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Review Article

Overview of Cell Death Mechanisms Induced by Rose Bengal Acetate-Photodynamic Therapy

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Photodynamic Therapy (PDT) is a non-invasive treatment for different pathologies, cancer included, using three key components: non-toxic light-activated drug (Photosensitizer, PS), visible light, and oxygen. Their interaction triggers photochemical reactions leading to Reactive Oxygen Species (ROS) generation, that mediate cytotoxicity and cell death. In the present paper, the most important findings about the synthetic dye Rose Bengal Acetate (RBAc), an emerging photosensitizer for its efficient induction of cell death, will be reported with the aim to integrate RBAc phototoxicity to novel therapeutic PDT strategies against tumour cells. After its perinuclear intracellular localization, RBAc causes multiple subcellular organelles damage, that is, mitochondria, Endoplasmic Reticulum (ER), lysosomes, and Golgi complex. Indeed, RBAc exerts long-term phototoxicity through activation of both caspase-independent and- dependent apoptotic pathways and autophagic cell death. In particular, this latter cell death type may promote cell demise when apoptotic machinery is defective. The deep knowledge of RBAc photocytotoxicity will allow to better understand its potential photomedicine application in cancer.

1. Introduction

The cancer cells acquire during tumorigenic process specific functional capabilities: self-sufficiency in growth signals, insensitivity to antigrowth signals, evading cell death, limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis [1]. Moreover, potential hallmark of cancer is immunosurvillance avoidance [2], allowing tumour cells to escape the innate and adaptive immunoresponse.

Conventional cancer therapies are based primarily on cell death induction, affecting not only cancer cells, but also immune cells, leading to immunosuppressive effects. In fact, chemotherapy and ionizing radiation delivered at doses sufficient to destroy neoplastic cells are toxic to the bone marrow, causing neutropaenia and other forms of myelosuppression. In parallel with destruction of tumour cells, the ideal cancer therapy should trigger an immune response directly to recognize and destroy all remaining tumour cells both in the primary tumour area and in distant micrometastases.

In this context, PDT is a promising cancer therapy for its efficiency in cell death induction and high selectivity for tumour cells. PDT is a binary therapy based on a two-step combination of two non-toxic elements in presence of O_2: the selective uptake of a photoactive drug (PS) by tumour cells and irradiation with appropriate wavelength light, easy directionable to get the therapeutic effect only on neoplastic lesion and able to trigger photochemical reactions leading to the generation of singlet oxygen ($^1O_2$) and other ROS [3].

Compared to traditional pharmacological or surgical cancer therapies, PDT presents important advantages: it is not invasive, does not require local or general anaesthesia, may be used to treat many superficial located or widespread lesions, can be applied independently on the age or concomitant diseases of the patient. The duration of treatment is short, no hospitalization is needed, and treatment can be repeated in case of recurrence.

2. Mechanisms of PDT

PDT is an emerging treatment used to eradicate premalignant and early-stage cancer and reduce the tumour size in end-stage cancers.

The first attempts to use photoactive drugs and light to treat skin diseases, such as vitiligo, rickets, psoriasis, and skin cancer date back to ancient Egyptians, Chinese, and Indians [4]. Over the last 50 years, starting from 1960 when Lipson and Baldes demonstrated regression of tumours after administration of porphyrins and visible light exposure [5], several studies were performed to understand the mechanisms involved in cell killing after photodynamic treatment. In 1978, Dougherty et al. demonstrated the successful application of PDT for cancer treatment [6], suggesting it as a very interesting experimental tool for the detection and treatment of lung, esophagus, colon, peritoneum, pleura, genitourinary tract, brain, eye, and skin tumours. In Canada, Japan, France, the Netherlands, Germany, and the United States (US), PDT is approved (in US by the Food and Drug Administration, FDA) for treatment of selected malignancies [7].

The mechanism of PDT action involves three key components: PS, light (wavelength appropriate for the PS), and tissue oxygen. The combination of these three components leads to the killing of tumour cells.

PDT-mediated tumour destruction is multifactorial: (1) direct tumour cell kill, (2) damage to the vasculature, and (3) rapid recruitment and activation of immune cells that can facilitate development of antitumour adaptive immunity [8–10] (Figure 1).

2.1. Photosensitizers, Light Source, and ROS Production. PSs are non-toxic drugs or dyes administrated either systemically, locally, or topically to patients bearing a lesion (frequently but not always cancer), which, by the illumination of the lesion with visible light in presence of oxygen, generate cytotoxic species leading to cell death and tissue destruction [11]. Efficient photosensitization primarily depends on physico-chemical properties of the PS, such as chemical purity, specific localization in neoplastic cells, sufficiently long residence time, short time interval between the administration of the drug and its accumulation in tumour cells, rapid clearance from normal tissues, activation at wavelength with optimal tissue penetration, high quantum yields for the generation of singlet oxygen, and lack of dark toxicity.

Hematoporphyrin derivative (HPD) or Photofrin was the first studied PS for clinical PDT. The majority of PSs, used for both clinical and experimentation, are derived from the tetrapyrrole aromatic nucleus present in natural pigments, such as heme chlorophyll and bacteriochlorophyll. A second group of PSs is represented by Phtalocyanines (PC). Another group of potential PSs includes completely synthetic conjugated pyrrolic ring systems, that comprise structures such as texaphyrins [12], porphycenes [13], and sapphyrins [14]. A last group of PSs are non-tetrapyrrole-derived naturally occurring (e.g., hypericin) or synthetic dyes (toluidine blue O and Rose Bengal) [15–17].

Photodynamic treatment strictly depends on the light source and light delivery. The choice of light source is, in turn, affected by the location of the tumour, the light dose delivered, and the PS used. Light sources employed in PDT are lasers, lamps, and Light Emitting Diodes (LED). In contrast to lamps, lasers allow the exact selection of wavelengths and the precise application of light. On the other hand, LED would offer several advantages for clinical and laboratory use: the choice of emission wavelength ranges from UVA (350 nm) to near infrared (1100 nm), while the band-width is 5–10 nm, and the power output can provide up to 150 mW/cm^2 over an area of approximately 20 cm^2. The main characteristics of LED use are price and versatility in light delivery on difficult anatomic area [18].

The efficacy of photosensitization is directly related to the amount of oxygen within the tumour and its environment that, in turn, depends on the concentration of oxygen in the tissue [19].

Upon irradiation, the PSs create a photodynamic reaction based on photophysical and photochemical reactions [3]. In particular, the PS in the ground state absorbs light and is activated to the single excited state with a short half-life ranging from 10^{-6} to 10^{-9} seconds. The singlet excited PS either decays back to the ground state, giving off energy in form of fluorescence or vibrational energy (photophysical reaction) or undergoes intersystem crossing to the longer lived (10^{-3} seconds) triplet excited state (photochemical reaction). The interaction of the triplet sensitizer with surrounding molecules results in two types of photooxidative reactions exploited in photodynamic treatment. In type I pathway, the triplet excited PS reacts with a substrate, such as plasma membrane and transfers an electron or hydrogen atom producing radical forms. These intermediates may react with oxygen to form peroxides, superoxides ions, and hydroxyl radicals (known as ROS), which initiate free radical chain reactions. Alternatively, type II reactions involve the transfer of triplet PS energy directly to molecular oxygen to form excited-state singlet oxygen, the most important reactive specie in PDT-mediated cytotoxicity [20]. Type I and II reactions can occur simultaneously and their ratio depends on the type of PS, substrate and oxygen concentration.

ROS can also be a byproduct of cellular metabolism involved in cell development, growth, survival, cell death, aging, drug metabolism, and cancer development [21, 22]. Under these physiological conditions, a series of antioxidative defense systems overcomes the ROS potential toxicity: intracellular SuperOxide Dismutase (SOD), catalase, and glutathione peroxidase [23]. The balance between ROS generation and antioxidative defense level is crucial for cell viability.

It is known that the cancer cells are frequently deficient in antioxidative defense systems reflecting a high vulnerability of tumour cells to ROS [24]. This characteristic is exploited by conventional chemotherapy associated with unexpected or systemic side effects since one of its main disadvantages is the little selectivity. Conversely, as above reported, PDT has a higher cancer therapeutic effect than chemotherapy because it involves the administration of a drug which not only preferentially localizes in cancer cells, but also is activated upon irradiation leading to photooxidative reactions.

2.2. Rose Bengal Acetate. Rose Bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodo-fluorescein disodium or RB) is a well-known type II photosensitizer and, thanks to the presence of several chlorines and iodines on the xanthene rings,

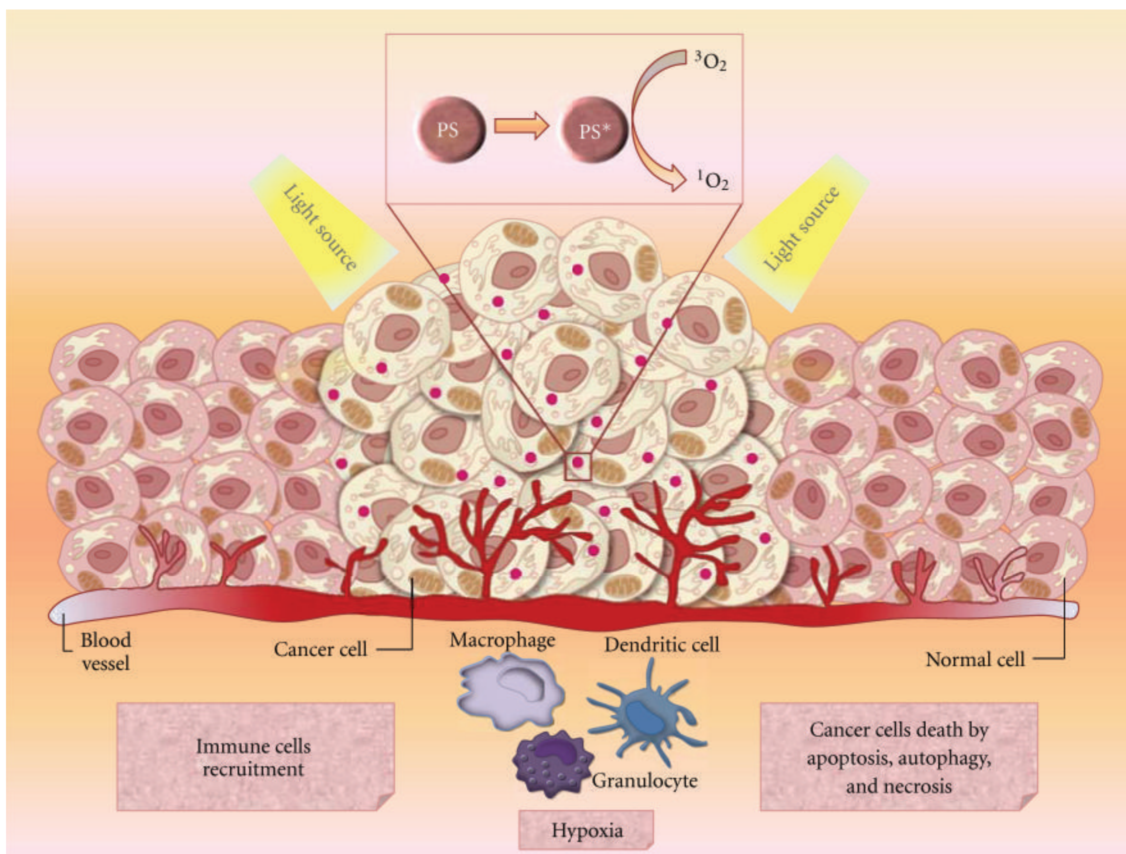


Figure 1: The mechanism of PhotoDynamic Therapy action on cancer cells. Upon irradiation with visible light of a specific wavelength, the Photosensitizer (PS) is excited from a ground state to an excited state, which transfers its energy to tissue ground state triplet oxygen producing singlet oxygen ($^1O_2$), a very reactive chemical specie able to destroy a tumour by multifactorial mechanisms. PDT can directly trigger tumour cells death by induction of apoptosis, autophagy, and necrosis, damage the vasculature, induce hypoxia, and rapidly recruit immune cells, such as macrophages, dendritic cells, and granulocytes.

exhibits facile photocatalytic conversion of triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2{}^*$) [25–27]. This property is achieved upon irradiation with green light [28], since it has an extremely large cross-section in the green (E_M = 99,800 M^{-1} cm^{-1} at 549 nm in water) [29] that is only mildly affected by local environment [26].

As a photosensitizer, RB can be used to kill microorganisms such as viruses [30, 31], Gram-positive bacterial species [32], and protozoa [33]. It can also induce photodynamic effects *in vitro* on red blood cells [34], cardiomyocytes [35], and retinal pigment epithelial cells [36] and *ex vivo* in nerve axon [37], corneal endothelium [38], heart [39], and pancreatic acini [40].

Because of its anionic nature, at low concentrations, RB is inhibited from crossing cell membranes and entering cells in the absence of a carrier [41]. Thus, to favour its intracellular accumulation, several RB hydrophobic derivatives (e.g., acetate or phosphate) have been developed [42].

Addition of acetate groups to the xanthene ring converts the molecule RB into a fluorogenic substrate derivative, RBAc (Figure 2), making it more hydrophobic and improving the molecule's ability to enter the cells.

At the same time, the photophysical (fluorescence emission) and photochemical (photosensitizing) properties of the native PS are quenched. Once inside the cells, the acetate groups are removed by cytoplasmatic carboxylic esterases restoring the native structure as well as the fluorescence and photosensitizing properties of RB. The intracellular accumulation of RBAc depends on the equilibrium between

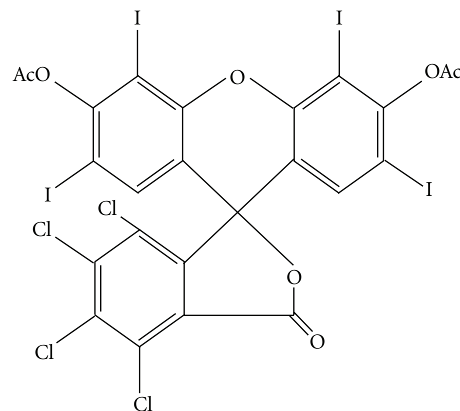


Figure 2: Rose Bengal diacetate structure.

three processes, that is, inactive RBAc influx, active RB restoration, and RB efflux [17].

Restored photoactive RB molecules long persist inside the cells, localizing in endosomes and then undergoing intracellular redistribution, firstly, in the perinuclear region, and finally in the Golgi apparatus and endoplasmic reticulum (ER) except in mitochondria [17, 43–48]. Intracellular RB localization was studied by using fluorescence confocal imaging and colocalization experiments based on organelle-specific dyes in mouse B16 and in human A2780 cells [46] as well as in C6 rat glioma cells and human HeLa cells [48].

In particular, colocalization of RB and Lucifer Yellow, the marker of the fluid phase endocytosis, suggests that, within a few minutes after treatment, RB is found first in endosomal compartment [44, 47] and after 30 minutes diffuses to perinuclear-polar localization. Simultaneously, restored RB molecules were observed in ER as demonstrated by colocalization with $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) an ER marker [47]. The pattern of RB localization does not change after longer incubation times. A dynamic equilibrium in intracellular restored RB distribution is achieved at 60 minutes of incubation, time chosen for *in vitro* RBAc-PDT.

ROS production by RB is achieved upon irradiation by visible green light, whose wavelength ranges between 530 and 560 nm. The minimally penetrating nature of such green light makes RB particularly useful in many cutaneous lesions and dermatological diseases.

3. Cell Death after RBAc-PDT

Following irradiation, PSs can induce organelle photodamage leading to Programmed Cell Death (PCD) (apoptosis, autophagy, and necrosis) in relation to PDT parameters, that is, PS type, its concentration and subcellular localization, and the light dose [49].

HeLa cells photosensitized with RBAc-PDT at 10^{-5} M and 1.6 J/cm^2 green light die by multiple cell death mechanisms (i.e., apoptosis and autophagy) (Figure 3).

3.1. Apoptosis. Apoptosis is the best-studied form of Programmed Cell Death (PCD), playing a pivotal role in pathological and physiological conditions, such as development, cellular homeostasis, and cancer [50]. It is an ATP-dependent process with well-defined morphological and biochemical features. At the morphological level, an apoptotic cell is characterized by chromatin condensation and fragmentation, cell shrinkage, plasma membrane blebbing, apoptotic bodies formation without plasma membrane breakdown, phosphatidylserine exposure on the outer leaflet of the plasma membrane [51]. Apoptotic cells exhibit several biochemical modifications, such as protein cleavage (e.g., PARP cleavage), protein cross-linking, DNA breakdown (characteristic electrophoretic ladder), and phagocytic recognition [52].

A group of cysteine proteases called caspases leads the apoptotic process, linking the initiating stimuli to the final demise of the cell. All caspases, 14 members in human, are synthesized as proenzymes or zymogens and are activated in response to an apoptotic signal. Activated caspases cleave cellular substrates, leading to the biochemical and morphological changes characteristic of apoptosis. Caspases cleavage is an important hallmark of apoptotic death: the activation of initiator caspases (e.g., caspase 8 and caspase-9) leads to the activation of effector caspases (e.g., caspase-3, -6, and -7) and to the extensive morphological modifications [53, 54].

The apoptotic changes in morphology following RBAc-PDT have been largely documented [47, 48, 55, 56].

Rat C6 glioma and human HeLa cells treated with 10^{-5} M RBAc and irradiated with 1.6 J/cm^2 green light produce filopodia, and cytoplasm is vacuolised. In parallel, in HeLa cells, extensive surface blebbing and loss of microvilli occur. Cell morphology changes correlate with cytoskeleton components rearrangement in photosensitized HeLa cells. In particular, microtubules reorganize to form thick bundles concentrated inside the blebs at longer recovery times; on the other hand, also microfilaments form bundles parallel to the plasma membrane and progressively thicker, especially at the cell periphery for increasing post-irradiation times, allowing cell detachment [57].

Moreover, photosensitized HeLa cells show enlarged, swollen, and densely packed ER cisternae, clustered free ribosomes, condensed chromatin, and fragmented nuclei, around which mitochondria cluster becoming rounder and larger with more densely packed cristae and enlarged inner space. The Outer Mitochondrial Membrane (OMM) breaks down, leaving a single layer of membrane with disorganized cristae [48, 55].

Apoptotic mechanisms are very complex and sophisticated, involving an energy-dependent cascade of molecular events. To date, apoptosis can be activated by several pathways: extrinsic or death receptor pathway, intrinsic or mitochondrial pathway, ER stress-mediated pathway, caspase-independent pathway, and caspase-12-dependent pathway.

3.1.1. Extrinsic or Death Receptor Pathway. The extrinsic pathway involves the binding of death ligands to their specific cell surface death receptors (e.g., FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4, and Apo2L/DR5) [58–62]. Death receptors are normally found in monomeric form on the membrane, and the binding with their specific ligands determines the trimerization. The formation of the trimer recruits, at intracytoplasmic level, several molecules of pro-caspase 8 (also known as FLICE) through the formation of a complex called DISC (Death Inducing Signaling Complex). The recruitment of pro-caspase 8 to DISC activates caspase 8 becoming able to directly cleave caspase 3, an effector protein, to complete the death program.

3.1.2. Intrinsic or Mitochondrial Pathway. The intrinsic pathway is triggered in response to both internal insults, such as DNA damage, and extracellular signals or in the absence of growth factors. It requires the Mitochondrial Outer Membrane Permeabilization (MOMP) leading to release of proapoptotic factors, such as cytochrome c. Once released into the cytosol, cytochrome c oligomerizes with Apaf-1 (Apoptotic Protease Activating factor-1) and, in the presence

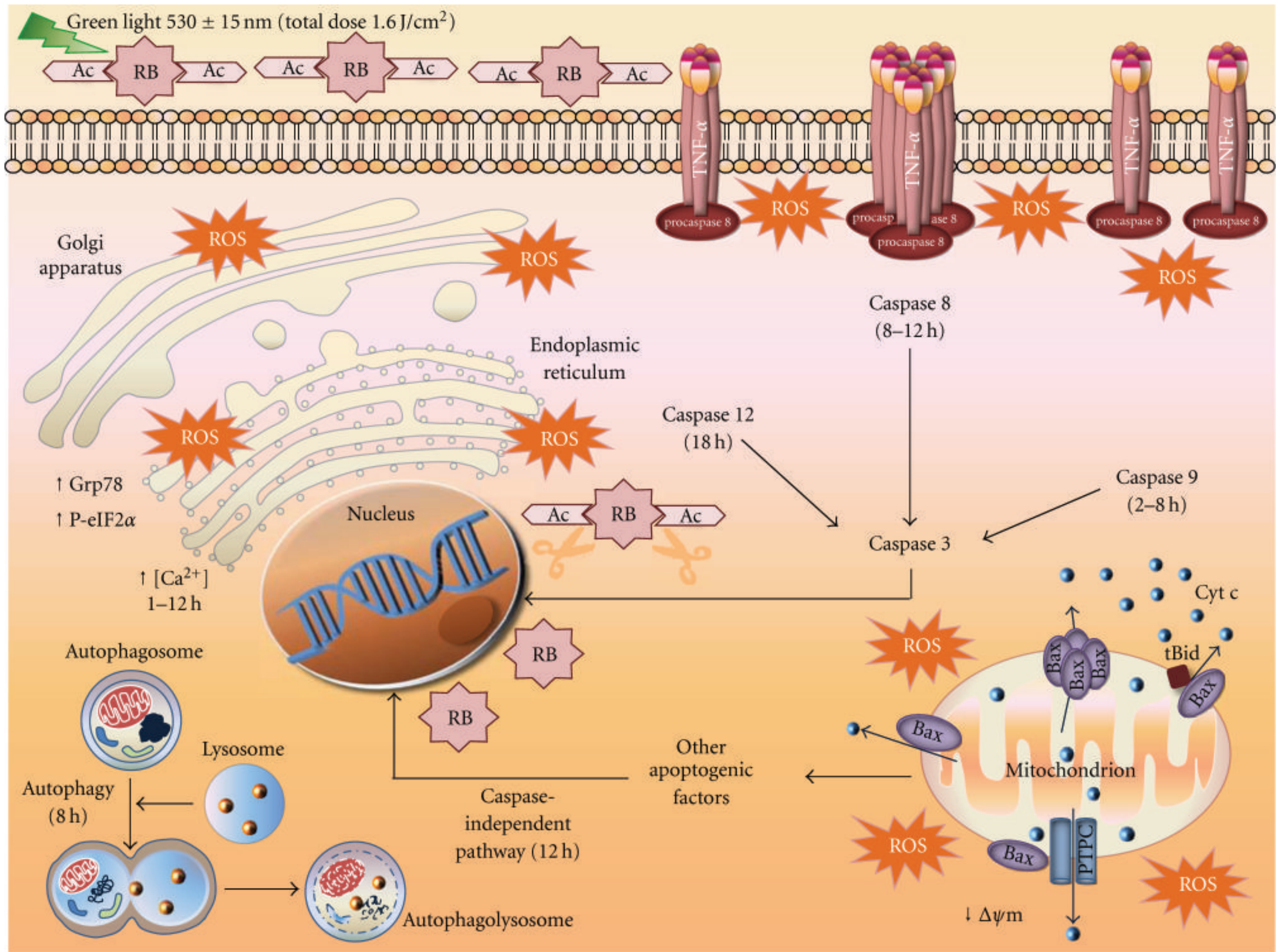


Figure 3: RBAc-PDT-mediated photocytotoxicity in HeLa tumour cells. RBAc (Rose Bengal Acetate) crosses the plasma membrane localizing in perinuclear region. Cytoplasmic esterases remove acetate groups and restore photoactive Rose Bengal molecules, producing ROS when excited by visible green light at 530 ± 15 nm (Total dose 1.6 J/cm^2). RBAc treatment induces cell death in HeLa cells by apoptosis and autophagy in a time-related manner. The onset of apoptosis is sustained by four different pathways. (1) Mitochondrial or intrinsic pathway: Caspase 9 is the first activated caspase (2–8 h after irradiation). Cytosolic Bax monomers translocate to the OMM (Outer Mitochondrial Membrane), $\Delta\psi$m (mitochondrial transmembrane potential) collapses and cytochrome c is released into the cytosol through pores formed by Bax oligomers, Bax-associated PTPC (Permeability Transition Pore Complex), Bax-mediated lipid bilayer destabilization, and Bax-tBid association. (2) Extrinsic pathway: ROS-mediated trimerization of death receptors (such as TNF-α) activates caspase 8 (8–12 h after irradiation). (3) ER stress-mediated pathway: ER (Endoplasmic Reticulum) stress, marked by Grp78 (glucose-regulated protein 78) upregulation and eIF2α (eukaryotic Initiation Factor 2 alpha) phosphorilation, early (1 h) after irradiation, mediates $[Ca^{2+}]_i$ increment at 12 h and caspase 12 cleavage at 18 h after irradiation. (4) Caspase-independent pathway: release of apoptogenic factors from mitochondria, following $\Delta\psi$m drop, triggers chromatin fragmentation without caspase 3 involvement. Autophagic cell death occurs at 8 h after irradiation.

of dATP or ATP, leads to the activation of pro-caspase 9 in a complex called "apoptosome." Finally, caspase 9 is released from the complex Apaf-1/cytochrome c and activates downstream caspases, such as 3, 6, and 7 [50].

3.1.3. ER Stress and Caspase 12-Dependent Pathway. Caspase 12-dependent pathway appears to be triggered by various stimuli that activate ER stress [63]. Caspase 12 is localized at the cytoplasmic face of the ER and is cleaved by the Ca^{2+}-dependent protease m-calpain. Once cleaved, caspase 12 activates caspase 9 without formation of apoptosome [64, 65] or may interact with pro-apoptotic protein Bap31, a 28 kDa integral ER membrane protein containing a cytoplasmic domain that preferentially associates with caspase 8 [66].

3.1.4. Caspase-Independent Pathway. Mitochondria also release proapoptotic proteins, for example, AIF (Apoptosis-Inducing Factor) and EndoG (Endonuclease G), able to trigger apoptosis without caspase involvement (caspase-independent pathway) by translocating to the nucleus where they generate DNA fragmentation [67, 68].

3.1.5. Apoptosis Regulation by Bcl-2 Family Members and Ca^{2+}. Caspases activation is regulated by a variety of factors, among which Bcl-2 family plays a pivotal role [69]. Bcl-2 family comprises anti- and pro-apoptotic, central regulators of the intracellular apoptotic signalling cascades [70]. Due to their ability to form homo- and heterodimers, these proteins function either independently or together in the regulation of apoptosis [71].

MOMP, in apoptosis, is primarily controlled by the Bcl-2 proteins [50, 72]. The antiapoptotic Bcl-2 and Bcl-X_L proteins prevent the release of cytochrome c, while the proapoptotic Bax, Bak, Bad, Bid, and Bim proteins favour its release. Particularly, Bid is cut by caspase 8 in a truncated form (tBid), which translocates from the cytosol to the OMM where, together with Bax and Bak, it induces the release of cytochrome c and other mitochondrial pro-apoptotic factors [73, 74].

Apoptosis can be brought about by a loss of calcium homeostatic control but can also be finely regulated, positively or negatively, by changes in Ca^{2+} distribution in intracellular compartments. The calcium content of the ER, the main store of intracellular calcium, determines the cell sensitivity to apoptotic stress, that, in turn, depends on the cells ability to transfer Ca^{2+} from the ER to the mitochondria. In physiological conditions, Ca^{2+} is pumped into the ER by SERCA ATPases and is released by the opening of inositol 1,4,5-triphosphate receptors ($InsP_3$) [75] and continuously cycles between the ER and mitochondria [76]. In fact, the mitochondrial release of cytochrome c promotes Ca^{2+} conductance through $InsP_3$. Calcium release triggers, in turn, a massive exit of cytochrome c from all mitochondria in the cell, committing the apoptotic process [77].

Bcl-2 family members orchestrate apoptotic machinery also by interfering with calcium flux between ER and mitochondria. Particularly, Bcl-2 located in the ER and in mitochondria enhances the store of Ca^{2+} likely by up-regulating SERCA gene expression. On the other hand, Bax and Bad promote opening of the voltage-dependent anion channel, a component of the Permeability Transition Pore Complex (PTPC), contributing to the release of cytochrome c [78].

3.1.6. RBAc-PDT-Induced Apoptosis. The onset of the intrinsic, extrinsic, ER stress, and caspase-independent pathways during PDT-induced apoptotic cell death has been largely documented (reviewed in [8]).

RBAc-PDT is able to induce apoptosis in HeLa cells through both caspase-dependent and independent pathways [56, 79]. Moreover, during a time course of 72 hours (h) post-irradiation, RBAc-PDT induces the independent activation of multiple apoptotic pathways. Apoptosis occurs as soon as 1 h after PDT by activation of intrinsic pathway, regulated by Bcl-2 family members. Particularly, by opening transition pore complex on the OMM, Bcl-2 pro-apoptotic proteins promote the loss of mitochondrial membrane potential ($\Delta\psi$m) and the consequent release, into the cytosol, of cytochrome c, ending in the cleavage cascade of caspase 9 and 3. The loss of $\Delta\psi$m, examined by MitoTracker Green and JC-1, fluorescent probes exhibiting potential dependent accumulation in mitochondria, occurs as early as 1–4 h after PDT [55, 57, 80], although mitochondria are not the primary target of RBAc. Relocation of Bax from the cytosol to the OMM mediates the drastic $\Delta\psi$m drop, inducing mitochondrial membrane permeabilization (MMP), a crucial lethal event after PDT. Particularly, Bax monomers inserted in OMM form openings both homodimerizing and engaging a close molecular cooperation with proteins of PTPC, such as Adenine Nucleotide Translocator (ANT) or Voltage-Dependent Anion Channel (VDAC), and destabilizing lipid bilayer [81].

Since Bcl-2 can prevent redistribution of Bax to mitochondrial sites, a signal for the loss of $\Delta\psi$m in HeLa cells could be the dramatic and specific RBAc-photo-induced oxidation of Bcl-2 with consequent loss of its function, as suggested by decrement of Bcl-2 in the cytosol [79].

Bid also regulates the intrinsic pathway, acting as molecular cross-talk between extrinsic and intrinsic pathways. Indeed, in HeLa cells committed to apoptosis by RBAc-PDT, activation of caspase 8 occurs from 12 to 72 h when tBid forms increase in membrane proteins pool and simultaneously Hsp70, negative regulator of the intrinsic pathway, largely increases in the cytosol, displacing pro-caspase 9 from apoptosome.

A caspase 12-dependent pathway is also induced in HeLa cells. Caspase 12-dependent apoptosis is triggered by ER stress, whose onset is proved by two ER stress sensors, that is, eIF2α (eukaryotic Initiation Factor 2 alpha) phosphorylation and GRP78 (Glucose Regulated Protein 78) up-regulation [79]. ER stress acts as a critical control point in several apoptotic pathways activated by stimuli causing Ca^{2+} overload or its homeostasis perturbation [82]. In HeLa cells $[Ca^{2+}]_i$, increment confirms the ER key role in RBAc-PDT [79].

Finally, RBAc-PDT triggers apoptosis by a caspase-independent pathway since incubation of RBAc-photosensitized HeLa cells with the pan-caspase inhibitor z-VAD does not completely prevent apoptosis induction [79]. Accordingly, Bottone and coworkers reported in the same experimental system the AIF translocation from the mitochondria to the nucleus from 24 to 72 h after irradiation [56].

The damage of cellular components and the triggering of several signalling pathways depend on the amount and the site of ROS generation [83]. Particularly, cytotoxicity of RBAc-PDT is ROS-mediated, and their generation is upstream to all apoptotic events. In fact, in HeLa cells, the amount of ROS after RBAc-PDT is threefold over that of non-photosensitized ones soon after RBAc Photodynamic treatment [79]. ROS photogeneration provokes delocalization and insertion of Bax on OMM [84] mediating mitochondrial damage and the activating of the intrinsic apoptotic pathway. Moreover, ROS play a role in the trimerization of membrane receptors, favoured by binding of RBAc to plasma membrane and the very short distance of diffusion of singlet oxygen [85].

Interestingly, inhibition of one apoptotic pathway, that is, caspase 9 (Z-LEHD-FMK), caspase 8 (Z-IETD-FMK), and pan-caspases (Z-VAD-FMK), does not impair the activation

of the others, suggesting the independent onset of these pathways ensuring long-term photokilling [79].

3.2. Autophagy. Autophagy or Type II PCD is a self-degradative process for the removal and turnover of damaged organelles and misfolded or aggregated proteins *via* the endosomal-lysosomal system. Although autophagy is generally thought of as a cell survival strategy, it has also been linked to PCD triggered by the metabolic and bioenergetic collapse. On the basis of morphological studies, autophagic cell death is characterized by the absence of chromatin condensation and massive cytoplasmic vacuolization [86]. Autophagic vacuole formation and lysosome delivery define three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. The first type, conserved from yeast to mammals, is mediated by double-membrane-bounded vacuoles, termed autophagosomes [87, 88]. The early autophagosomes derived from specialized membrane cisternae of not yet clarified origin, called phagophore, contributed by the ER and/or the trans-Golgi and endosomes [89, 90]. The phagophore expands to enclose cellular cargo, such as aggregated or misfolded proteins and damaged organelles, resulting in the formation of the late autophagosome. Then, the outer membrane of autophagosome fuses with a lysosome to form an autolysosome, leading to the degradation of the sequestred cytoplasmic material [91]. The whole process is ATP dependent and requires cytoskeletal proteins to permit the movement of the vacuolar system [92, 93].

In microautophagy, the lysosome itself takes up cytosolic components through invagination of the lysosomal membrane [94]. Finally, Chaperone-Mediated Autophagy (CMA) involves the direct translocation of cytosolic proteins containing the pentapeptide motif KFERQ across the lysosomal membrane in a complex with chaperone proteins recognized by the Lysosomal-Associated Membrane Receptor 2A (LAMP-2A) [95].

The most well-known inducer of autophagy is nutrient starvation, but it can also be activated by other physiological stress stimuli (e.g., hypoxia, energy depletion, ER stress, and high temperature), hormonal stimulation, and pharmacological agents [96].

The role of autophagy in PDT-treated cells is controversial, but several studies suggest the relationship, probably based on ROS generation, between autophagy and cell death [97]. In fact, due to the high reactivity of photogenerated ROS, autophagy is initiated to remove and degrade oxidatively damaged organelles and aggregated proteins produced by photochemical reactions [98]. However, ROS can stimulate autophagy both to protect the cells or to commit them to their final demise [99]. Autophagic cell death occurs in RBAc photosensitized HeLa cells probably to eliminate damaged mitochondria or ER, as demonstrated by TEM analysis showing characteristic double membrane autophagosomes. To date, the microtubule-associated protein Light Chain 3 (LC3), a mammalian homologue of yeast Atg8, is the widely used marker for autophagosome: induction of autophagy in RBAc-treated HeLa cells is supported by the conversion of LC3BI (localized in the cytosol) to LC3BII (localized on autophagosome membranes) at 8 h after irradiation [79, 100].

It has been recently reported that intrinsic pathway-induced apoptosis and ER damage-induced autophagy can be simultaneously induced by PDT [101–103]. Interestingly, in RBAc-PDT, the two forms (apoptosis and autophagy) are activated independently at different time points, ensuring cell death when one of them is inactivated [79].

3.3. Necrosis. Necrosis or type III PCD is morphologically characterized by cell and organelles volume swelling, cytoplasm vacuolization, plasma membrane breakdown, and subsequent intracellular content loss resulting in an inflammatory reaction. Necrosis is considered as an accidental and uncontrolled cell death modality, but recent evidence indicates that it can also be regulated by specific signal transduction pathways initiated by death domain and Toll-like receptors [104, 105]. If apoptosis is the preferential cell death modality during PDT, a shift from apoptosis to necrosis strictly depends on PDT dose (e.g., light dose and PS concentration increment). Particularly, RBAc 10^{-5} M in HeLa cells induces negligible necrosis only due to the absence of apoptotic cell clearance by phagocytes. On the other hand, limited necrotic cell death depends probably also on the removal of ROS damage by autophagy [79].

4. Conclusions

Recent evidence indicates that PDT can evoke apoptosis, autophagy, and necrosis, which could explain why, in some experimental protocols, the specific inhibition of one death mechanism is not sufficient to block PDT-mediated cell death.

In the attempt to design new fluorogenic substrates useful in PDT, it is the goal to select PSs efficient in inducing cell death. The photosensitizer RB efficiently triggers multiple cell death types, that is, apoptosis, autophagy, and necrosis, occurring independently from each other. It is already well known that RB has minimal side effects, such as prolonged photosensitivity, due to its photobleaching property [17] and facile photocatalytic conversion [26] upon irradiation with minimally penetrating green light [24]. This latter property is particularly relevant for treatment of many dermatologic conditions, such as psoriasis and actinic keratosis [106]. Recent findings suggest RB as a very promising PDT agent since it ensures long-term cytotoxic effects by inducing, at different time points, both apoptotic and autophagic cell death. Moreover, considering that apoptosis is the preferred mechanism to cell death in RBAc-PDT-treated HeLa cells, the independent temporal activation of the different apoptotic pathways ensures cell death when one or several of them are inactivated. Then, the development of new therapeutic protocols in PDT strictly depends on knowledge of the molecular differences and cross-communication between the different cell death programs. For the clinical application of PDT, it is crucial to set up the conditions inducing apoptosis rather than necrosis. In fact, apoptotic programs entail the rapid and safe clearance of dead cells by phagocytes;

conversely, the removal of necrotic cells may be less effective and leads to the inflammatory response.

In addition, the clearance of killed cells is crucial for *in vivo* PDT application, since a defective removal of dead cells may be responsible for the alteration of immune system responses leading to the onset of autoimmune diseases. Moreover, the efficiency of clearance is strictly dependent on cell death type induced. Thus, the knowledge of how cancer cells die after PDT could also clarify the impact of different cell death PDT induced on the immune system responses and therapeutic outcome. In conclusion, on the basis of all above-reported data, RBAc is a powerful cytotoxic PDT agent since it induces a long-lasting and time-related cell death originating from or converging on multiple damaged organelles, that is, mitochondria, lysosomes, Golgi apparatus, and ER, independently from its first perinuclear intracellular localisation (Figure 3).

References

[1] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[2] G. P. Dunn, A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber, "Cancer immunoediting: from immunosurveillance to tumor escape," *Nature Immunology*, vol. 3, no. 11, pp. 991–998, 2002.

[3] K. Plaetzer, B. Krammer, J. Berlanda, F. Berr, and T. Kiesslich, "Photophysics and photochemistry of photodynamic therapy: fundamental aspects," *Lasers in Medical Science*, vol. 24, no. 2, pp. 259–268, 2009.

[4] Z. Luksiene, "Photodynamic therapy: mechanism of action and ways to improve the efficiency of treatment," *Medicina (Kaunas, Lithuania)*, vol. 39, no. 12, pp. 1137–1150, 2003.

[5] R. L. Lipson and E. J. Baldes, "The photodynamic properties of a particular hematoporphyrin derivative," *Archives of Dermatology*, vol. 82, no. 4, pp. 508–516, 1960.

[6] T. J. Dougherty, J. E. Kaufman, A. Goldfarb et al., "Photoradiation therapy for the treatment of malignant tumors," *Cancer Research*, vol. 38, no. 8, pp. 2628–2635, 1978.

[7] T. J. Dougherty, C. J. Gomer, B. W. Henderson et al., "Photodynamic therapy," *Journal of the National Cancer Institute*, vol. 90, no. 12, pp. 889–905, 1998.

[8] E. Buytaert, M. Dewaele, and P. Agostinis, "Molecular effectors of multiple cell death pathways initiated by photodynamic therapy," *Biochimica et Biophysica Acta*, vol. 1776, no. 1, pp. 86–107, 2007.

[9] P. Castano, P. Mroz, and M. R. Hamblin, "Photodynamic therapy and anti-tumour immunity," *Nature Reviews Cancer*, vol. 6, no. 7, pp. 535–545, 2006.

[10] M. Korbelik, "PDT-associated host response and its role in the therapy outcome," *Lasers in Surgery and Medicine*, vol. 38, no. 5, pp. 500–508, 2006.

[11] P. Castano, T. N. Demidova, and M. R. Hamblin, "Mechanisms in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization," *Photodiagnosis and Photodynamic Therapy*, vol. 1, no. 4, pp. 279–293, 2004.

[12] M. R. Detty, S. L. Gibson, and S. J. Wagner, "Current clinical and preclinical photosensitizers for use in photodynamic therapy," *Journal of Medicinal Chemistry*, vol. 47, no. 16, pp. 3897–3915, 2004.

[13] R. M. Szeimies, S. Karrer, C. Abels et al., "9-acetoxy-2,7,12,17-tetrakis-(β-methoxyethyl)-porphycene(ATMPn), a novel photosensitizer for photodynamic therapy: uptake kinetics and intracellular localization," *Journal of Photochemistry and Photobiology B: Biology*, vol. 34, no. 1, pp. 67–72, 1996.

[14] V. Král, J. Davis, A. Andrievsky et al., "Synthesis and biolocalization of water-soluble sapphyrins," *Journal of Medicinal Chemistry*, vol. 45, no. 5, pp. 1073–1078, 2002.

[15] P. Agostinis, A. Vantieghem, W. Merlevede, and P. A. M. de Witte, "Hypericin in cancer treatment: more light on the way," *International Journal of Biochemistry and Cell Biology*, vol. 34, no. 3, pp. 221–241, 2002.

[16] J. C. Stockert, A. Juarranz, A. Villanueva, and M. Cañete, "Photodynamic damage to HeLa cell microtubules induced by thiazine dyes," *Cancer Chemotherapy and Pharmacology*, vol. 39, no. 1-2, pp. 167–169, 1996.

[17] G. Bottiroli, A. C. Croce, P. Balzarini et al., "Enzyme-assisted cell photosensitization: aproposal for an efficient approach to tumor therapy and diagnosis. The Rose Bengal fluorogenic substrate," *Photochemistry and Photobiology*, vol. 66, no. 3, pp. 374–383, 1997.

[18] L. Brancaleon and H. Moseley, "Laser and non-laser light sources for photodynamic therapy," *Lasers in Medical Science*, vol. 17, no. 3, pp. 173–186, 2002.

[19] B. W. Henderson and T. J. Dougherty, "How does photodynamic therapy work?" *Photochemistry and Photobiology*, vol. 55, no. 1, pp. 145–157, 1992.

[20] A. Greer, "Christopher Foote's discovery of the role of singlet oxygen [$^1o_2(^1\Delta_g)$] in photosensitized oxidation reactions," *Accounts of Chemical Research*, vol. 39, no. 11, pp. 797–804, 2006.

[21] K. J. Davies, "Oxidative stress: the paradox of aerobic life," *Biochemical Society Symposium*, vol. 61, pp. 1–31, 1995.

[22] M. Sundaresan, Z. X. Yu, V. J. Ferrans, K. Irani, and T. Finkel, "Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction," *Science*, vol. 270, no. 5234, pp. 296–299, 1995.

[23] B. Demple and L. Harrison, "Repair of oxidative damage to DNA: enzymology and biology," *Annual Review of Biochemistry*, vol. 63, pp. 915–948, 1994.

[24] J. Fang, T. Seki, and H. Maeda, "Therapeutic strategies by modulating oxygen stress in cancer and inflammation," *Advanced Drug Delivery Reviews*, vol. 61, no. 4, pp. 290–302, 2009.

[25] J. Paczkowski, J. J. M. Lamberts, B. Paczkowska, and D. C. Neckers, "Photophysical properties of Rose Bengal and its derivatives (XII)," *Journal of Free Radicals in Biology and Medicine*, vol. 1, no. 5-6, pp. 341–351, 1985.

[26] D. C. Neckers, "Rose Bengal," *Journal of Photochemistry and Photobiology*, vol. 47, no. 1, pp. 1–29, 1989.

[27] I. E. Kochevar, C. R. Lambert, M. C. Lynch, and A. C. Tedesco, "Comparison of photosensitized plasma membrane damage caused by singlet oxygen and free radicals," *Biochimica et Biophysica Acta*, vol. 1280, no. 2, pp. 223–230, 1996.

[28] P. C. Lee and M. A. J. Rodgers, "Laser flash photokinetic studies of Rose Bengal sensitized photodynamic interactions of nucleotides and DNA," *Photochemistry and Photobiology*, vol. 45, no. 1, pp. 79–86, 1987.

[29] N. Houba-Herin, C. M. Calberg-Bacq, J. Piette, and A. van de Vorst, "Mechanisms for dye-mediated photodynamic action: singlet oxygen production, deoxyguanosine oxidation and phage inactivating efficiencies," *Photochemistry and Photobiology*, vol. 36, no. 3, pp. 297–306, 1982.

[30] J. Lenard, A. Rabson, and R. Vanderoef, "Photodynamic inactivation of infectivity of human immunodeficiency virus and

other enveloped viruses using hypericin and Rose Bengal: inhibition of fusion and syncytia formation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 1, pp. 158–162, 1993.

[31] J. Chodosh, M. C. Banks, and W. G. Stroop, "Rose Bengal inhibits herpes simplex virus replication in vero and human corneal epithelial cells in vitro," *Investigative Ophthalmology and Visual Science*, vol. 33, no. 8, pp. 2520–2527, 1992.

[32] T. A. Dahl, W. R. Midden, and D. C. Neckers, "Comparison of photodynamic action by Rose Bengal in gram-positive and gram-negative bacteria," *Photochemistry and Photobiology*, vol. 48, no. 5, pp. 607–612, 1988.

[33] F. S. Cruz, L. A. Lopes, and W. de Souza, "The photodynamic action of Rose Bengal on *Trypanosoma cruzi*," *Acta Tropica*, vol. 41, no. 2, pp. 99–108, 1984.

[34] D. P. Valenzeno and J. P. Pooler, "Cell membrane photomodification: relative effectiveness of halogenated fluoresceins for photohemolysis," *Photochemistry and Photobiology*, vol. 35, no. 3, pp. 343–350, 1982.

[35] L. ver Donck, J. van Reempts, G. Vandeplassche, and M. Borgers, "A new method to study activated oxygen species induced damage in cardiomyocytes and protection by Ca^{2+}-antagonists," *Journal of Molecular and Cellular Cardiology*, vol. 20, no. 9, pp. 811–823, 1988.

[36] I. A. Menon, P. K. Basu, S. D. Persad, S. Rosatone, and J. D. Wiltshire, "A study on the sequence of phototoxic effects of Rose Bengal using retinal pigment epithelial cells in vitro," *Experimental Eye Research*, vol. 49, no. 1, pp. 67–73, 1989.

[37] J. P. Pooler and D. P. Valenzeno, "Kinetic factors governing sensitized photooxidation of excitable cell membranes," *Photochemistry and Photobiology*, vol. 28, no. 2, pp. 219–226, 1978.

[38] D. S. Hull, E. C. Strickland, and K. Green, "Photodynamically induced alteration of cornea endothelial cell function," *Investigative Ophthalmology and Visual Science*, vol. 18, no. 12, pp. 1226–1231, 1979.

[39] D. J. Hearse, Y. Kusama, and M. Bernier, "Rapid electrophysiological changes leading to arrhythmias in the aerobic rat heart. Photosensitization studies with Rose Bengal-derived reactive oxygen intermediates," *Circulation Research*, vol. 65, no. 1, pp. 146–153, 1989.

[40] E. K. Matthews and Z. J. Cui, "Photodynamic action of Rose Bengal on isolated rat pancreatic acini: stimulation of amylase release," *FEBS Letters*, vol. 256, no. 1-2, pp. 29–32, 1989.

[41] A. C. Croce, E. Wyroba, and G. Bottiroli, "Distribution and retention of Rose Bengal and disulphonated aluminium phthalocyanine: a comparative study in unicellular eukaryote," *Journal of Photochemistry and Photobiology*, vol. 16, no. 3-4, pp. 318–330, 1992.

[42] D. K. Luttrull, O. Valdes-Aguilera, S. M. Linden, J. Paczkowski, and D. C. Neckers, "Rose Bengal aggregation in rationally synthesized dimeric systems," *Photochemistry and Photobiology*, vol. 47, no. 4, pp. 551–557, 1988.

[43] G. Bottiroli, A. C. Croce, P. Baglioni et al., "Fluorogenic substrates for diagnosis and photodynamic treatment of tumors," 2000, US Patent 6036941.

[44] G. Bottiroli, A. C. Croce, M. Biggiogera et al., "Photosensitizer damage targets in Rose Bengal acetate treated cells," *Lasers in Surgery and Medicine*, no. 173, pp. 13–41, 2001.

[45] A. C. Croce, D. Locatelli, M. Monici et al., "Application of Xuorogenic substrates to photodynamic therapy: Rose Bengal acetate," *Lasers in Surgery and Medicine*, no. 121, pp. 9–26, 1997.

[46] A. C. Croce, R. Supino, K. S. Lanza, D. Locatelli, P. Baglioni, and G. Bottiroli, "Photosensitizer accumulation in spontaneous multidrug resistant cells: a comparative study with Rhodamine 123, Rose Bengal acetate and Photofrin," *Photochemical and Photobiological Sciences*, vol. 1, no. 1, pp. 71–78, 2002.

[47] C. Soldani, M. G. Bottone, A. C. Croce, A. Fraschini, G. Bottiroli, and C. Pellicciari, "The Golgi apparatus is a primary site of intracellular damage after photosensitization with Rose Bengal acetate," *European Journal of Histochemistry*, vol. 48, no. 4, pp. 443–448, 2004.

[48] C. Soldani, M. G. Bottone, A. C. Croce et al., "Apoptosis in tumour cells photosensitized with Rose Bengal acetate is induced by multiple organelle photodamage," *Histochemistry and Cell Biology*, vol. 128, no. 5, pp. 485–495, 2007.

[49] N. L. Oleinick and H. H. Evans, "The photobiology of photodynamic therapy: cellular targets and mechanisms," *Radiation Research*, vol. 150, supplement 5, pp. S146–S156, 1998.

[50] N. N. Danial and S. J. Korsmeyer, "Cell death: critical control points," *Cell*, vol. 116, no. 2, pp. 205–219, 2004.

[51] G. Häcker, "The morphology of apoptosis," *Cell and Tissue Research*, vol. 301, no. 1, pp. 5–17, 2000.

[52] M. O. Hengartner, "The biochemistry of apoptosis," *Nature*, vol. 407, no. 6805, pp. 770–776, 2000.

[53] D. W. Nicholson, "Caspase structure, proteolytic substrates, and function during apoptotic cell death," *Cell Death and Differentiation*, vol. 6, no. 11, pp. 1028–1042, 1999.

[54] I. N. Lavrik, A. Golks, and P. H. Krammer, "Caspase: pharmacological manipulation of cell death," *Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2665–2672, 2005.

[55] M. G. Bottone, C. Soldani, A. Fraschini et al., "Enzyme-assisted photosensitization with Rose Bengal acetate induces structural and functional alteration of mitochondria in HeLa cells," *Histochemistry and Cell Biology*, vol. 127, no. 3, pp. 263–271, 2007.

[56] M. G. Bottone, C. Soldani, A. Fraschini et al., "Enzyme-assisted photosensitization activates different apoptotic pathways in Rose Bengal acetate treated HeLa cells," *Histochemistry and Cell Biology*, vol. 131, no. 3, pp. 391–399, 2009.

[57] E. Panzarini, B. Tenuzzo, F. Palazzo, A. Chionna, and L. Dini, "Apoptosis induction and mitochondria alteration in human HeLa tumour cells by photoproducts of Rose Bengal acetate," *Journal of Photochemistry and Photobiology B*, vol. 83, no. 1, pp. 39–47, 2006.

[58] Y. Chicheportiche, P. R. Bourdon, H. Xu et al., "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis," *Journal of Biological Chemistry*, vol. 272, no. 51, pp. 32401–32410, 1997.

[59] A. Ashkenazi and V. M. Dixit, "Death receptors: signaling and modulation," *Science*, vol. 281, no. 5381, pp. 1305–1308, 1998.

[60] M. E. Peter and P. H. Krammer, "Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis," *Current Opinion in Immunology*, vol. 10, no. 5, pp. 545–551, 1998.

[61] A. Suliman, A. Lam, R. Datta, and R. K. Srivastava, "Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways," *Oncogene*, vol. 20, no. 17, pp. 2122–2133, 2001.

[62] F. Rubio-Moscardo, D. Blesa, C. Mestre et al., "Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes," *Blood*, vol. 106, no. 9, pp. 3214–3222, 2005.

[63] T. Nakagawa, H. Zhu, N. Morishima et al., "Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-β," *Nature*, vol. 403, no. 6765, pp. 98–103, 2000.

[64] N. Morishima, K. Nakanishi, H. Takenouchi, T. Shibata, and Y. Yasuhiko, "An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12," *Journal of Biological Chemistry*, vol. 277, no. 37, pp. 34287–34294, 2002.

[65] R. V. Rao, A. Peel, A. Logvinova et al., "Coupling endoplasmic reticulum stress to the cell death program: role of the ER chaperone GRP78," *FEBS Letters*, vol. 514, no. 2-3, pp. 122–128, 2002.

[66] F. W. Ng, M. Nguyen, T. Kwan et al., "p28 Bap31, a Bcl-2/Bcl-X(L)- and procaspase-8-associated protein in the endoplasmic reticulum," *Journal of Cell Biology*, vol. 139, no. 2, pp. 327–338, 1997.

[67] S. A. Susin, E. Daugas, L. Ravagnan et al., "Two distinct pathways leading to nuclear apoptosis," *Journal of Experimental Medicine*, vol. 192, no. 4, pp. 571–580, 2000.

[68] L. Y. Li, X. Luo, and X. Wang, "Endonuclease G is an apoptotic DNase when released from mitochondria," *Nature*, vol. 412, no. 6842, pp. 95–99, 2001.

[69] A. Burlacu, "Regulation of apoptosis by Bcl-2 family proteins," *Journal of Cellular and Molecular Medicine*, vol. 7, no. 3, pp. 249–257, 2003.

[70] T. Möröy and M. Zörnig, "Regulators of life and death: the Bcl-2 gene family," *Cellular Physiology and Biochemistry*, vol. 6, no. 6, pp. 312–336, 1996.

[71] Y. Tsujimoto, "Cell death regulation by the Bcl-2 protein family in the mitochondria," *Journal of Cellular Physiology*, vol. 195, no. 2, pp. 158–167, 2003.

[72] B. Leber, J. Lin, and D. W. Andrews, "Embedded together: the life and death consequences of interaction of the Bcl-2 family with membranes," *Apoptosis*, vol. 12, no. 5, pp. 897–911, 2007.

[73] H. Puthalakath and A. Strasser, "Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins," *Cell Death and Differentiation*, vol. 9, no. 5, pp. 505–512, 2002.

[74] C. Martinou and D. R. Green, "Breaking the mitochondrial barrier," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 1, pp. 63–67, 2001.

[75] M. J. Berridge, P. Lipp, and M. D. Bootman, "The versatility and universality of calcium signalling," *Nature Reviews Molecular Cell Biology*, vol. 1, no. 1, pp. 11–21, 2000.

[76] R. Rizzuto, M. Brini, M. Murgia, and T. Pozzan, "Microdomains with high Ca^{2+} close to IP3-sensitive channels that are sensed by neighboring mitochondria," *Science*, vol. 262, no. 5134, pp. 744–747, 1993.

[77] M. P. Mattson and S. L. Chan, "Calcium orchestrates apoptosis," *Nature Cell Biology*, vol. 5, no. 12, pp. 1041–1043, 2003.

[78] S. Shimizu, M. Narita, and Y. Tsujimoto, "Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC," *Nature*, vol. 399, no. 6735, pp. 483–487, 1999.

[79] E. Panzarini, V. Inguscio, and L. Dini, "Timing the multiple cell death pathways initiated by Rose Bengal acetate photodynamic therapy," *Cell Death and Disease*, vol. 2, no. 6, article e169, 2011.

[80] E. Panzarini, B. Tenuzzo, and L. Dini, "Photodynamic therapy-induced apoptosis of HeLa cells," *Annals of the New York Academy of Sciences*, vol. 1171, pp. 617–626, 2009.

[81] D. R. Green and G. Kroemer, "The pathophysiology of mitochondrial cell death," *Science*, vol. 305, no. 5684, pp. 626–629, 2004.

[82] N. Demaurex and C. Distelhorst, "Cell biology: apoptosis—the calcium connection," *Science*, vol. 300, no. 5616, pp. 65–67, 2003.

[83] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," *Nature*, vol. 408, no. 6809, pp. 239–247, 2000.

[84] L. Sun, T. Chen, X. Wang, Y. Chen, and X. Wei, "Bufalin induces reactive oxygen species dependent Bax translocation and apoptosis in ASTC-a-1 Cells," *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, 12 pages, 2011.

[85] S. Zhuang and I. E. Kochevar, "Ultraviolet a radiation induces rapid apoptosis of human leukemia cells by fas ligand-independent activation of the fas death pathway," *Photochemistry and Photobiology*, vol. 78, no. 1, pp. 61–67, 2003.

[86] G. Kroemer, L. Galluzzi, P. Vandenabeele et al., "Classification of cell death: recommendations of the nomenclature committee on cell death 2009," *Cell Death and Differentiation*, vol. 16, no. 1, pp. 3–11, 2009.

[87] D. J. Klionsky, A. M. Cuervo, and P. O. Seglen, "Methods for monitoring autophagy from yeast to human," *Autophagy*, vol. 3, no. 3, pp. 181–206, 2007.

[88] D. J. Klionsky, H. Abeliovich, P. Agostinis et al., "Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes," *Autophagy*, vol. 4, no. 2, pp. 151–175, 2008.

[89] E. L. Axe, S. A. Walker, M. Manifava et al., "Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum," *Journal of Cell Biology*, vol. 182, no. 4, pp. 685–701, 2008.

[90] A. Simonsen and S. A. Tooze, "Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes," *Journal of Cell Biology*, vol. 186, no. 6, pp. 773–782, 2009.

[91] N. Mizushima, "Autophagy: process and function," *Genes and Development*, vol. 21, no. 22, pp. 2861–2873, 2007.

[92] M. Fengsrud, M. L. Sneve, A. Overbye et al., "Structural aspects of mammalian autophagy," in *Autophagy*, D. Klionsky, Ed., pp. 11–25, Landes Bioscience, Georgetown, Tex, USA, 2004.

[93] P. Codogno and A. J. Meijer, "Signaling pathways in mammalian autophagy," in *Autophagy*, D. Klionsky, Ed., pp. 26–47, Landes Bioscience, Georgetown, Tex, USA, 2004.

[94] C. W. Wang and D. L. Klionsky, "Microautophagy," in *Autophagy*, D. Klionsky, Ed., pp. 107–125, Landes Bioscience, Georgetown, Tex, USA, 2004.

[95] P. Saftig, W. Beertsen, and E. L. Eskelinen, "LAMP-2: a control step fot phagosome and autophagosome maturation," *Autophagy*, vol. 4, no. 4, pp. 510–512, 2008.

[96] W. Bursch, A. Karwan, M. Mayer et al., "Cell death and autophagy: cytokines, drugs, and nutritional factors," *Toxicology*, vol. 254, no. 3, pp. 147–157, 2008.

[97] D. Kessel and N. L. Oleinick, "Initiation of autophagy by photodynamic therapy," *Methods in Enzymology*, vol. 453, pp. 1–16, 2009.

[98] M. Dewaele, W. Martinet, N. Rubio et al., "Autophagy pathways activated in response to PDT contribute to cell resistance against ROS damage," *Journal of Cellular and Molecular Medicine*, vol. 15, no. 6, pp. 1402–1414, 2011.

[99] R. Scherz-Shouval and Z. Elazar, "ROS, mitochondria and the regulation of autophagy," *Trends in Cell Biology*, vol. 17, no. 9, pp. 422–427, 2007.

[100] L. Dini, V. Inguscio, B. Tenuzzo, and E. Panzarini, "Rose Bengal acetate photodynamic therapy-induced autophagy," *Cancer Biology and Therapy*, vol. 10, no. 10, pp. 1048–1055, 2010.

[101] J. J. Reiners Jr, P. Agostinis, K. Berg, N. L. Oleinick, and D. Kessel, "Assessing autophagy in the context of photodynamic therapy," *Autophagy*, vol. 6, no. 1, pp. 7–18, 2010.

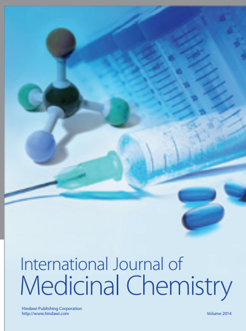
[102] D. Kessel, "Protection of Bcl-2 by salubrinal," *Biochemical and Biophysical Research Communications*, vol. 346, no. 4, pp. 1320–1323, 2006.

[103] D. Kessel, M. G. Vicente, and J. J. Reiners Jr, "Initiation of apoptosis and autophagy by photodynamic therapy," *Lasers in Surgery and Medicine*, vol. 38, no. 5, pp. 482–488, 2006.

[104] P. Golstein and G. Kroemer, "Cell death by necrosis: towards a molecular definition," *Trends in Biochemical Sciences*, vol. 32, no. 1, pp. 37–43, 2007.

[105] N. Festjens, T. V. Berghe, and P. Vandenabeele, "Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response," *Biochimica et Biophysica Acta*, vol. 1757, no. 9-10, pp. 1371–1387, 2006.

[106] E. Wachter, C. Dees, J. Harkins et al., "Topical Rose Bengal: pre-clinical evaluation of pharmacokinetics and safety," *Lasers in Surgery and Medicine*, vol. 32, no. 2, pp. 101–110, 2003.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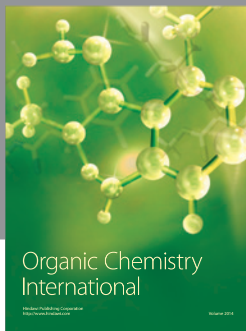
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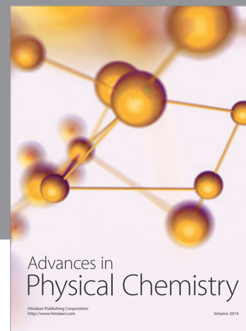
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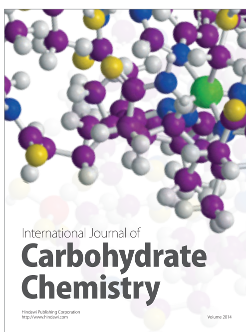
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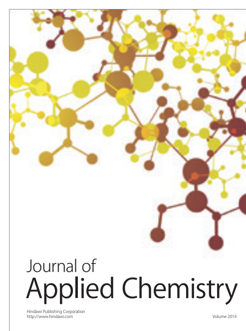
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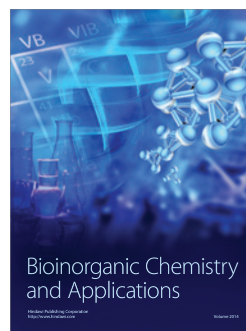
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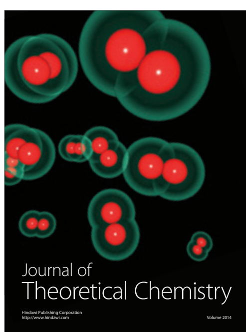
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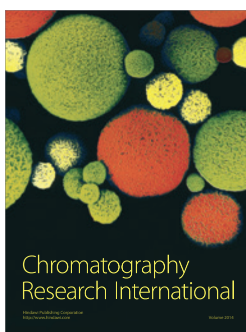
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