2005**, *37*, 165–70.

(79) Brown, W. J.; Chambers, K.; Doody, A. Phospholipase A2 (PLA2) enzymes in membrane trafficking: mediators of membrane shape and function. *Traffic* **2003**, *4*, 214–21.

(80) Hendrickx, N.; Dewaele, M.; Buytaert, E.; Marsboom, G.; Janssens, S.; Van Boven, M.; Vandenheede, J. R.; de Witte, P.; Agostinis, P. Targeted inhibition of p38alpha MAPK suppresses tumor-associated endothelial cell migration in response to hypericin-based photodynamic therapy. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 928–35.

(81) Wei, M. C.; Lindsten, T.; Mootha, V. K.; Weiler, S.; Gross, A.; Ashiya, M.; Thompson, C. B.; Korsmeyer, S. J. tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes Dev.* **2000**, *14*, 2060–71.

(82) Eskes, R.; Desagher, S.; Antonsson, B.; Martinou, J. C. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol. Cell. Biol.* **2000**, *20*, 929–35.

(83) Puthalakath, H.; O'Reilly, L. A.; Gunn, P.; Lee, L.; Kelly, P. N.; Huntington, N. D.; Hughes, P. D.; Michalak, E. M.; McKimm-Breschkin, J.; Motoyama, N.; Gotoh, T.; Akira, S.; Bouillet, P.; Strasser, A. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* **2007**, *129*, 1337–49.

(84) Nylandsted, J.; Gyrd-Hansen, M.; Danielewicz, A.; Fehrenbacher, N.; Lademann, U.; Hoyer-Hansen, M.; Weber, E.; Multhoff, G.; Rohde, M.; Jaattela, M. Heat shock protein 70 promotes cell survival by inhibiting lysosomal membrane permeabilization. *J. Exp. Med.* **2004**, *200*, 425–35.

(85) Boatright, K. M.; Salvesen, G. S. Mechanisms of caspase activation. *Curr. Opin. Cell Biol.* **2003**, *15*, 725–31.

In a recent work,[86] on Jurkat cells, the involvement of death receptors in hypericin-mediated photocytotoxicity was investigated, using neutralizing monoclonal antibodies against Fas, FasL, TNF-R1 and a polyclonal rabbit antihuman TRAIL antiserum.

Following hypericin incubation and photoactivation, the anti-TRAIL antibody was found to specifically inhibit apoptosis in a dose dependent manner. The anti-Fas and anti-FasL antibodies, although efficient in inhibiting apoptosis associated with the chemotherapeutic agent paclitaxel, had no effect on hypericin photoinduced apoptosis. Finally the anti-TNF-R1 mAb had no effect on either paclitaxel or hypericin-PDT mediated apoptosis.

Elsewhere,[87] the specific involvement of Fas in hypericin photoinduced apoptosis was shown in two human nasopharyngeal carcinoma cell lines (CNE2 and TWO-1), as well as colon (CCL-220.1) and bladder (SD) carcinoma cells. More specifically, following hypericin photoactivation, Fas/FasL expression was found to be upregulated and Fas-FADD-signaling to cell death was triggered promoting caspase-8 processing and Bid cleavage, in all tumor cell lines tested.

In contrast to the above observations, hypericin-mediated photokilling was unaffected in HeLa cells expressing the potent viral caspase-1 and -8 inhibitor CrmA[88] which fully blocked TNF-induced apoptosis. Moreover, measurements of actual caspase-8 activity in various human and murine cancer cell lines[88−90] revealed negligible or poor caspase-8 activation by hypericin PDT.

Although these results are difficult to reconcile, it is possible that in certain cell types, and under conditions favoring partial plasma membrane retention of hypericin, a part of photogenerated ROS causes rapid perturbation of membrane-bound receptor activities resulting in TRAIL-R or Fas activation leading to apoptosis.

All documented processes involved in either intrinsic or extrinsic apoptosis following hypericin photosensitization are illustrated in Figure 5.

Autophagy and Autophagic Cell Death

Recent studies show that hypericin-mediated photosensitization may induce nonapoptotic cell death associated with an aberrant induction of macroautophagy.[38,77] Macroautophagy, or simply autophagy, is a dynamic and highly regulated process of self-digestion conserved in all eukaryotic organisms normally occurring at low rate as a major homeostatic mechanism. It can, nevertheless, be stimulated and accelerated in conditions of nutrient deprivation or cellular stress, in order to remove damaged organelles, toxic metabolites or intracellular pathogens and generate new building blocks through recycling of cytoplasmic material. Albeit it is unlikely that autophagy is a *bona fide* cell death mechanism, aberrant stimulation of autophagy can eventually promote cell death.[91] The functional contribution of this catabolic process in cell death is still uncertain; however, recent studies suggest that autophagy may regulate cancer development and progression as well as the response to cytotoxic therapies.[92]

A first report on hypericin-mediated autophagy revealed that hypericin PDT enhanced stimulation of this catabolic pathway on apoptosis-deficient (e.g., $Bax^{-/-}Bak^{-/-}$ DKO) MEFs, which died assuming a necrotic morphology.[38] These cells exhibited the classical biochemical hallmarks of autophagy, including accumulation of the lipidated form of LC3 II/Atg8, redistribution of GFP-LC3 from a cytosolic smooth pattern into a punctate one, and the ultrastructural features of autophagy,[38] i.e. organelle sequestration by double membrane vacuoles, autophagosomes (see Figure 6).

Aberrant autophagy stimulation under conditions of caspase inhibition was found to be involved in cell death propagation, since, in apoptosis-deficient ($Bax^{-/-}Bak^{-/-}$ DKO) cells, the pharmacological blockade of autophagy by phosphatidylinositol 3-kinase (PI3K) class III inhibitor wortmannin was cytoprotective.[38] Stimulation of autophagy by hypericin-mediated ER photodamage was not instigated by secondary mitochondrial malfunctions triggering cell death, as suggested in other paradigms,[93] since the loss of mitochondrial transmembrane potential or cytochrome *c* release was not detected in DKO cells.[38]

Autophagy initiation by hypericin PDT is not restricted to apoptosis-deficient cells since ongoing investigations suggest that both apoptosis and autophagy are simultaneously initiated in various human and murine cancer cell lines as

(86) Schempp, C. M.; Simon-Haarhaus, B.; Termeer, C. C.; Simon, J. C. Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. *FEBS Lett.* **2001**, *493*, 26–30.

(87) Ali, S. M.; Chee, S. K.; Yuen, G. Y.; Olivo, M. Hypericin induced death receptor-mediated apoptosis in photoactivated tumor cells. *Int. J. Mol. Med.* **2002**, *9*, 601–16.

(88) Assefa, Z.; Vantieghem, A.; Declercq, W.; Vandenabeele, P.; Vandenheede, J. R.; Merlevede, W.; de Witte, P.; Agostinis, P. The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. *J. Biol. Chem.* **1999**, *274*, 8788–96.

(89) Hendrickx, N.; Volanti, C.; Moens, U.; Seternes, O. M.; de Witte, P.; Vandenheede, J. R.; Piette, J.; Agostinis, P. Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. *J. Biol. Chem.* **2003**, *278*, 52231–9.

(90) Vantieghem, A.; Xu, Y.; Declercq, W.; Vandenabeele, P.; Denecker, G.; Vandenheede, J. R.; Merlevede, W.; de Witte, P. A.; Agostinis, P. Different pathways mediate cytochrome c release after photodynamic therapy with hypericin. *Photochem. Photobiol.* **2001**, *74*, 133–42.

(91) Levine, B.; Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **2008**, *132*, 27–42.

(92) Rubinsztein, D. C.; Gestwicki, J. E.; Murphy, L. O.; Klionsky, D. J. Potential therapeutic applications of autophagy. *Nat. Rev. Drug Discovery* **2007**, *6*, 304–12.

(93) Elmore, S. P.; Qian, T.; Grissom, S. F.; Lemasters, J. J. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J.* **2001**, *15*, 2286–7.

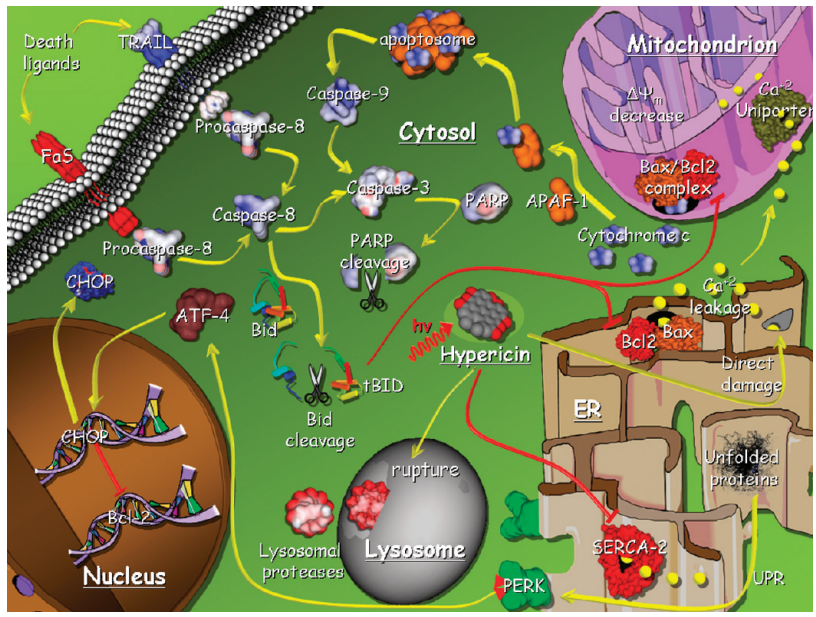

Figure 5. Apoptotic pathways activated by hypericin PDT. Upon accumulation in cancer cells, depending on drug concentration and exposure time, hypericin shows affinity for different intracellular sites, including mitochondria, ER/Golgi, and lysosomes. Light activation of hypericin can launch the mitochondrial pathway of apoptosis, which is engaged by the release of cytochrome *c* in the cytosol. Binding of cytochrome *c* to apoptotic peptidase activating factor 1 (Apaf-1) results in the formation of the apoptosome, the molecular platform for activation of the initiator caspase-9, which in turn processes and activates the effector caspases-3/7. Either through an autocrine mechanism involving the release of the death ligands TRAIL and Fas or because of possible death-receptor activation by plasma membrane associated hypericin, photosensitization can result in the activation of the initiator procaspase-8. Caspase-8 subsequently leads to the proteolytic activation of the main effector caspases-3/7 and can generate truncated Bid (tBid), which can, in turn, bind to Bcl-2, thereby inhibiting its antiapoptotic function. Light activation of ER-localized hypericin ER causes SERCA2 pump photodamage and ER Ca^{2+} depletion, thus initiating Bax-dependent mitochondrial apoptosis. The loss of ER Ca^{2+} homeostasis instigates the accumulation of unfolded proteins in the ER, which subsequently triggers the activation of proximal effectors of the unfolded protein response (UPR), including PERK. In the latter pathway, induction of the transcription factor CHOP via the PERK-ATF-4 axis can repress Bcl-2 expression, favoring mitochondrial apoptosis. Photoactivation of lysosome-associated hypericin may result in lysosomal membrane permeabilization and concomitant release of lysosomal proteases. These can cleave Bid into its proapoptotic form (tBid), thereby amplifying mitochondrial apoptosis. Ultimately, these converging signalling pathways result in the activation of the effector caspases which are responsible for the morphological and biochemical features of apoptotic cell death, including internucleosomal DNA fragmentation, membrane blebbing, and cell shrinkage.

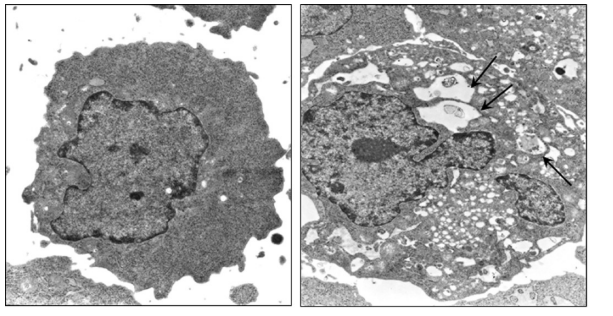

Figure 6. Autophagy induction 6 h following hypericin PDT on $Bax^{-/-}$ $Bak^{-/-}$ murine embryonic fibroblasts (DKO MEFs) as evident from the transmission electron microscopy (TEM) image on the right. Arrows indicate vacuoles with detectable content. A TEM photomicrograph of an untreated cell is also shown for comparison (left).

well as in untransformed cells (M. Dewaele and P. Agostinis, unpublished results).

Stimulation of autophagy and apoptosis has been also reported following light-activation of other ER/mitochondria associated photosensitizers in murine leukemia L1210 cells[94] and human prostate Bax-deficient DU-145 cells,[95,96] thus supporting the view that both signals are activated as a consequence of organellar photodamage. Interestingly, au-

(94) Kessel, D.; Reiners, J. J., Jr. Apoptosis and autophagy after mitochondrial or endoplasmic reticulum photodamage. *Photochem. Photobiol.* **2007**, *83*, 1024–8.

(95) Kessel, D.; Vicente, M. G.; Reiners, J. J., Jr. Initiation of apoptosis and autophagy by photodynamic therapy. *Lasers Surg. Med.* **2006**, *38*, 482–8.

(96) Xue, L. Y.; Chiu, S. M.; Azizuddin, K.; Joseph, S.; Oleinick, N. L. The death of human cancer cells following photodynamic therapy: apoptosis competence is necessary for Bcl-2 protection but not for induction of autophagy. *Photochem. Photobiol.* **2007**, *83*, 1016–23.

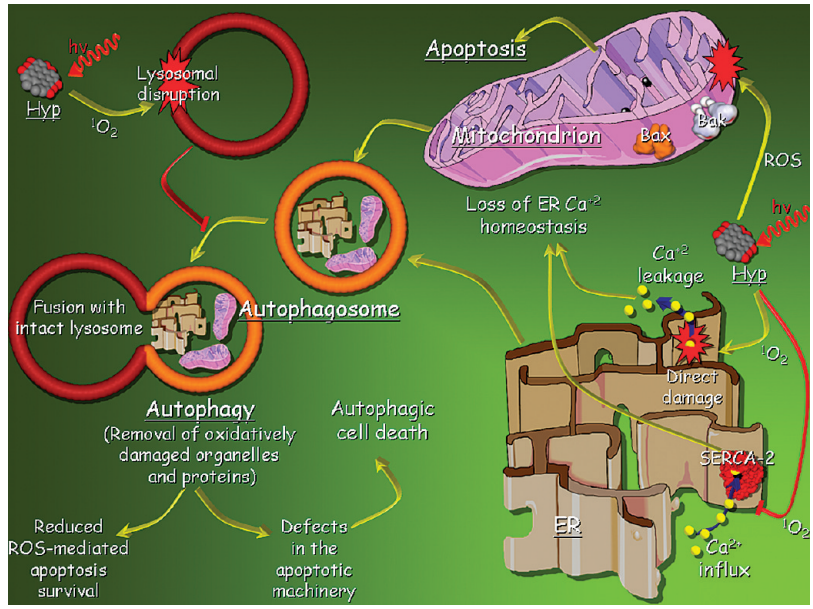

Figure 7. Autophagy in hypericin PDT. Photodamage to key organelles like the ER and mitochondria following light activation of hypericin localized at these sites may stimulate autophagy as part of a survival pathway in an attempt to remove dysfunctional organelles or oxidized proteins. In apoptosis-deficient cells, for example, cells lacking the pro-apoptotic multidomain Bax and Bak proteins and/or caspase signaling (see text), aberrant autophagy stimulation causes the induction of an autophagic cell death pathway, whose molecular elements have not been revealed yet. Under conditions leading to enhanced lysosomal photodamage by hypericin, inhibition of the fusion of autophagosome with lysosomes and consequent blockage of the recycling process (e.g., autophagic flux) could occur. The accumulation of unprocessed autophagosomes, and their possible breakdown in the cytosol, could then favor apoptotic cell death.

tophagy induction in these PDT paradigms was associated with rapid photodamage of Bcl-2[97] leading to the suggestion that autophagy after PDT could be instigated by disrupting the association between Bcl-2 with the key autophagy regulator Beclin 1 at the ER, a molecular event which has been shown to positively regulate autophagy.[98] In contrast to these observations, light activation of hypericin does not result in direct damage to either Bcl-2[99] or Beclin-1 (M. Dewaele and P. Agostinis, unpublished results). Thus while the available data indicate that autophagy stimulation is a common phenomenon associated with PDT-mediated cell injury, the molecular mechanism triggering this degradative pathway appears to be photosensitizer-specific and involves distinct primary targets of photogenerated ROS. Autophagy after PDT is probably stimulated in an attempt to remove nonfunctional, oxidatively modified proteins, and/or damaged organelles, which cannot be removed by the ubiquitin-proteasome system or other degradative mechanisms. Perpetuation of autophagy may further lead to irreparable intracellular degradation, metabolic collapse and ultimately necrosis-like death. However, whether autophagic cell death is generally a backup death mechanism in PDT treated cells with defective apoptosis signaling should be further explored.

Primary damage to the mitochondria following direct photosensitization by hypericin may instigate their sequestration into autophagic vacuoles (mitophagy) in order to maintain cellular integrity and function. Likewise, in cases where the ER have sustained the main photodamage, they could be the preferred target of autophagic degradation (reticulophagy). Recent studies have established that there are instances where the ER is preferentially targeted for autophagic degradation to support survival in yeast.[100]

While it is very likely that hypericin-PDT mediated photodamage to different targets instigates autophagy, the exact role of this catabolic process in PDT still awaits further investigations. A schematic overview of the possible connection between organellar photodamage, autophagy and cell death is shown in Figure 7.

(97) Kessel, D.; Arroyo, A. S. Apoptotic and autophagic responses to Bcl-2 inhibition and photodamage. *Photochem. Photobiol. Sci.* **2007**, *6*, 1290–5.

(98) Pattingre, S.; Tassa, A.; Qu, X.; Garuti, R.; Liang, X. H.; Mizushima, N.; Packer, M.; Schneider, M. D.; Levine, B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **2005**, *122*, 927–39.

(99) Vantieghem, A.; Xu, Y.; Assefa, Z.; Piette, J.; Vandenheede, J. R.; Merlevede, W.; De Witte, P. A.; Agostinis, P. Phosphorylation of Bcl-2 in G2/M phase-arrested cells following photodynamic therapy with hypericin involves a CDK1-mediated signal and delays the onset of apoptosis. *J. Biol. Chem.* **2002**, *277*, 37718–31.

(100) Yorimitsu, T.; Nair, U.; Yang, Z.; Klionsky, D. J. Endoplasmic reticulum stress triggers autophagy. *J. Biol. Chem.* **2006**, *281*, 30299–304.

Conclusions and Outlook

Hypericin is a potent photosensitizer exhibiting a multifaceted photocytotoxicity. It operates in a "chameleon" fashion exploiting its unique chemical properties to induce cell death through the engagement of different pathways resulting primarily in apoptosis and/or necrosis. While necrosis is mainly the result of direct damage to key subcellular organelles, apoptosis is orchestrated by the activation of caspase dependent or independent pathways, followed often by secondary necrosis. Aberrant stimulation of autophagy may serve as a backup death pathway in apoptosis resistant cells.

Moreover, hypericin engages a multitude of subcellular targets resident in different organelles (mitochondria, ER or lysosomes). Key mitochondrial targets of hypericin include complex III of the ETC, aconitase and hexokinase, whereas in the ER hypericin photoactivation results in fast and selective photodamage to the SERCA2 pump.

Much remains to be elucidated about the photodynamic action of hypericin. In particular, the Golgi apparatus needs to be thoroughly studied as a hypericin PDT target. Other points also await clarification, such as: How does hypericin photoactivation precipitate the extrinsic apoptosis pathway? What is the full role of the UPR, which is engaged in response to ER stress, in hypericin PDT? What is the exact role of autophagy in cells undergoing apoptosis after hypericin-mediated PDT and what are the molecular mechanisms that turn it into a death pathway when apoptosis is defective? Given that stimulation of ER stress pathways and autophagy in response to anticancer therapy may paradoxically contribute to both cell survival and cell death, PDT with hypericin or other photosensitizers stimulating these cellular processes may become an interesting paradigm for further investigations.

As more of these questions are investigated and answered, the complete magnitude of the photocytotoxic diversity of hypericin will emerge. We believe however that the present review sets solid foundations for directing future research.

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