Combination of Photodynamic Therapy with Anti-Cancer Agents

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Abstract: Degenerative diseases such as cancer usually involve more than one pathological process. Therefore, attempts to combat such diseases with monotherapeutic approaches may not always do so efficiently. For this reason, the use of combination therapy with modalities that target different disease pathways represents an alternative strategy. Photodynamic therapy (PDT) has already been established as an alternative therapy for the treatment of various types of malignant disorders, including oesophageal, lung and bladder cancer as well as other degenerative diseases. This technique involves the administration of a tumor localizing photosensitizer followed by its activation with light of a specific wavelength. In the presence of tissue oxygen, the photoactive sensitizer triggers a series of photochemical and photobiological processes that may lead to direct cancer cell damage, tumor microvascular occlusion and host immune response. Due to these multiple actions, PDT has increasingly gained recognition as a potential adjuvant for conventional cancer treatments. Several preclinical studies and some clinical trials suggest that the use of PDT in combination with established treatments or with newly-developed modalities may be of benefit as compared to the individual modalities. In this review, we briefly introduce the reader to the main photobiological aspects of PDT, and then discuss the use of PDT in combination with other pharmacological approaches for the treatment of cancer.

Keywords: Combination therapy, photodynamic therapy, chemotherapy, immunotherapy, angiogenesis inhibitors, cancer, synergies.

INTRODUCTION

Most diseases of interest for contemporary drug development involve multiple, distinct pathological processes [1]. Therefore, the use of combination therapies with different mechanisms of action might offer potential advantages over a single therapy. This may explain why despite the pharmaceutical industry's crusade for single molecule blockbusters [2], combination therapies have increasingly received attention in the last few years. In this context, the ultimate goal of an association of two different therapeutic approaches is an enhancement or even synergistic effect as compared to each individual treatment without increasing the number of side effects. An extensive review on calculations of synergistic or additional effects based on experimental data has been published by Greco *et al.* [3]. Assessing the nature and intensity of agent interactions is universal and especially critical in the treatment of many human diseases. There are various approaches to determining synergy, antagonism, or additional effects of therapeutic compounds and the reader is referred to the above-mentioned reference. In the literature, most concentration effect models and curves are based on monotonically increasing effects. In clinical as well as preclinical studies, apparent toxic effects and, therefore, decreasing efficacy with increasing drug dose will make the assessment of synergy more complicated and will not be discussed in this review. Assuming that in a combinational treatment a drug is not interacting with itself, the problem of drug interaction is a three dimensional problem with the treatment efficacy as a function of two individual variables, i.e. the doses of each drug applied. In this context, most concepts are based on methodologies developed by Loewe and Muischnek in 1926 [4]. Graphically, these methodologies can be explained by so-called isobolograms (see Fig. (**1**)). In these, each axis represents the concentration of each drug and the isoline in this plot represents the line of a given therapeutic effect, e.g. 50% growth inhibition, 25% of induction of protein expression, etc. As a prime example, Fig. (**1**) shows the graphical representation of such an isobologram for two hypothetical drugs that inhibit the growth of cancer cells *in vitro*. The isoline is the projection of 50% growth inhibition. From Fig. (**1**), it becomes clear that drugs 1 and 2 have a single effective concentration for 50% growth inhibition of 20 mg/ml and 100 mg/ml, respectively. If no interaction between both drugs exists, any combination of drug concentrations that fall on the connecting line between these two values should give a 50% growth inhibition (e.g. 10 mg /ml of Drug 1 and 50 mg/ml of Drug 2). However, if the efficacy of the combined treatment is higher or lower, the isoline for this combination should fall under the straight line or vice versa, indicating synergy or antagonism, respectively. These graphic considerations can be put into a basic mathematical assessment approach:

$$1 = \frac{D_1}{ID_{X,1}} + \frac{D_2}{ID_{X,2}}$$

Whereas D_1 and D_2 represent the concentrations of each drug in the mixture, and $ID_{X,1}$ and $ID_{X,2}$ are the concentrations of each drug that result in X% of the inhibition when given alone. When the right hand side of the equation (equal to the combination index (CI) of Berenbaum [5] is less than 1, then synergism is indicated. However, if this value is higher than one, antagonism is indicated.

Photodynamic therapy (PDT) has been recently evaluated as an adjuvant therapy to other therapeutic modalities, including surgery, hyperthermia, radiotherapy, immunotherapy, and chemotherapy as new approaches for the treatment of a variety of cancers and non-malignant disorders [6-8].

In PDT, two individually non-toxic components are combined to induce cellular effects in an oxygen-dependent manner [9]. The first component consists of a photosensitive molecule –a photosensitizer- that preferentially localizes to a target cell and/or tissue. The second involves the administration of light of a specific wavelength that activates the sensitizer. The excited sensitizer generates highly reactive singlet oxygen and other reactive oxygen species that trigger a complex cascade of photochemical reactions and photobiological events that eventually cause injury and death of targeted cells (see Fig. (**2**)).

In this review we first summarize the photobiological processes induced by PDT in order to provide the reader with the fundamentals for the use of PDT in a combinational approach. Then, we present state-of-the-art preclinical and clinical studies on combinations of PDT with other pharmacological approaches for cancer treatment with emphasis in four main categories: (1) PDT and chemotherapy; (2) PDT and pro-oxidant or oxidant enhancers; (3) PDT and angiogenesis inhibitors; and (4) PDT and immunotherapy.

1. PHOTOBIOLOGY OF PDT

The response of tumors to PDT depends on several factors, including those related to the experimental protocol (photosensitizer,

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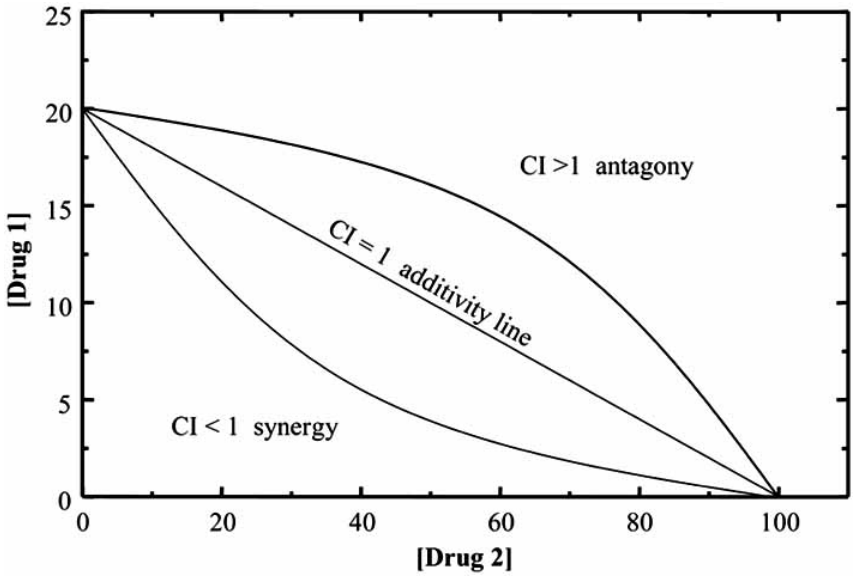


Fig. (1). Determination of treatment interactions by isobolographic analysis. The predicted additivity of two drugs is calculated using a geometrical construct in which the predicted efficacy of two treatments together, to produce an identical effect, is graphically presented (additivity line). This line is constructed by connecting the two individual drug doses that produce a given effect (the isoeffect). Each point on the additivity line represents the issoeffect and correlates with a particular dose of both drugs. Each corresponding dose would give a certain level of effect, which if added together, would give the isoeffect, had the treatments occurred independently. From the isobologram, additivity of the observed treatment can be assumed if the treatment efficacy of any combination of drug concentrations falls on the additivity line. If the observed efficacy falls below the additivity line, the combined treatments are 'synergistic' and 'antagonistic' if they fall above.

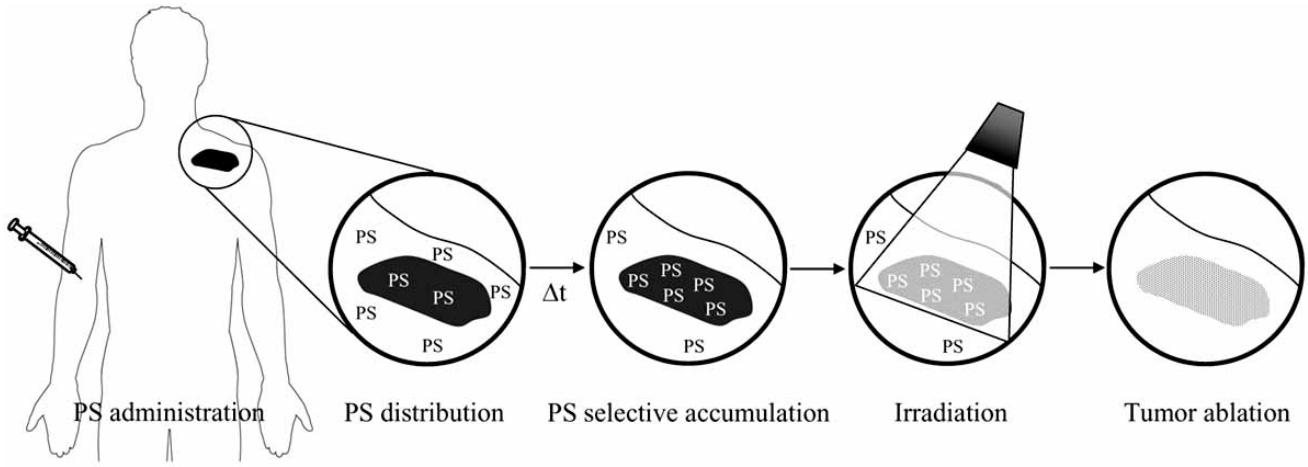


Fig. (2). The principles of PDT. A photosensitizer (PS) is administered systemically (or topically). After a period to allow the PS to accumulate in the target tissue, irradiation with non-thermal light activates the PS. In the presence of cellular/tissular oxygen, the photoexcited sensitizer triggers the production of highly reactive singlet oxygen and/or other free radicals that may lead to cell/tissue damage.

dose, light dose and drug light interval) and those related to the tumor (cell type and its genetic and metabolic phenotype). Different characteristics of the photosensitizer, e.g. its chemical nature, hydrophobicity, overall charge, charge-to-mass ratio, and cellular uptake mechanisms, generally determine its biodistribution and intracellular localization [10]. In turn, this, together with the photosensitizer concentration, irradiation conditions and oxygen level in the tissue, will determine the biological response of tumoral cells. Upon excitation of the photosensitizer with light two principle photochemical pathways, known as Type I and Type II, will trigger this biological response (see Fig. (**3**)).

In the Type I, the photosensitizer interacts with a biomolecule (or oxygen) resulting in hydrogen atom (or electron) transfer that leads to the production of radicals. In the Type II, singlet oxygen is generated as a result of energy transfer from the triplet exited state of the photosensitizer to the triplet ground state of molecular oxygen. Although these two pathways occur in the photosensitization process, it appears that the Type II predominates over the Type I, highlighting singlet oxygen as the principal actor for PDT-induced damage. Scheme **1**, illustrates how different sorts of biomolecules react readily with this highly reactive molecule.

Targets of PDT include tumor cells, vasculature of the tumor and normal tissue, as well as the host's immune system [6]. Therefore, the response to PDT commonly involves three main mechanisms (see Fig. (**4**)) whose contributions are dependent on the numerous factors previously mentioned [9-11].

First, PDT can kill tumor cells directly. Second, PDT can produce profound changes in the tumor vasculature leading to tumor infarction. Third, PDT can induce an inflammatory and immune

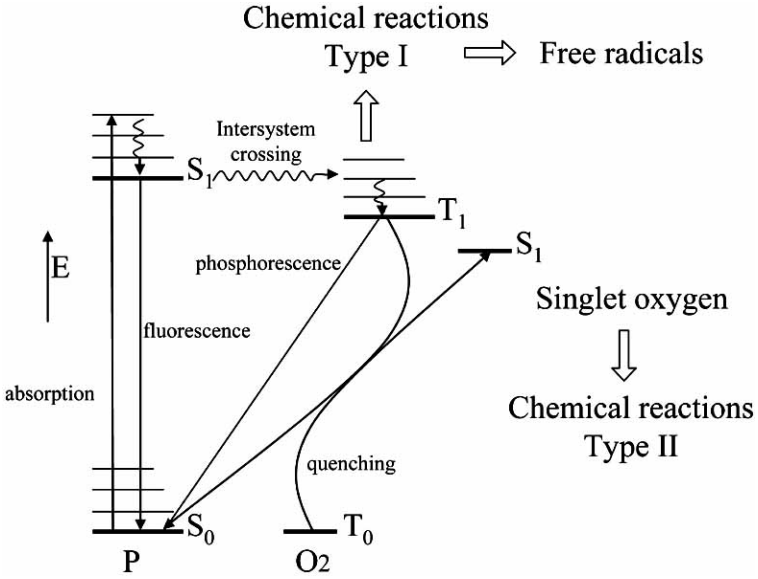
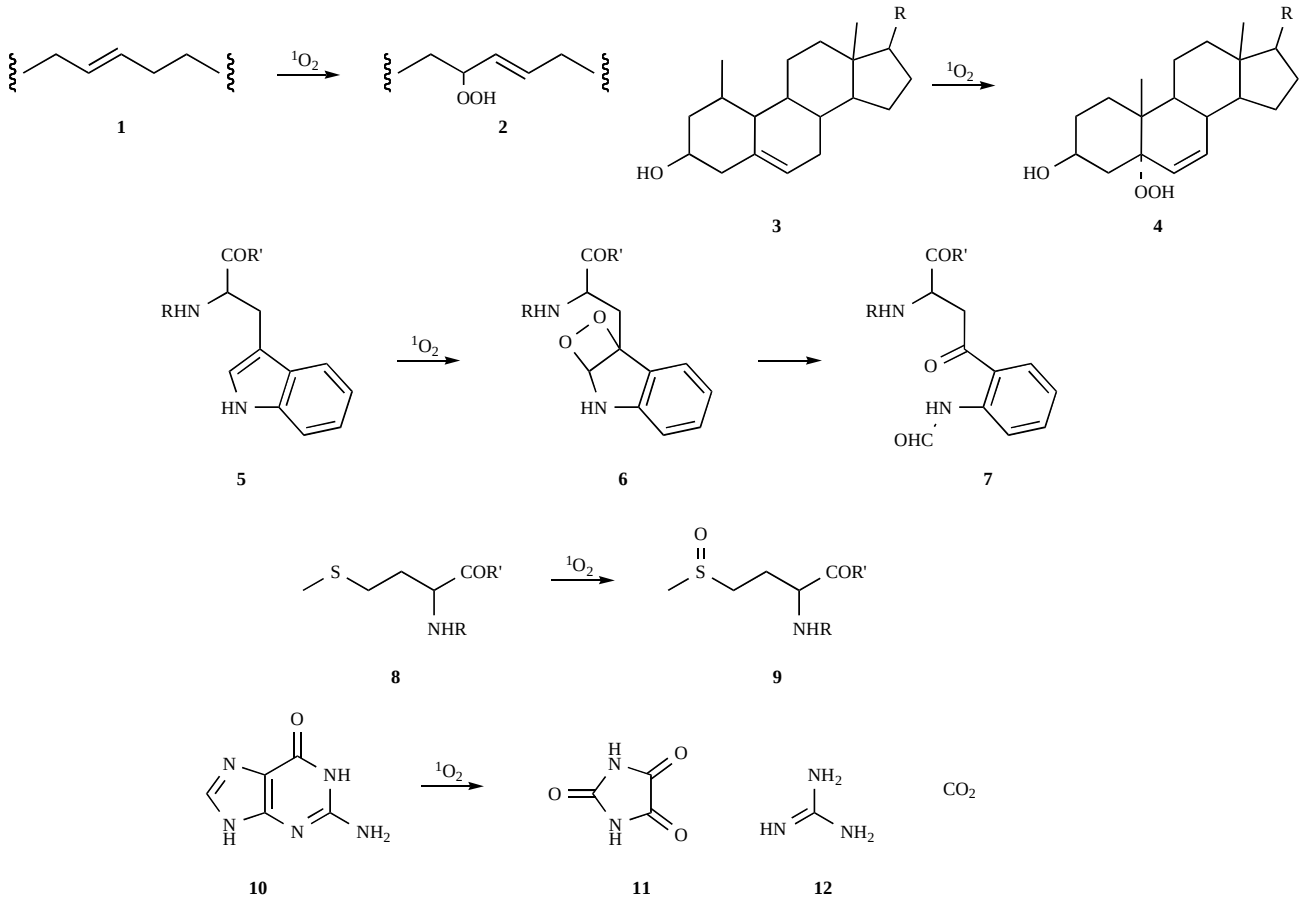


Fig. (3). Modified Jablonski diagram of PDT sensitization. Irradiation with light of appropriate wavelength causes excitation of the photosensitizer from the ground state S_0 to higher energy levels, e.g. the first excited singlet state S_1. The singlet excited photosensitizer can relax back to the ground state through fluorescence photon emission or internal conversion, or can undergo spin-forbidden intersystem crossing into the triplet state (T_1). From these two excited states (S_1, T_1) the excited sensitizer can react with a biomolecule or oxygen to produce radicals (Type I reaction). From the triplet state, the photoexcited molecule returns to the ground state *via* phosphorescent photon emission, reacts with biomolecules or oxygen to produce radicals (Type I reaction) or interacts with triplet ground state molecular oxygen, generating highly toxic singlet oxygen $^1O_2$ which, in turn triggers a cascade of reactive oxygen species producing reaction that can cause cellular damage.



Scheme (1). Typical reactions of singlet oxygen with selected biomolecules: unsaturated lipid (**1**), cholesterol (**3**), tryptophan (**5**), methionine (**8**), and guanine (**10**).

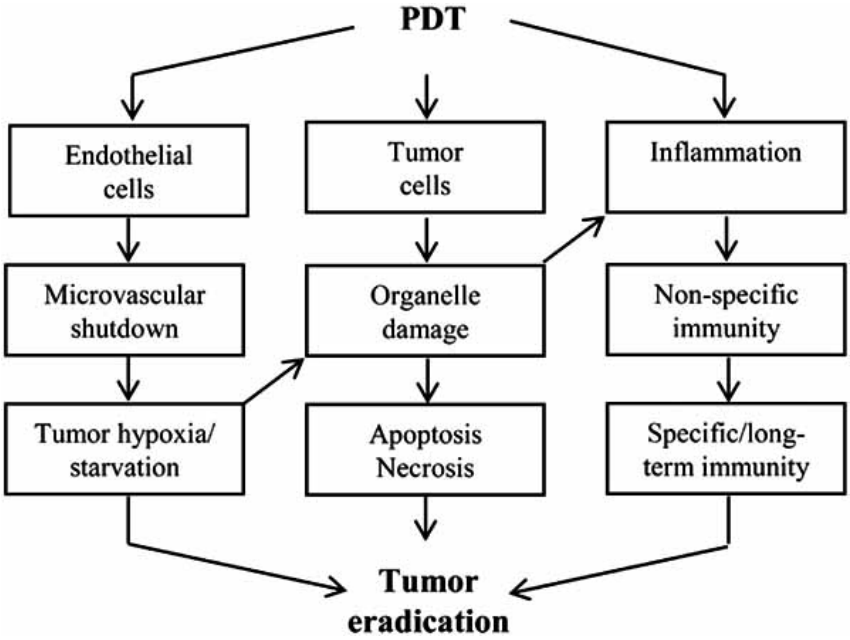


Fig. (4). Mechanisms of PDT-mediated tumor destruction. PDT can kill tumor cells directly by apoptosis and/or necrosis, and indirectly through damage of the tumor associated vasculature and/or activation of the immune response against tumor cells.

response against tumor cells. The complex interaction of these components is required for long-term tumor control. Moreover, the response to PDT strictly depends on the localization of the photosensitizer within the cell following exposure, which, in turn, is dominated by the photosensitizer's structure and hydrophobicity. Fig. (**5**) shows the chemical structures of some photosensitizers that are relevant for the present review.

1.1. Direct Damage and Cell Death

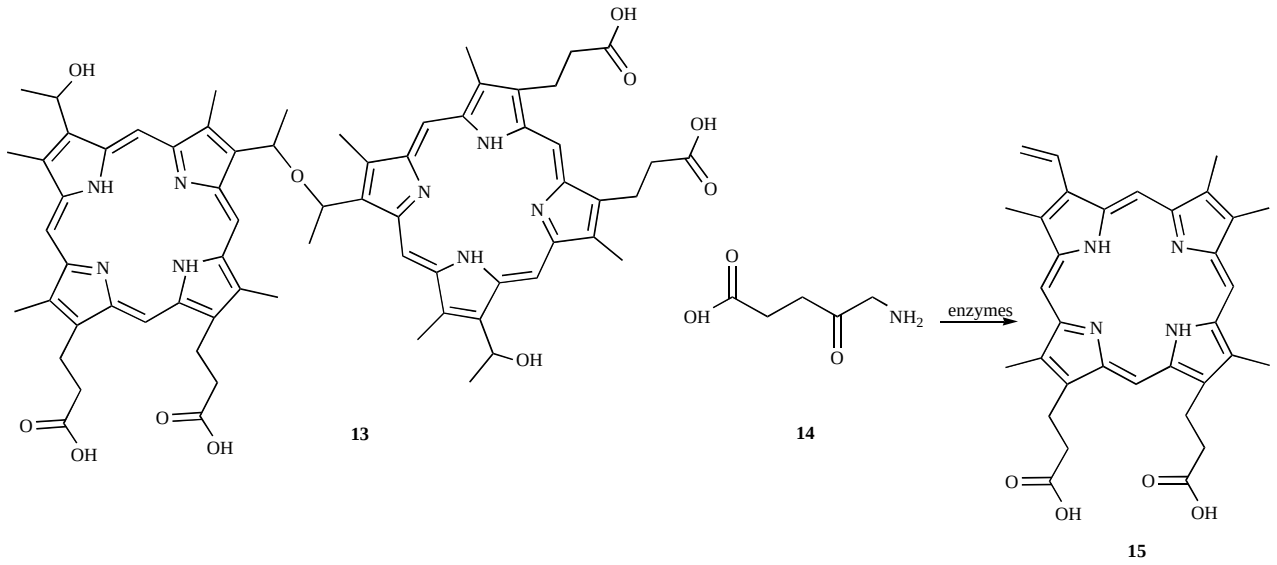
Photodynamic therapy *in vivo* reduces the number of clonogenic tumor cells through direct photodamage of cellular components, but complete tumor eradication is often not achieved, predominantly due to non-homogeneous distribution of the photosensitizer within the tumor and the limited availability of oxygen within the target tissue during irradiation [6,12]. Cellular damage seems to occur preferentially in the plasma membrane, membranous organelles, and in particular, endoplasmic reticulum, golgi apparatus, lysosomes and mitochondria, microtubules, and the nucleus [10,13]. Direct photo-oxidation of membrane lipids may not be directly cytotoxic, but these oxidized lipids may serve as potent signals that trigger cell death. Lysosomal damage caused by PDT may not always be lethal since cytosolic inhibitors can inactivate enzymes released subsequent to lysosome disruption. However, this disruption may also release the photosensitizer initially localized in lysosomes, allowing its relocalization to more susceptible organelles such as the mitochondrial membrane or the nucleus. Mitochondria have been considered to be very susceptible targets for photodynamic damage because ATP, required to power cellular functions, is produced in these organelles [13]. Furthermore, mitochondrial damage has been closely related to the apoptotic effect of PDT due to the release of cytochrome c that triggers the onset of apoptosis [14,15]. As a consequence to PDT, the outer mitochondrial membrane potential has been shown to decrease, and subsequently, cytochrome c is released from the intermembrane site. Then, along with other apoptotic activating factors present in the cytoplasm, it directly activates the cascade of caspases that carry out the final stages of apoptosis. Microtubules are also vulnerable to PDT. However, irreversible depolymerization of tubulin, as well as microfilament disruption, is only observed at higher PDT doses. It has been suggested that DNA damage is not initially a major contributor to the PDT response presumably due to initial photosensitizer localization in the nuclear membrane. However, DNA lesions, such as single-strand breaks and/or alkali-labile sites, have been observed in various cells after PDT, which may imply the relocalization of the photosensitizer during illumination [10].

Tumor cells directly damaged by PDT undergo at least two types of cell death: apoptosis, and necrosis [11,13-15]. However, recent studies also suggest induction of autophagy as a third mechanism of PDT to kill cells [16]. Apoptosis is a normal physiological process controlled by intra- and extracellular signals that control tissue development and involution as well as tissue homeostasis. It is characterized by a common sequence of morphological and biochemical changes including condensation of chromatin and formation of membrane-enclosed vesicles. These vesicles confine a residual cell component, which, in turn, limits leakage of intracellular material, and thus prevents the onset of inflammation. Eventually, these apoptotic bodies are scavenged by phagocytes and cells die under immunological control. In contrast, necrosis is a violent and quick form of degeneration that results from high levels of cell damage. It is characterized by the destruction of organelles and disruption of the plasma membrane, leading to the release of intracellular content into the extracellular compartment and tissue inflammation. Autophagy was originally characterized as a survival response to cellular stress. However, there are also experimental evidence that it is implicated as a death pathway [16]. In authophagy, cytosol and organelles become encased in vacuoles which fusionate with lysosomes for breakdown and eventual recycling of the resulting macromolecules.

Although PDT can lead to apoptosis, necrosis, autophagy, or a combination of these three outcomes, in many cases, PDT is highly efficient in inducing apoptosis [10]. However, it is not still clear if apoptosis induced by PDT *in vivo* is the result of direct damage, secondary to vascular occlusion and inflammation or a combination of both [10]. Furthermore, some studies suggest that apoptosis is not the process by which PDT-treated cells die, but rather the process by which lethally damaged cells are dismantled [14]. According to Kessel *et al.* [16], a similar role may hold for autophagy.

1.2. Vascular Damage

Besides direct damage of disease associated cells, PDT can induce severe damage to tumor microvasculature leading to persistent



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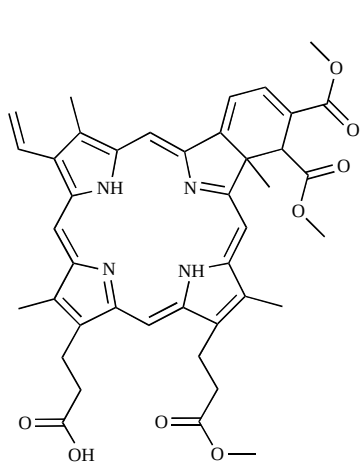

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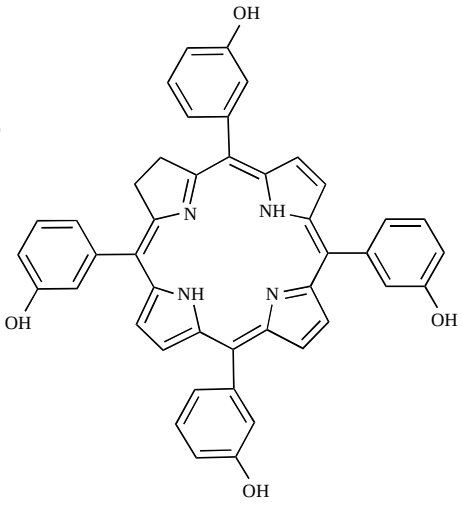

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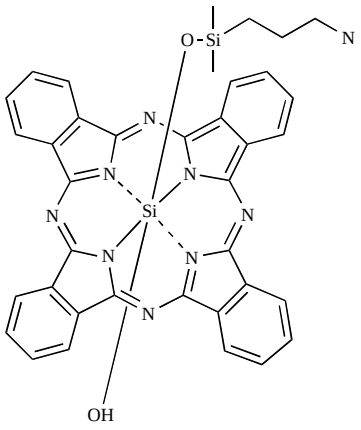

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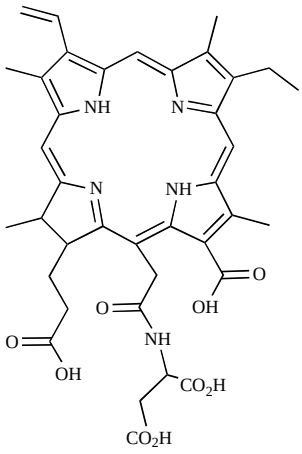

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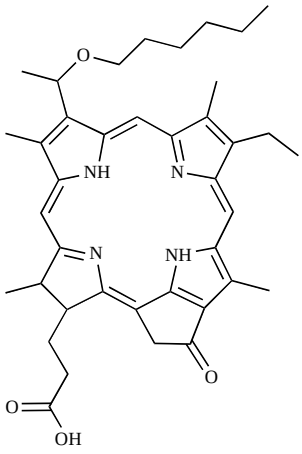

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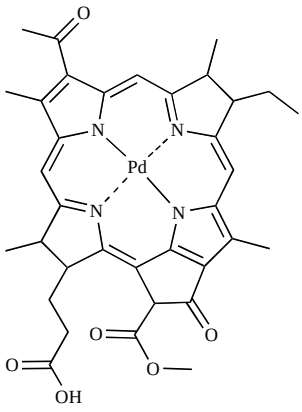

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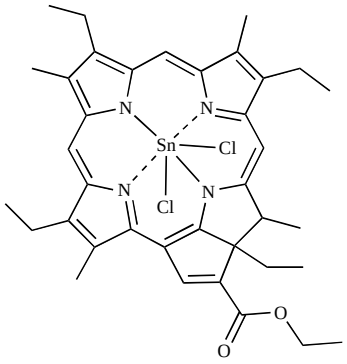

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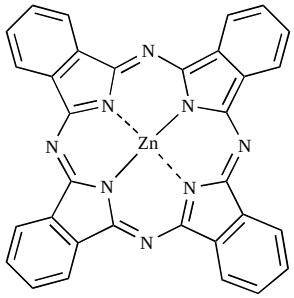

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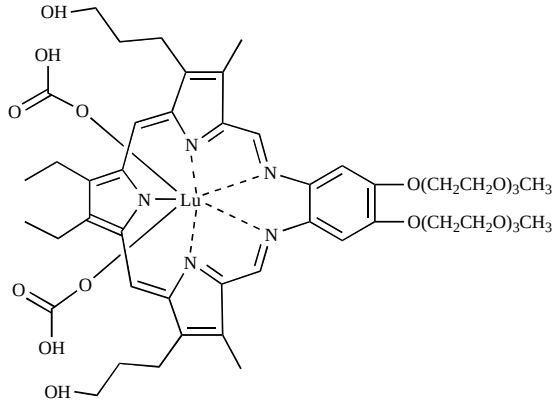

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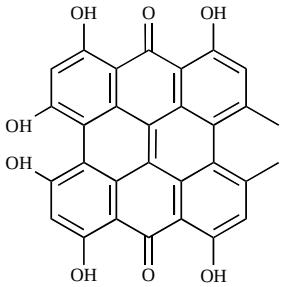

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Fig. (5). Structures of selected photosensitizers either clinically approved or in trials, which have been studied in combination therapy. Hematoporphyrin derivative (Photofrin®) (**13**), 5-aminolevulinic acid (Levulan®) ((**14**) is a prodrug of protoporphyrin IX (**15**), benzoporphyrin derivative (Visudyne®) (**16**), *m*-tetrahydroxyphenyl chlorine (Foscan®) (**17**), silicon phthalocyanine (**18**), monoaspartyl chlorine e6 (**19**), hexylpyropheophorbide a (**20**), palladium bacteriopheophorbide a (**21**), tin etiopurpurin (**22**), zinc phthalocyanine (**23**), lutetium texaphyrin (**24**), and, hypericin (**25**).

post-PDT tumor hypoxia/anoxia and nutrient deficiency, which in turn is lethal to the tumor. The mechanism underlying the vascular occlusion by PDT differs widely with different photosensitizers [6,13,17], and has been recently reviewed by Krammer [18]. However, some of these effects include vessel constriction/collapse, macromolecular leakage, leukocyte adhesion, blood flow stasis, and thrombus formation. These are apparently linked to platelet activation and release of thromboxane, as well as damage to the vascular endothelium and production of nitric oxide by the endothelium. Endothelial cells normally produce a balance of vasodilating (i.e. prostacyclin and endothelium derived growth factors) and vasoconstrictive (endothelin-1) mediators that maintain a healthy vascular tone. PDT exposes the basement membrane to the blood serum, inducing cascades of eicosanoids and other inflammatory agents that switch the balance towards vasoconstriction. Platelet and neutrophils adhere to the vessel wall, roll toward the constriction and aggregate, at which point they migrate into the surrounding tissues following chemokine gradients. Conversely, expression of potent angiogenic factors, such as vascular endothelial growth factors (VEGF), cyclooxygenase-2 (COX-2), and metalloproteinases (MMPs), can be upregulated during PDT [9,19-21]. VEGFs are endothelial-cell-specific mitogens and survival factors that also cause increased vascular permeability and recruit progenitor endothelial cells from the bone marrow [22]; COX-2 is an inducible isoform of prostaglandin-endoperoxide synthase considered an early response gene involved in inflammation and mitogenesis that mediates the production of prostaglandins (PG), which are powerful angiogenic mediators [23]; MMPs are zinc-containing endopeptidases that function in both physiological and pathological conditions, and are involved in tumor angiogenesis, invasion, and metastasis [24]. Although the mechanism involved in the upregulation of these three types of molecules is not completely clear, oxidative stress and tumor tissue hypoxia induced by PDT are thought to activate a number of transcription factors and signaling pathways that regulate their transcription [19,20,25]. The hypoxia inducible factor (HIF)-1α induced by PDT has been closely related to the activation of the *VEGF* gene [19], while the nuclear transcription factor (NF)-κB seems to play a major role in the PDT-induced COX-2 [20,25]. PDT has been shown to increase the expression of the extracellular matrix metalloproteinase inducer (EMMPRIN), and to block the tissue inhibitor of metalloproteinase (TIMP)-1, which results in an efficient stimulation of the production of MMPs [26].

1.3. Inflammatory and Immune Effect of PDT

Photodynamically induced changes in the plasma membrane and the membrane of cellular organelles can trigger different events with far-reaching consequences [6,27]. PDT stimulates multiple signal transduction pathways simultaneously for both cell death and survival. Thus, cell fate is likely to be determined by the interaction of these pathways [10]. In fact, PDT is presumed to induce the release of a variety of lipid and secondary messengers (phospholipases C, A2, sphingomyelinase, PGE_2, NO), as well as calcium ions from intracellular stores. As a result, several protein kinase signaling cascades are activated, some of which seem to lead to cell death through apoptosis and inflammatory/immune responses, whereas others seem to promote cell survival. PDT is also a strong inducer

of the expression of a range of stress response genes that are thought to enhance survival after oxidative stress through regulation of apoptotic cell death. Upregulation of glucose-regulated proteins, such as GRP78, can either protect or sensitize cells exposed to PDT, depending on the subcellular localization of the photosensitizer. Furthermore, the expression of cytokines - tumor necrosis factor (TNF); interleukin (IL) and granulocyte-colony-stimulating factor (G-CSF) - may promote either cell death or cell survival or an immune response *in vivo*.

PDT-mediated immune reactions are presumably a key contributor to final tumor eradication. In addition, differences in the nature and intensity of the inflammatory response between normal and cancerous tissues may contribute to the selectivity of PDT-induced damage [6,9]. Although inflammation is frequently accompanied by immunosuppressive effects, PDT appears to be able to tip the balance of agents that regulate the immune system towards either activation or suppression by the release of specific cytokines [13]. This is probably controlled by many complex factors including, but not limited to, the nature of the photosensitizer, light dose, and dose rate [13]. The mechanism by which PDT induces tumor immunity has been suggested by Korbelik [28]. The inflammatory signaling accompanied by the release of large quantities of cell debris, cytokines and other chemotactic agents, initiates and maintains the recruitment of leukocytes from the blood and amplifies their activity. Within minutes of light treatment, a massive regulated invasion of neutrophils is produced. Neutrophils can remain within tumor blood vessels and degranulate, releasing myeloperoxidases, lysosomal enzymes and toxic oxygen radicals that destroy endothelial and tumor cells. As neutrophils die, the release of their cellular contents induces chemotaxis of new waves of immune cells. Mast cells flood into the damaged tissue and release granules containing vasoactive agents and cytokines. In addition, monocytes and macrophages invade, proliferate, collect cell debris, and preferentially recognize and destroy pockets of surviving tumor cells. At the end of the inflammatory response, macrophages and monocytes can secrete immunosuppressive factors as a result of a transient reduction in the delayed-type contact hypersensitivity response, which downregulates the response and may hamper any future, specific immune response. Otherwise, macrophages and/or dendritic cells can be prompted to phagocytize large numbers of cancer cells damaged through photodynamic action. These tumor-associated cells serve as antigen presenting cells processing tumor-specific peptides and presenting them on their membranes in the context of major histocompatibility class II molecules. Presentation of tumor peptides, accompanied by intense accessory signals, creates conditions for the recognition of tumor antigens by $CD4^+$ helper T lymphocytes. These lymphocytes rapidly expand and become activated and, in turn, sensitize cytotoxic $CD8^+$ T cells to tumor-specific epitopes leading to fully developed tumor immunity. It is reported that B lymphocytes and natural killer cells (NK) also become activated and may contribute to PDT-elicited immune responses, but the role of these cells is still unclear [6]. Specific immune cells can, under reduced tumor burden, eliminate small foci of viable cancer cells that have escaped other PDT mediated anti-tumor effects [27]. Furthermore, their activity is not limited to the original PDT treated site, but can include disseminated and metastatic lesions of the same cancer [28], which may be decisive in attaining long-term tumor control.

Due to its multiple physiological effects, PDT may be successfully combined with a variety of pharmacological protocols for achieving substantial gains in tumor destruction and long-term tumor control.

2. PDT AND CHEMOTHERAPY

Cisplatin, doxorubicin and mitomycin C are three of the most extensively studied chemotherapeutic drugs used as single agents in the management of various cancers [29]. It is believed that their main mechanism to kill cancer cells relies on their binding to DNA and interfering with subsequent processes in cell replication, eventually leading to cell death. Despite the wide use of these drugs, their dose-limiting toxicities and the emergence of resistance have lead to the search for new approaches that minimize these two drawbacks. In this context, some *in vitro* and *in vivo* studies have revealed promising results. One of the first studies was performed by the group of Nahabedian [30]. They treated nude mice bearing RIF-1 and EMT-6 tumors with cisplatin, doxorubicin, hematoporphyrin derivative-mediated (HpD)-PDT or a combination of each drug and PDT. The RIF-1 tumors were only sensitive to doxorubicin and no additional anti-tumor effect was observed when combined with PDT. On the other hand, the EMT-6 tumors were moderately sensitive to PDT and mildly sensitive to both cisplatin and doxorubicin. While combining PDT and cisplatin did not result in an enhanced anti-tumor effect, the combination PDT and doxorubicin significantly enhanced the effect of PDT alone. The authors suggested that this enhancement corresponds either to an increased activity of doxorubicin alone, due to a photochemical reaction of the drug during PDT or to a secondary effect to the mild hyperthermia generated by irradiation. Although, their results did not evidence synergistic effects, a good tolerability for the combination regimen was demonstrated. Later, Canti *et al.* [31] administered the same cytotoxic agents followed by PDT with aluminum phthalocyanine (AlS_2Pc) to mice bearing L1210 leukemia and P388 lymphoma. Non-therapeutic drug doses were used. As expected, low doses of either doxorubicin or cisplatin were ineffective; however, in combination with PDT, a significant additive anti-tumor effect was observed. Furthermore, the combination of light treatment and drugs did not result in increased anti-tumor activity. The tumoricidal effect of interstitial PDT using Photofrin® combined with mitomycin C was also investigated in RIF-1 tumors by Baas and co-workers [32]. While each treatment alone induced a small but significant tumor growth delay, the combination of mitomycin C with PDT further increased this delay. In these experiments, the light dose to obtain the same effect of Photofrin® alone was reduced by a factor of two when the cytostatic agent was given prior to PDT. In contrast, mitomycin C given immediately after illumination did not improve the effect of PDT. In another study, different photosensitizers, meso-tetrahydroxyphenylchlorin (*m*-THPC), bacteriochlorin a (BCA), and Photofrin® were compared in terms of tumor regrowth and cures in combination with mitomycin C [33]. The combination of mitomycin C with either *m*-THPC or BCA without illumination was not significantly different from the anti-neoplastic agent given alone. However, in agreement with the previously performed study, the use of *m*-THPC-PDT combined with mitomycin C allowed considerable reduction in the light or photosensitizer dose without affecting the therapeutic effect compared to *m*-THPC-PDT alone. For *m*-THPC-PDT, however, mitomycin C given immediately after illumination was equally effective as mitomycin C given 15 minutes before illumination, in contrast to the results found for PDT with Photofrin®, where the maximum benefit from the combination was obtained when the chemotherapeutic drug was given before illumination. This difference can be attributed to vascular occlusion during PDT with Photofrin®, which inhibits the access of mitomycin C to the tumor mass. BCA-PDT combined with mitomycin C did not result in a greater tumor response compared with PDT alone when the photosensitizer was given 15 minutes before illumination. However, when BCA was injected 1 hour before illumination, the association with mitomycin C resulted in a significant decrease in regrowth. Because both photosensitizer and chemotherapeutic agents were injected at the same time in these experiments, the BCA distribution within the tumor tissue was probably suboptimal for the shortest time interval.

On the other hand, several studies have evaluated the combination of anti-neoplastic drugs and PDT in various *in vitro* cell culture models [34-36] using different techniques to assess either cell vi-

ability or cell proliferation after treatment. The group of Kopecek [37] has assessed the interaction between doxorubicin and mesochlorin e_6 monoethylene diamine (Mce_6) on human ovarian epithelial carcinoma OVCAR-3 *in vitro*. According to the isobolographic approach (see Fig. (**6**)), doxorubicin and Mce_6-PDT appeared to act independently (additively) at doses above their ED_{50} and synergistically when both agents were administered at 50% of their ED_{50}. Both the dose and effect isobole analyses confirmed the continuous action of doxorubicin, in contrast to the single effect of Mce_6-PDT, which is consistent with their suggested mechanisms. However, because *in vitro* studies do not allow the assessment of either vascular or inflammatory/immune effects of PDT, these findings do not discard multiple mechanisms when Mce_6 is used as photosensitizer. In fact, this drug is one of the examples of a photosensitizer causing vascular damage leading to stasis shortly after the initiation of light treatment [17].

Nonaka and co-workers [34] studied the cytotoxic and apoptotic effect of a combination of cisplatin and PDT with Photofrin® on L5178 mouse lymphoma cells. A significantly increased number of apoptotic cells was demonstrated for the combined treatment compared to either treatment alone. This enhanced effect was shown to be synergistic and related to the effect of caspase-3 activity induced by both PDT and cisplatin through different pathways. Crescenzi *et al.* [35] investigated the effect of indocyanine green-(IG)-mediated-PDT combined with cisplatin on MCF-7 breast cancer cells. Single treatments were not lethal for MCF-7 cells, whereas in combination, the overall lethal output was potentiated. Isobolographic assessment of the combined treatments revealed additive and quasi-synergistic responses depending on cell viability tests. Later, the same group [36] used similar conditions for H1299 lung cancer cells. In these experiments, monotherapies reduced the cell viability by approximately 50%, whereas the combined therapies did so by 92% as a result of their additive effects.

Cell culture models have also been used for the assessment of the cytotoxicity of Photofrin II, a purified version of HpD, or 5-aminolaevulinic acid (5-ALA)-mediated PDT combined with mitomycin C. Some examples consist of human colon adenocarcinoma and bladder cancer cell lines [38-40]. The group of Ma [38,39] investigated the cytotoxic effects of mitomycin C in cultured WiDr human colon adenocarcinoma cell lines and then compared this single treatment to a combination treatment with Photofrin II-PDT. The cytostatic agent additively increased the cell sensitivity to PDT. Upon increasing the concentration of the antineoplastic drug, the combined effect changed from additive to synergistic, as judged by isobologram analysis. This result can be related to the change in cell accumulation from late S and early G2 phase to mid and early S phase with increasing mitomycin C dose. Datta and co-workers [40] studied the effect of 5-ALA-mediated-PDT in combination with mitomycin C on the J82 bladder cancer cell line and a mitomycin C-resistant counterpart (J82/MMC). Cell viability assays demonstrated that the J82/MMC was not cross-resistant to PDT and suggested a higher sensitivity of J82/MMC to PDT than the parent cell line. For both cell lines, an enhanced effect occurred only when the cytostatic agent was given first. The type of interaction and the sequence of mitomycin C and 5-ALA-mediated-PDT was more deeply investigated by the same group [41] and will be discussed later.

Analogous results from *in vitro* and *in vivo* models have been reported, revealing a reduction in cell viability and tumor surface, respectively. Brophy and Keller [42] worked with Photofrin II as the photosensitizer, doxorubicin as the cytotoxic agent, and H-MESO-1 cells and BDF1 hybrid male mice as the model. *In vitro*, PDT with Photofrin II alone resulted in a 23% decrease in cell viability, while the addition of increasing doses of doxorubicin to PDT reduced cell viability by more than 60%. *In vivo*, a 50% reduction of tumor surface with further regrowth was observed for PDT alone, while no impact on tumor growth was evidenced for doxorubicin alone. The combination of both treatments resulted in 100% tumor necrosis with no tumor regrowth suggesting a more rapid, intense and prolonged response compared to each treatment given alone. Similarly, Kirveliene *et al.* [43] worked with MH-22A murine hepatoma cells and nude mice models and *m*-THPC-PDT. Single treatment with either *m*-THPC-PDT or doxorubicin resulted in increasing dose-dependent cytotoxicity against MH-22A cells. Cell viability after PDT in combination with doxorubicin was significantly reduced under most conditions compared to individual treatments. Furthermore, *in vivo* data showed a significant reduction in tumor volume by both single treatments compared to the control group. *m*-THPC-PDT and doxorubicin in combination were more effective in inhibiting tumor growth than any of these two treatments alone. These two studies show evidence of a good correlation between *in vivo* and *in vitro* data: a higher anti-tumor activity of combined regimens against transplanted cancer cells corresponded with a higher cytotoxicity *in vitro*. However, the degree of statisti-

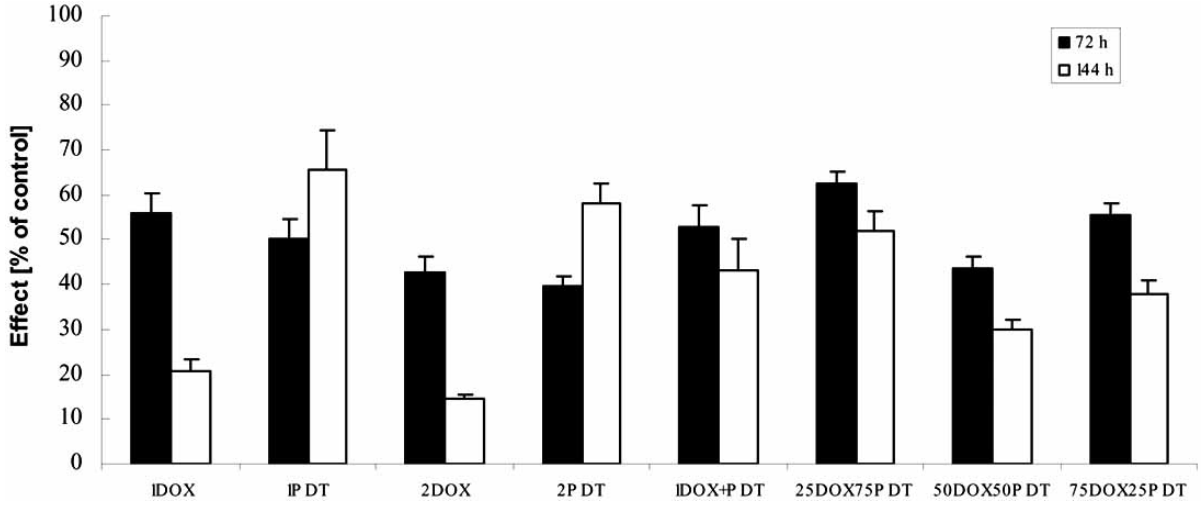

Fig. (6). Dose-addition isobole analysis for the interaction between doxorubicin and PDT with Mce_6. MTT assay 72- and 144-hour assays. One ED_{50} doxorubicin (1DOX) and one ED_{50} Mce_6-PDT (1PDT) and twice the ED_{50} of doxorubicin (2DOX) and Mce_6-PDT (2PDT) are depicted for comparison. The expected results for the combination of 50% ED_{50} doxorubicin with 50% ED_{50} Mce_6-PDT = 100% ED_{50} are depicted as 1DOX+PDT. For the combination 25% ED_{50} doxorubicin with 75% ED_{50} Mce_6-PDT (25DOX75PDT) antagonism was noted. Synergy was observed for the combination 50% ED_{50} doxorubicin with 50% ED_{50} Mce_6-PDT (50DOX50PDT). Additivity persisted for 75% ED_{50} doxorubicin with 25% ED_{50} Mce_6-PDT (25DOX75PDT). (Adapted from reference [37]).

cal significance between the effects *in vivo* and *in vitro* differed to some extent.

There is good support that the combination of a cytotoxic drug and PDT might improve the overall outcome of the treatment of cancer. In spite of that, the relevance of the sequencing of the combining agents to the combination effect is still under evaluation. Table **1** summarizes the type of interaction found *in vitro* for cisplatin, doxorubicin, or mitomycin C in combination with PDT. French *et al.* [41] investigated the interaction of mitomycin C and 5-ALA-mediated PDT in both the J82 bladder cancer cell line and its mitomycin C-resistant counterpart, J82/MMC. Mitomycin C increased 5-ALA-induced protoporphyrin IX (PpIX) fluorescence in both cell lines in a dose-related manner. This interaction was greater in the J82/MMC than in the J82 cell line, presumably due to the higher mitochondrial density of the mitomycin C-resistant cell line. Isobolograms for both cell lines showed that the interaction was marginally synergistic for J28/MMC, while it was not necessarily additive for J28. The administration of mitomycin C after PDT resulted in an antagonistic effect compared to single treatments, while the opposite sequence appeared to be synergistic.

Uehara *et al*. [44] treated C3H/HeNCrj mice transplanted with R-S1 mouse squamous cell carcinoma with Photofrin®-PDT in combination with cisplatin administered either after or before PDT. At suboptimal cisplatin doses, histological findings showed that the administration of cisplatin 3 hours before PDT was significantly different from other combinations such as cisplatin 1 hour before PDT and cisplatin immediately after PDT, as well as from each therapy alone. Compared to the control group, administration of cisplatin 3 hours before PDT was the only treatment resulting in a significant reduction of tumor volume at three, seven and 10 days. Therefore, the interval of administration between the anti-cancer drug and PDT greatly influences the therapeutic outcome.

In contrast to cisplatin, doxorubicin seems to enhance the PDT response against tumor cells when this cytostatic agent is administered after PDT. Kirveliene *et al.* [43] observed that the antitumoral activity against MH-22A was more pronounced when *m*-THPC-PDT was followed by doxorubicin. *In vitro* data showed that the difference between cytotoxic effects of doxorubicin and the combined treatment with doxorubicin before PDT was approximately 40%, while it exceeded 60% when doxorubicin was added after PDT. The contribution of combination was evaluated by analysis of variance. This analysis revealed an antagonistic component when doxorubicin was given first. This negative interaction was mainly explained in terms of reduction of the cellular uptake of the photosensitizer caused by the cytotoxic agent. On the other hand, when doxorubicin was added after PDT, the combined action resulted from the addition of the individual cytotoxicities of the anti-neoplastic agent and *m*-THPC-PDT. *In vivo*, the anti-tumor activity of PDT 15 minutes before doxorubicin regimen was higher than that of doxorubicin 24 hours before PDT. However, the statistical significance between these two sequences was not as high as it was observed *in vitro*.

Because of the few studies comparing data *in vitro* and *in vivo*, it is difficult to ascertain the best sequence of combining the treatments *in vivo*. *A priori*, the effect of either mitomycin C or cisplatin in combination with PDT is superior when the cytostatic agent is administered first, while the effect of combining doxorubicin plus PDT is superior when doxorubicin is subsequently applied to PDT. These phenomena can be explained by different considerations, i.e. increase (mitomycin C) or decrease (doxorubicin) in photosensitizer uptake in the presence of the anti-cancer drug, sufficient distribution of the cytostatic agent (cisplatin) at the moment PDT is performed, and the action of both therapeutic approaches on cells in different phases of their cell cycle (mitomycin C, cisplatin). However, the complexity of the *in vivo* situation may lead to inconsistencies with *in vitro* findings in particular when factors such as sequencing and dosing of combined treatments are involved.

Similar to mitomycin C, other bioreductive drugs, which are produgs converted into potent cytotoxins under metabolic conditions of either low oxygen tension or in the presence of high levels of specific reductases [45], have been studied in combination with PDT. The association of such chemotherapeutic agents with PDT might circumvent the tumor resistance to PDT in hypoxic regions of tumors where insufficient oxygen is present. At the same time, this approach exploits the local hypoxia induced by PDT, therefore, enhancing the anti-tumor response of individual treatments.

Misonidizole, one of the oldest bioreductive agents, was used by Gonzolez *et al.* [46] as an adjunctive therapy with HpD-PDT to treat Dunning rat prostatic cancers. In these studies, the light dose in the PDT also produced a significant hyperthermia. An enhanced effectiveness on tumor growth was observed by combining PDT and hyperthermia induced effects with misonidizol compared to PDT or misonidizole alone. Later, Henry and Isaacs [47] studied the effect of PDT associated with a new generation of more potent bioreductive agents represented by the compound RSU1164 in the same model. Combination of RSU1164 with HpD-PDT was found to synergistically delay tumor growth. In fact, the bioreductive drug alone produced no significant effect on tumor growth suggesting an insufficient degree of tumor hypoxia to activate the agent. While PDT alone delayed tumor growth, the tumor size at 24 days in

Table 1. ***In Vitro*** **Interactions Between Conventional Chemotherapeutic Agents and PDT with Different Photosensitizers**

Chemotherapeutic	Photosensitizer	Sequence	Interaction	Cell line	Reference
Cisplatin	Photofrin	pre-PDT	synergistic	L5178	[34]
		pre-PDT	additive	H1299	[36]
	Indocyanine G	pre-PDT	from additive to synergistic	MCF-7	[35]
Doxorubicin	Photofrin II	pre-PDT	synergistic	H-MESO-1	[42]
	Mce_6	pre-PDT	from additive to synergistic	OVCAR-3	[37]
	m-THPC	pre-PDT	antagonistic	MH-22A	[43]
		post-PDT	additive	MH-22A	[43]
Mitomycin C	Photofrin II	pre-PDT	form additive to synergistic	WiDr	[39]
	5-ALA	pre-PDT	antagonistic	J82	[40]
		pre-PDT	synergistic	J82/MMC	[40]
		post-PDT	antagonistic	J82	[40]
		post-PDT	antagonistic	J82/MMC	[40]

combination with RSU1164 was reduced by 50%. Furthermore, AlS_2Pc-mediated PDT in combination with bioreductive analogs of the RSU1164, the RSU1069 and its prodrug RB6145, was performed by Bremner *et al.* [48]. In RIF-1 experimental murine tumors, RSU1069 and RB6145 potentiated the effect of PDT when light was administered between 30 minutes and 6 hours after photosensitizer administration. Tumor cures were observed under optimal conditions. Bioreductive drugs such as misonidazole, pimonidazole, metronidazole, nimorazole, RB6145, RSU1069, SR4233, mitomycin C, or RB90740 at their maximum tolerated doses were used in combination with PDT in the same model [49]. Although misonidazole also enhanced PDT, the results were less pronounced as compared to RSU1069 and RB6145. No potentiation of the PDT effect was observed in the combination with pimonidazole, metronidazole, and nimorazole. Due to systemic effects under anesthesia, the two latter were administered at lower doses than their reported maximum tolerated doses, which may partly explain this observations. For the agent SR4233, only a limited tumor growth inhibition effect was observed when tumor hypoxia was increased by either PDT or clamping in combination with AlS_2Pc or Photofrin-mediated PDT, presumably due to the rapid conversion of SR4233 into inactive metabolites under severely hypoxic conditions [50].

In a recent study, Hasan and co-workers [51] isolated and characterized cell lines resistant to ALA-mediated-PDT derived from a LM3 murine mammary adenocarcinoma. They found that the oxygen consumption was significantly increased in the resistant clones compared to LM3. Furthermore, subcutaneous injection of those clones to mice, showed a tumor growth delay and early necrosis in comparison to the parenteral cell line, which can be related to their increased oxygen consumption. According to the authors, not only PDT can induce *in vivo* chronic hypoxia due to the vascular shutdown but, also, surviving cells may be hypoxic by an independent mechanism and, therefore, can be preferential targets of bioreductive drugs.

Altogether, these studies suggest that combination therapy of bioreductive drugs and PDT may be of value in the treatment of cancer. Factors such as drug distribution within the tumor and the ability of the drugs to be reduced under the conditions caused by PDT have to be considered when choosing the bioreductive drug.

Another approach in the context of combination treatments consists of the use of conjugates between the photosensitizer and the chemotherapeutic agent. So-called porphyrin platinum conjugates (PPC) consist of a porphyrin derivative and a platinum fragment in the same molecule. The hypothesis for the use of such systems is based not simply on the combined effect of PDT and cytostatic activities, but also on the porphyrin-mediated targeting of tumors [52]. Brunner and co-workers [53,54] have obtained promising results with such conjugates compared to standard monotherapy and combination therapy. Initially, different PPCs were obtained from platinum complexes of a series of 1,2-diamines and hematoporphyrin or 13,17-bis(2-carbocyethyl)-3,8 [bis-(ethyleneglycolmonoethyl ether) oxyethyl]-2,7,12,18-tetramethylporphin. Then, their activity against MDA-MB-231 mammary carcinoma cells was evaluated and compared to that of cisplatin and PDT with Photofrin® either alone or in combination. Three of the tested conjugates were as active as or even more active than cisplatin in association with PDT, demonstrating the validity of this approach. One of these conjugates evaluated as a liposomal formulation showed a strongly concentration-dependent activity. A second generation of these conjugates (PPC II) was evaluated in two cell lines, the J82 bladder cancer cell line and the UROtsa established from a normal urothelium. One of these PPC II, a water-soluble conjugate, exceeded the sum of cell viability induced by HpD-PDT and cisplatin therapy. In addition, proliferation in J82 cells was more affected than in UROtsa cells, presumably due to the lower dark phototoxicity of this conjugate on the latter cell line enabling them to recover from the treatment.

Other strategies of selective tumor targeting in combined chemotherapy and PDT have been investigated by Kopecek and co-workers [37,55-57]. Based on the use of polymeric drug delivery systems for the improvement of the specificity of drug action, an N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer bound doxorubicin and a HPMA copolymer-Mce_6 were assessed as individual therapies and in combination using a human epithelial ovarian carcinoma (OVCAR-3) xenograft model [37,57]. The incorporation of HPMA copolymers extended the narrow margin of safety evidenced for free Mce_6 and demonstrated both safety and efficacy of HPMA-doxorubicin conjugates. The combination therapy of HPMA-doxorubicin and HPMA-Mce_6-PDT was non-toxic and resulted in complete tumor ablation, while none of the monotherapies showed a complete response. Further *in vitro* studies on OVCAR-3 [58] showed that both HPMA copolymers required a 10-fold increase in drug concentration to show equivalency with free drugs. At ED_{50}, the efficacy profile administration of HPMA-doxorubicin followed HPMA-Mce_6, resulting in an enhanced long-term inhibition of OVCAR-3 cells as compared to HPMA-Mce_6 alone. The opposite sequence was not significantly different from HPMA-doxorubicin alone in terms of efficacy. *In vivo*, an enhanced tumor accumulation was achieved for both HPMA-Mce_6 (4-fold) and HPMA-doxorubicin (2-fold) as compared to free drug. In the same study, several single and multiple treatments were evaluated. The prolonged retention time of the photosensitizer conjugate within the tumor made multiple PDT irradiations feasible. Single HPMA-Mce_6-PDT combined with multiple HPMA-doxorubicin treatments exhibited a significantly greater effect than multiple HPMA-doxorubicin treatments alone. Multiple HPMA-Mce_6 photodynamic treatments were better therapy than single HPMA-Mce_6-PDT treatment with multiple HPMA-doxorubicin injections. As expected, the best results were obtained when multiple HPMA-Mce_6-PDT were combined with multiple HPMA-doxorubicin treatments. Later, the same team also investigated the use of such metacrylamide-drug copolymers specifically targeted with monoclonal antibodies in a murine model [56]. The incorporation of the OV-TL 16 antibody enhanced accumulation in tumors by a factor of 13 with a concomitant increase in therapeutic efficacy of the combined immunoconjugate therapy. In addition, combined treatments of the antibody targeted drugs (HPMA-Mce_6-Ab-doxorubicin and HPMA-Mce_6-Ab-PDT) resulted in inhibition of tumor growth with more than three times longer remission in OVCAR-3 xenografts than was achieved with the corresponding non-targeted conjugates.

Due to their lack of selectivity, an undesirable consequence of the administration of conventional chemotherapeutic compounds is the observation of their toxicity in normal tissues with a high rate of cellular turnover [59]. Therefore, new anti-neoplastic agents have been developed to address this issue. An interesting new prototype is an ether lipid, edelfosine, a synthetic analogue of lysophosphatidylcholine that does not act directly on the formation and function of cellular replication, but modulates membrane properties and metabolism of phospholipids, resulting in a selective apoptotic response in tumor cells [60]. Both therapies, edelfosine and merocyanine 540 (MC540)-mediated-PDT, have been used separately as purging agents in clinical trials [61,62]. Recently, some authors reported their use in combination. Yamazaki and Sieber [63] evaluated their combined effect on L1210 murine leukemia cells, and, K562 and HL-60 human leukemia cells. A high degree of synergy between both treatment regimens was observed, in particular when the PDT preceded the anti-cancer lipid. Interestingly, edelfosine enhanced the antileukemic effect of MC540-PDT even on K562 cells, which are refractory to edelfosine as a single purging agent. Inactivation of normal murine granulocyte-macrophage progenitors was also enhanced. However, the potentiating effect of Edelfosine on the photoinactivation of normal bone marrow cells was small compared to that of leukemia cells. Siebe and co-workers [64,65] performed an initial assessment of the safety and efficacy of a simi-

lar two-step purging procedure (MC540-PDT followed by edelfosine). A variety of cell lines, moderately to minimally sensitive to MC540-PDT, were employed. The combination significantly depleted all tumor cells, while preserving a considerable percentage of normal cells. At low edelfosine concentration, the combined cytotoxic effect appeared to be additive, while at higher concentrations it led to synergistic effects. In tumor cells, the transition from an additive to synergistic effect occurred at lower concentrations of edelfosine than in normal haematopoietic stem and progenitor cells.

A different study on potential combinations was reported by Tsujino and colleagues [66]. They studied the effect of amifosine and/or amphotericin B, drugs usually used concomitantly with chemotherapy, in the anti-tumor effect of MC540-PDT over leukemia cells, wild-type small cell lung cancer cells and cisplatin-resistant small cell lung cancer cells. The use of non-toxic concentrations of amifosine and amphotericin B, either alone or in combination, enhanced the photoinactivation of cancer cells by MC540. Amphotericin B also enhanced the effect of PDT on normal granulocyte-macrophage progenitors, whereas amifosine protected them against the cytotoxic action of PDT.

Other anti-neoplastic agents, so far less studied in combination with PDT, are the so-called inhibitors of the cellular energy metabolism such as lonidamine and levamisol. These molecules exert a powerful inhibitory effect on oxygen consumption, aerobic glycolysis and lactate transport and accumulation of neoplastic cells [67,68]. Shevchuk and co-workers [69,70] showed a potentiating anti-tumor action of 5-ALA-based-PDT when administered together with these agents. V79 Chinese hamster lung fibroblast cells were preincubated with lonidamine or levamisol jointly with 5-ALA for 4 hours prior to irradiation, or first treated with 5-ALA-based-PDT followed by the incubation with any of the two inhibitors 24 hours post-irradiation. Only a minor dark cytotoxicity was observed by each inhibitor in combination with 5-ALA. However, both levamisol and lonidamine altered the efficacy of PDT most likely due to their interference with the biosynthetic pathway of heme. However, at low concentrations lonidamine synergistically enhanced the sensitivity of the cells to PDT, presumably due to its effect on either glycolysis or the respiration chain. On the other hand, levamisol induced a maximum of 1.5-fold increase in the PDT efficacy profile above which no further increase was induced. This synergistic interaction correlates well with the levamisol stimulation of 5-ALA-induced PpIX synthesis within the whole range of concentrations investigated. The administration of levamisol or lonidamine after PDT induced only an additive or slightly synergistic effect.

In spite of the promising results of these *in vitro* and *in vivo* studies, only a small number of clinical trials can be found in the literature. Jin and colleagues [71] reported a study in 144 patients with cardiac cancer in advanced stages. Patients were treated by HpD-PDT alone, Tegafur/uracil and mitomycin C in association (standard chemotherapy), or a combination of both PDT and standard chemotherapy. For PDT, an intravenous injection of the photosensitizer 48-72 hours prior to treatment was used and multiple exposures were occasionally needed. The dosages for combined chemotherapy were variable, but a common schedule was Tegafur/uracil every day and mitomycin C every week. In patients receiving PDT plus chemotherapy, treatments were preformed at the same time using the schedules mentioned above. No significant difference was observed with respect to the effect rate (complete remission plus partial remission) among the three groups. However, 19.5% of patients from the group with combination therapy showed complete remission compared to 5.5% and 8.3% for PDT and chemotherapy groups, respectively. None of the patients treated with PDT in combination with chemotherapy had liver or kidney function failure and only mild bone marrow depression was observed in this group. Moreover, no severe side effects, such as hemorrhage or perforation, were observed. This suggests that a combination of these two therapies with the proposed regimens is safe and may be beneficial.

In another trial, four patients with recurrent skin metastasis of a mammary carcinoma were administered low dose PDT using Photofrin® with or without previous infusion of a low dose mitomycin C [72]. The use of chemotherapy prior PDT allowed the reduction of the light dose by a factor of two, which may be useful to treat larger tumor areas. Moreover, the skin phototoxicity lasted maximally three weeks, which reduces the undesired prolonged cutaneous photosensitivity. A phase-1 study of sequential mitomycin C and 5-ALA-mediated-PDT administered to 22 patients with recurrent superficial bladder cancer was reported by Skyrme and colleagues [73]. Cumulative tumor recurrences were low, up to 11 at 24 months after PDT, compared to 29 in the 18 months before enrollment. No patient had evidence of progression to muscle-invasive disease. Additionally, the combined treatment was safe and well tolerated. No systemic or phototoxic side effects or reduction in bladder volume were reported. These results provide support of a new possibility for the treatment of superficial bladder cancer. Pass *et al.* [74] carried out a phase III randomized trial of surgery with and without intraoperative PDT and postoperative immunochemotherapy for malignant pleural mesothelioma (MPM). Surgical cryoreduction of the tumor was performed on 63 patients and those patients randomized to PDT received intraoperative PDT with Photofrin® as well. All patients received two cycles of immunochemotherapy after surgery (tamoxifen, interferon (IFN)-α and cisplatin). Intraoperative PDT did not result in any improvement of the treatment with respect to time to recurrence, recurrence patterns, and median survival, suggesting that first-generation intrapleural PDT is not beneficial to patients with MPM. Although this new therapeutic intervention was not successful in this study, it is far too early to discard it as a technique for intrapleural cancer control.

Methotrexate is an anti-cancer agent that is currently used in combination with other chemotherapeutic compounds for the treatment of many types of mostly invasive cancers [75]. It impedes tetrahydrofolate synthesis to competitive binding to dihydrofolate reductase, which, in turn, inhibits the synthesis of purines needed for the synthesis of DNA and RNA. Recently, it has been shown that this mechanism mainly contributes to the cell differentiation in colon cancer cells [76], which, then, can up-regulate heme biosynthesis in some cells [77]. It has been already shown that agents stimulating cell differentiation such as DMSO positively influence 5-ALA induced PpIX synthesis [78]. In this context, Sinha *et al.* [79] have shown that pre-incubation of a human prostate cancer cell line with methotrexate and subsequent 5-ALA mediated PDT increased the efficacy of the PDT treatment significantly. Similar effects were observed with differentiation inducers such as retinoic acid and vitamin D [80].

3. PDT AND PRO-OXIDANTS AGENTS OR OXIDANT ENHANCERS

The use of antioxidants in cancer prevention is mostly justified through prevention of oxidative damage of DNA [81]. However, one of the most important biological effects after PDT is oxidative stress through radical oxygen species resulting in cell death. Interestingly, a number of antioxidants can also exhibit pro-oxidant activities, especially in the presence of catalytic metals [82]. In fact, some studies have shown an enhanced activity of PDT by the concomitant use of certain antioxidants. A detailed review on this topic including different antioxidants has been recently published by Jakus and Farkas [83]. Early studies of the effect of ascorbate on the photosensitization of red blood cells by porphyrins [84] and phthalocyanines [85] evidenced an increased photohemolysis and rate of cell lysis in a dose-dependent manner, suggesting that ascorbate acts as a reactant and not as a catalyst. Buettner and coworkers [82,86] investigated the use of iron and ascorbate in com-

bination with Photofrin®-PDT on L1210 murine leukemia cells and human oral squamous cell carcinoma SCC-25 cells. PDT alone resulted in the production of membrane-derived free radicals. The pro-oxidant combination significantly enhanced the production of lipid radicals, 4- and 2-fold times when present during photosensitization or given after PDT, respectively. The dose effect curves of iron revealed that, with iron, an optimal concentration can be achieved, above which no further increase in radical production can be observed. The increased radical production correlated with a decrease in cell survival for both cell lines. Furthermore, the photosensitizer dose could be reduced to one-third when using the pro-oxidant combination. In contrast, Frank *et al*. [81] found a potent antioxidant activity of ascorbic acid, protecting mitochondria and other cell structures from oxidative cell injury induced by 5-ALA-mediated-PDT. Ascorbic acid was found to significantly inhibit the cellular protein oxidation and lipid peroxidation as well as the amount of necrotic factors, i.e. FasL and TNF-α mRNA expression generated by 5-ALA-mediated-PDT. According to these studies, ascorbate might act as pro-oxidant at low concentrations and as an antioxidant at higher concentrations. However, concentration may not be the only indication for ascorbates anti- or pro-oxidant activity. In a recent study, Kramarenko and colleagues [87] investigated the influence of ascorbate on Visudyne®-PDT on HL-60 and U937 human leukemia cells. Cells were preincubated with Visudyne® and ascorbate either in combination or alone, and then irradiated. For HL-60 cells, combined treatment resulted in a 65% decrease in cell survival compared to 50% for PDT alone. In contrast, using the same conditions as were used for HL-60 cells, only minimal changes in survival of U937 cells were observed. Furthermore, the growth rate of HL-60 cells was considerably reduced (2.5-fold) by the ascorbate and PDT combined treatment as compared to non-treated cells, whereas growth curves for treated and non-treated U937 cells were not significantly different. Both cell lines are from the same myelomonocytic origin and, therefore, similar results were expected. However, HL-60 cells showed a high level of myeloperoxidase, an enzyme which upon activation, i.e. by H_2O_2, induces the formation of more reactive oxidants. In U937 cells, this enzyme activity was below the limit of detection. Data clearly showed that ascorbate enhances the production of H_2O_2 associated with PDT using Visudyne®, and thus activation of myeloperoxidase in HL-60 cells, increasing the overall cytotoxic effect.

In the same way, the efficiency of PDT jointly administered with other widely used antioxidants, i.e. butyl hydroxyanisole (BHA), α-tocopherol has been investigated [88,89]. Shevchuk *et al*. [88] studied the effect of BHA in either HpD- or Mec_6-based-PDT on Ehrlich ascites carcinoma (EAC). Co-incubation of EAC cells with BHA and any of the two photosensitizers, followed by irradiation, caused an antagonistic interaction. However, upon increase of BHA concentration to toxic levels, the cytotoxic effect shifted towards an additive enhancement of the overall outcome. In contrast, EAC cells were 10-fold more sensitive to BHA when pretreated with HpD-PDT, showing a synergistic interaction between both treatments. Furthermore, *in vivo* studies showed an additive action of BHA on the tumoricidal effect of HpD-PDT presumably caused by impairment of mitochondrial respiration. In fact, incubation of cells with BHA alone resulted in a significant decrease (60%) in dehydrogenase activity and oxygen consumption as well as a significant increase (60%) in the rate of aerobic glycolysis and inhibition of the aerobic lactate production.

Bezdetnaya and co-workers [89] investigated the influence of α-tocopherol in the *m*-THPC-sensitized photoinactivation of both HT29 adenocarcinoma cells and MRC-25 normal fibroblasts. At low concentrations of α-tocopherol, no effect on *m*-THPC-induced photosensitization of HT29 cells was observed. At higher concentrations of α-tocopherol, however, efficacy of PDT was increased and the effect was synergistic at cytotoxic levels of the vitamin. No changes in the PDT-mediated cell survival profile of MRC-5 cells were observed by preincubation of cells with α-tocopherol prior PDT. These results were consistent with the effective incorporation of α-tocopherol by neoplastic cells and with the absence of its uptake by MRC-5 cells. The same group [90] carried out another study using a water-soluble α-tocopherol analogue, Trolox, in combination with *m*-THPC-PDT on HT29 xenografts in nude mice. The administration of Trolox before PDT resulted in a reduction of tumor growth by a factor of 1.5, whereas no significant differences were observed when administered post-PDT. Comparison of the contribution of the different pathways for the combination Trolox pre-PDT showed the singlet oxygen reaction due to PDT being dominant at the onset of the irradiation and then decreased as a result of the induced hypoxic conditions; this decrease is accompanied by an increase in the oxidized Trolox radical. Thus, the enhancement effect is likely due to the Trolox-mediated radical pathway working in concert with singlet oxygen while oxygen concentration is decreased in the course of PDT. An alternative to this photochemical mechanism may involve the capacity of antioxidants to induce tumor cell differentiation and to inhibit tumor growth.

Since tumor cells use the overexpression of glutathione (GSH) as a protective mechanism against PDT-induced formation of free radicals [91], decreasing intracellular GSH levels is another attractive option for increasing sensitization of tumor cells to PDT. *In vitro* studies by Miller and Henderson [92] showed an augmented response to PDT by buthionine sulfoximine (BSO), which inhibits the rate-limiting enzyme (γ-glutamylcysteine synthetase) in the pathway of glutathione biosynthesis. Concomitant BSO treatment given to four cell lines (CHO, V79, EMT6, RIF) resulted in an increase of PDT-induced cell death directly related to the GSH-depletion by BSO. Later studies with BSO demonstrated that this compound also enhanced the effect of PDT with Photofrin® *in vivo* [93,94]. Drugs were administrated 24 hours prior to irradiation. The combined treatment resulted in a significant increase in tumor regression. Additionally, when animals were treated with BSO, the GSH level in neoplastic cells was half of that in normal cells, suggesting that BSO selectively enhances the tumor response to PDT.

4. PDT AND ANGIOGENESIS INHIBITORS

As shown previously, PDT mechanisms include apoptotic and necrotic responses and indirect cell death through microvascular injury, leading to inflammation and hypoxia. On the other hand, PDT also induces expression of angiogenic and cell survival signals including VEGF, COX-2, and MMP [19,20,25,26]. This might ultimately lead to neovascularization and in some instances tumor recurrence. Therefore, researchers have investigated the potential impact of inhibitors of these stress signals on the therapeutic outcome of PDT.

Recently, we have shown that PDT-induced vascular occlusion is partially reversible and can lead to reperfusion of previously occluded blood vessels and formation of new blood vessels [95]. Conversely, this relapse was found to be reduced by the coadministration of compounds acting against VEGF (see Fig. (**7**)), which clearly demonstrates the benefit of PDT in association with VEGF inhibitors.

Ferrario and co-workers [19] confirmed that PDT with Photofrin® induced the expression of HIF-1α and increased the protein target levels of HIF-1α target gene - *VEGF* - within a transplantable BA mouse mammary carcinoma. In the same study, tumor-bearing mice were treated with PDT followed by 10 daily doses of two anti-angiogenic peptides, either IM862 or EMAP-II. The latter induces apoptosis in growing capillary cells and prevents vessel in-growth, while IM8662 inhibits the production of VEGF and activates NK cells. Both combinations resulted in the potentiation of tumoricidal action of PDT. In fact, the anti-angiogenic agents on their own did not produce any tumor cure or regression, but increased the response of cancer cells to PDT by a factor of two. A recent study of

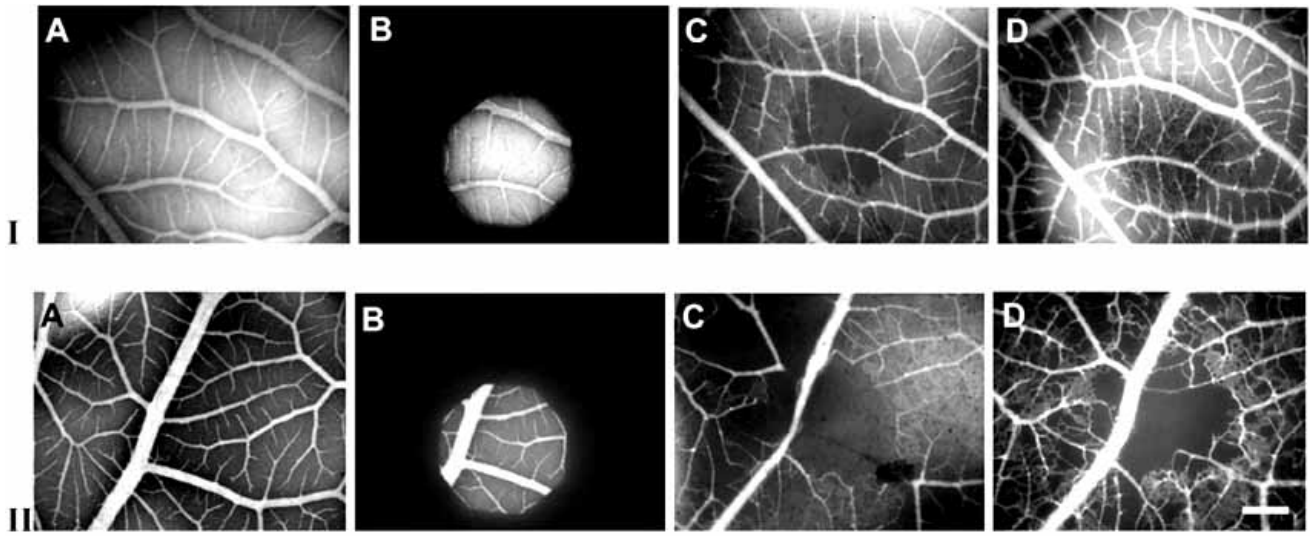

Fig. (7). Comparative damage on chorioallantoic membrane vasculature produced by PDT alone and PDT in combination with anti-angiogenic treatment. (**I**) PDT using intravenously applied BPD-MA (0.25 mg/Kg embryo), (**II**) PDT using intravenously applied BPD-MA (0.25 mg/Kg embryo) followed by anti-VEGF therapy using topically applied sFlt-1 (1μg/embryo) 6 hours post-PDT. (**A**) Photosensitizer fluorescence angiography before irradiation (BPD-MA: λ_{ex} = 400–440 nm; λ_{em} > 610 nm). (**B**) Photosensitizer fluorescence angiography during BPD-MA-PDT (λ_{ex} = 400–440 nm; λ_{em} > 610 nm). Diameter of the irradiated area: 1.8 mm; irradiation condition: 30 J/cm^2, 1 minute after photosensitizer injection. (**C**) Sulforhodamine 101 fluorescence angiography (λ_{ex} = 510–560 nm; λ_{em} = 625-675 nm) 24 hours after PDT. (**D**) Sulforhodamine 101 fluorescence angiography (λ_{ex} = 510–560 nm; λ_{em} = 625-675 nm) 48 hours after PDT. Contrast medium (Lipidem®) injected under the chorioallantoic membrane. Damage score on the irradiated area after 24 h: (**IC**) BPD-MA-PDT: 4, (**IIC**) BPD-MA-PDT/sFlt-1: 5; after 48 h: (**ID**) BPD-MA-PDT: 0, (**IID**) BPD-MA-PDT/sFlt-1: 4. White bar = 500 μm.

Ferrario and Gomer [96] studied the effect of Avastin, a novel anti-angiogenic monoclonal antibody already approved for the treatment of colon and rectal cancer, on the sensitization of malignant cells to PDT with Photofrin®. Avastin was chronically administered to tumor-bearing mice immediately after irradiation. An overexpression of human VEGF rather than host cell derived mouse VEGF was detected within tumors after PDT implying that tumor cells were the origin of most of the detectable VEGF. Avastin combined with PDT resulted in a statistically significant increased number of long-term tumor cures compared to individual treatments. A tumor cure rate of 55% was obtained with the combined treatment regimen, whereas PDT alone and Avastin alone showed only 22% and 10% cure rates, respectively. Interestingly, this enhancement of anti-tumor activity was not accompanied by any observable increase in normal tissue toxicity.

Dimitroff *et al.* [97] evaluated the anti-angiogenic and anti-tumor efficacy of two tyrosine kinase (RTK) inhibitors, PD166285 and PD173074, in C3H mice transplanted with murine mammary 16c tumor cells. The anti-cancer activity after oral administration of RTK inhibitors following PDT with hexylether pyropheophorbide-a (HPPH) was compared to that of PDT alone. PDT treatment showed no significant decrease in tumor growth when compared to control mice. However, combining HPPH-PDT and follow-up treatment for 14 days with PD166285 displayed a dramatic increase in tumor-free interval. In addition, both RTK inhibitors significantly decreased the tumor regrowth (from 2- to 3-fold) when combined with PDT. Only mice from the combination groups exhibited tumor cures. In general, tumor regrowth was observed by one week after withdrawal from the treatment, suggesting the need for treatment maintenance. Some side effects, i.e. hyperkeratosis and neurotoxicity at high doses of PD166285, were observed for the combination with HPPH-PDT, while no noticeable toxicities were manifested with HPPH-PDT combined with PD173074 treatment. Zhou *et al.* [98] evaluated other RTK inhibitors, SU5416 and SU6668S, in combination with hypericin-mediated-PDT on human nasopharyngeal carcinoma CNE2 xenografts. Although, differences in tumor growth among individual and combined treatments were not statistically significant, SU6668 in combination with PDT seemed to be more effective. This observation suggests that SU6668 works better than SU5416 to inhibit tumor regrowth, which may be explained by the fact that SU6668 blocks VEGF, FGF and, PDGF receptors while SU5416 is highly specific for VEGF-receptor 2. Conversely, after withdrawal, the tumor regrowth rate was higher for SU6668 than for SU5416, which is in agreement with previous *in vitro* studies showing the latter as a long-lasting inhibitor of VEGF-dependent proliferation in cells [99].

Hasan and co-workers [100] studied the effect of TNP-470, an anti-angiogenic peptide which strongly inhibits vascular endothelial cell proliferation and migration by blocking methionyl aminopeptidase-2, on subcurative benzoporphyrin derivative monoacid ring A (BPD-MA)-PDT. This study showed that subcurative PDT in an orthotopic model of prostate cancer increases not only VEGF secretion but also the incidence of lymph node metastases. Prostate weight and prostate volume were significantly reduced by the chronic administration of TNP-470 after PDT compared to the control group. TNP-470 given before PDT did not result in any significant differences. Interestingly, animals in the control group and animals receiving either PDT alone, TNP-470 alone, or PDT pretreated with TNP-470 had a weight loss, whereas the weight of animals receiving TNP-470 after PDT did not change. These results showed that, if the angiogenic action of VEGF is blocked by TNP-470, tumor growth, lymph node metastasis, and, disease-related toxicity are reduced.

Another potent vascular targeting agent, the 5,6 Dimethylxanthenone-4-acetic acid (DMXAA), has been also shown to selectively enhance PDT activity against mouse tumors [101,102]. Experimental evidence suggests that DMXAA increases tumor vascular permeability both directly and through the induction of other vasoactive mediators, such as TNF-α [103]. In a first study on PDT combined with DMXAA, Bellnier *et al.* [101] investigated the effect of administering a low dose DMXAA prior to PDT with Photofrin® in a transplanted murine RIF-1 tumor model. Cultured RIF-1 cells were mostly resistant to DMXAA and TNF-α. However, the combinational treatment with PDT resulted in the reduction of tu-

mor size as well as in a significant delay in regrowth and was both timing and sequence dependent. DMXAA was ineffective when administered after PDT, but effective when given a few hours before PDT. Furthermore, at low doses of Photofrin®, the effect on normal tissue of DMXAA combined with PDT was similar to that of PDT alone. According to these studies, DMXAA and not PDT was responsible for the increased expression of TNF-α after treatment indicating that this factor was primary responsible for the enhanced anti-tumor activity of PDT at low photosensitizer doses.

In a later study, the same research group [102] treated BALB/c mice bearing Colon-26 tumors with HPPH-PDT and DMXAA. Four different treatment regimens inducing different patterns of cellular, immune, and vascular responses were compared. The photosensitizer and the vascular permeability enhancer were administered 24 and 2 hours before irradiation, respectively. This time, an *in vivo* assessment of the tumor vascular response by magnetic resonance imaging and the fluorescein exclusion assay were included. PDT and DMXAA significantly enhanced the permeability of tumors when given as single treatments. However, the changes in vascular permeability induced by PDT did not predict tumor curability. In contrast, high-dose DMXAA or the combination of PDT and low-dose DMXAA, showed a common pattern of vascular permeability that was associated with long-term cure rates of more than 70%. Moreover, the therapeutic effects of DMXAA alone or in combination with PDT were largely confined to the tumor, whereas treatments with PDT increased the vascular permeability in both the tumor and peritumoral tissue. Although the doses of DMXAA and PDT in the combination regimen had virtually no anti-tumor activity when used separately, the combination of both at ineffective doses seemed to induce significant vascular damage which correlated with the enhanced tumor cure rate.

Due to its important role in inflammation and mitogenesis [104], PDT-induced expression of COX-2 would potentially impede the efficacy of PDT. Thus, COX-2 inhibition following PDT represents another alternative for the treatment of cancer. In addition, COX-2 overexpression has been reported for some types of cancer, such as colon, lung, and breast cancer [105-107]. Therefore, considerable attention has been paid to the potential effect of selective COX-2 inhibitors in combination with PDT against malignant cells in recent years. Ferrario and co-workers [20] showed the efficacy of PDT using Photofrin® followed by administration of multiple doses of the COX-2 inhibitor NS-398 in RIF tumors in C3H/HeJ mice. The tumor response was not affected by the administration of NS-398 alone or low PDT doses. However, combination of PDT with the inhibitor treatment resulted in statistically significant increases in tumor cures even at low PDT doses. Although the mechanism by which COX-2 inhibition enhances PDT responsiveness remains unclear, the NS-398 reduced the induction of PGE_2 and VEGF synthesis after PDT. These results are in agreement with Harvey and co-workers [108], who tested the effect of mono-L-aspartyl chlorine e6 (NPe_6)-PDT followed by NS-398 treatment on C57BL/6NCr mice bearing Colon-38 tumors. Unexpectedly, NS-398 did not potentiate the effect of PDT using a fractionated dosing regimen of the photosensitizer, presumably because the observed effect was already maximal with PDT alone.

In a subsequent paper, Ferrario *et al*. [109] showed that COX-2 also decreased the PDT-induced expression of the proinflammatory mediators IL-1β and TNF-α, and increased PDT-treated tumor levels of the anti-inflammatory cytokine, IL-10. PDT with Photofrin® together with NS-398 or celecoxib, a FDA approved COX-2 specific inhibitor, was tested on mouse mammary carcinoma cells and tumor-bearing mice. Both inhibitors, at non-cytotoxic concentrations, increased *in vitro* cellular photosensitization in a dose-dependent manner. This increase was directly related to an increased level of apoptosis. *In vivo*, multiple doses of either NS-398 or celecoxib in combination with PDT quadrupled the cure rate of PDT alone. In contrast, the enhancement to PDT responsiveness was predominantly assigned to the inhibition of expression of angiogenic and inflammatory molecules by COX-2 inhibitors and not to an increase of the apoptotic activity as it was seen in the cell culture studies.

In contrast, Makowski *et al*. [110] found that neither NS-398 nor other COX-2 inhibitors (rofecoxib and nimesulide) were capable of sensitizing C-26 tumor cells to Photofrin®-mediated-PDT-induced damage. According to the authors, because NS-398 used by Ferrario *et al*. [20] was given chronically after PDT, it seems possible that the potentiating effects were indirect and resulted from independent anti-tumor effects of the inhibitor. Later experiments allowed them to confirm that the administration of COX-2 inhibitors before PDT did not influence the effectiveness of PDT and that effectively superior vascular and tumor damage is obtained with the chronic administration of COX-2 inhibitors after PDT. Differences between sequencing suggest that COX-2 inhibitors potentiate the anti-tumor effects of PDT through inhibition of angiogenesis interacting with the reconstruction of blood vessels damaged by PDT.

Expression of COX-2 in lesions of the skin and oral cavity was reported by Akita *et al*. [111]. The authors studied the effect of 5-ALA-mediated-PDT in combination with nimesulide in two human oral squamous cell carcinoma cell lines, HSC-2 and HSC-4. The inhibitory effect of the combined treatment was superior to the individual treatment for the HSC-2, which overexpresses COX-2, while in HSC-4 cells no statistically significant differences were observed, presumably due to low levels of COX-2 expression. In fact, the interaction of 5-ALA-mediated-PDT with nimesulide in HSC-2 was demonstrated to be synergistic, supporting the hypothesis of independent mechanism of action.

Yee *et al*. [112] evaluated multiple-dose celecoxib as adjuvant treatment to improve the anti-tumor responsiveness of nasopharyngeal carcinoma bearing mice. Whereas PDT alone, inhibitor alone and the inhibitor given 24 hours post-PDT regimen were not significantly different in terms of tumor growth, the difference between PDT alone and in combination with the inhibitor given already 6 hours post-PDT was of borderline significance. Furthermore, simultaneous downregulation of COX-2, HIF-1α, VEGF A isoforms 165 and 121 occurred *in vivo* only when celecoxib was administered 6 hours post-PDT. Again, these results implicate that timing and sequence of the administration is crucial for effective tumor control in combination therapy.

There is experimental evidence of upregulation of inducible COX-2 after hypericin-mediated PDT induced by the selective activation of the mitogen-activated protein kinase (MAPK) p38 α and β at the protein and mRNA levels [113]. Therefore, an early blocking of the PG release through p38 MAPK inhibition might be useful as adjunctive therapy to PDT. Furthermore, the inhibition of the alpha isoform has been reported to block the release of VEGF and suppress tumor-promoted endothelial cell migration [114]. Hendrickx and colleagues [113] showed that the use of PD169316, a pyridinyl imidazole p38α MAPK inhibitor, improved the effectiveness of hypericin-PDT against human cervix carcinoma (HeLa) cells and human transitional cell carcinoma of the bladder (T-24), by blocking upregulation of COX-2 as well as by sensitizing cells to apoptosis. Unlike COX-2 inhibitors, the p38 MAPK inhibitor also interfered with apoptotic cell death in photodamaged cells. However, this difference does not exclude the validity of the use of COX-2 inhibitor for improving the anti-cancer efficacy of PDT as the inhibition of the COX-2-dependent synthesis of growth-promoting factors clearly surpasses the anti-apoptotic role of the enzyme. In the same study [114], the response of HY-based PDT combined with either the COX-2 inhibitor NS398 or the p38 MAPK inhibitor PD169316 were compared. Although endothelial cell migration was blocked to a similar extent by both inhibitors, inhibition of p38α MAPK pathway was more effective in suppressing VEGF synthesis. Moreover, experiments including wild type

and p38α knockout mouse embryonic fibroblasts clearly showed a balance towards cell death for p38α-deficient cells, which was not achievable by selective COX-2 inhibition by NS398. Altogether, these results imply that inhibition of p38 MAPK might be a more promising cancer treatment strategy than COX-2 inhibition.

An additional, but less studied, strategy consists of the administration of PDT in combination with MMP inhibitors since such enzymes are reported to be expressed after PDT and related to tumor angiogenesis, growth, invasion, and metastatic potential [115]. Ferrario *et al.* [26] evaluated the anti-tumor activity to Photofrin®-based-PDT with subsequent chronic administration of prinomastat, a potent synthetic MMP inhibitor, in BA bearing mice. The combination of PDT plus MMP inhibitor resulted in a significant difference in long-term cure rate compared to PDT alone. Tumors treated with prinomastat alone exhibited a modest reduction in growth, but no decrease in tumor size or long-term cures. Furthermore, skin damage by PDT with or without the MMP inhibitor was similar, which suggests that MMP expression does not modulate PDT-mediated normal skin phototoxicity. This study shows preliminary evidence of the potential advantageous therapeutic outcome for cancer treatment by using PDT and MMP inhibitors.

5. PDT AND IMMUNOTHERAPY

As mentioned above, anti-tumor immunity has been shown to be stimulated after PDT by the acute inflammatory response, generation of tumor-specific antigens, and induction of heat-shock proteins [116]. Treatment regimens using PDT and immunostimulating treatments are therefore likely to constitute an effective combination for various types of cancer. A common strategy to such combination is to sustain and/or amplify PDT-induced immunity against the treated cancerous lesion. Table **2** summarizes the predominant mechanisms of interaction between PDT and immunotherapy. Herein, the term "immunotherapy" will be used for all biological therapeutic agents that use the body's immune system, either directly or indirectly, to fight cancer or to lower treatment-associated side effects. Combinations of PDT with some biological therapies including cytokine therapy, microbial adjuvants, and regulatory T cells and adoptive cellular therapies have already been addressed in a recent review on PDT and anti-tumor immunity [117].

Table 2. Potential Mechanisms of Interaction Between PDT and Immunotherapy

• Upregulation of leukocyte adhesion molecules
• Potentiation of neutrophils and macrophages
• Induction of secondary cytokines
• Activation of dendritic cells, facilitation of the presentation of tumor antigens released following PDT, and induction of adaptative immunity
• Activation of $CD4^+$ helper T-lymphocytes and sensitization of $CD8^+$ cytotoxic T-lymphocytes
• Downregulation of $CD4^+CD25^+$ T-regulatory cells and potentiation of immunity
• Activation of B lymphocytes and natural killer cells
• Inactivation of remaining viable tumor cells through adaptative immunity
• Decrease of immunosuppressive effect induced by PDT

Combination of PDT with several cytokines has shown to enhance PDT anti-tumor effect. Hall *et al.* [118] found a synergistic interaction of PDT with Photofrin® concomitant with IFN-α in T24 human bladder cancer cells. They showed that the PDT doses had to be doubled in order to achieve the same amount of cell death of IFN-α given 24 hours post-PDT. Moreover, at high photosensitizer doses, the combined treatment allowed to reduce the light dose by a factor of three. Although the effect of IFN-α alone was dose-dependent, an increase of the IFN-α dose in the combination treatment did not result in a significant improvement. Altogether, these results suggest that the use of combined PDT and IFN-α may be able to significantly reduce the dose of light, which in turn may reduce the side effects without decreasing the efficacy. Dima and colleagues [119] evaluated IL-2 together with cyclophosphamide, another immunostimulant, in combination with Photofrin II-PDT. Wistar rats with ascites tumor cells were treated with both individual and combination therapies. Fractionated irradiation and multiple doses of IL-2 and cyclophosphamide entrapped in liposomes were used. PDT in combination with low doses of cyclophosphamide and IL-2 regimen was found to considerably reduce not only the tumor volume, but also the mortality of animals by more than 1.5-fold compared to local treatment with only PDT. Furthermore, the mitogenic response and cytotoxic activity of spleen lymphocytes were significantly increased by the associated therapy compared to PDT, IL-2, or cyclophosphamide alone. In the same way, Photofrin II-PDT has been investigated in association with recombinant human TNF-α in SM-F adenocarcinoma DBA/2 bearing mice [120]. Administration of TNF-α a few hours before suboptimal PDT doses resulted in a tumor area reduction of 57% compared to 21% of TNF-α alone. However, when standard doses of PDT were used, the interaction between PDT and the biologically active agent appeared to be additive. The administration of TNF-α immediately before irradiation seemed to be less effective than its administration a few hours before. No difference in normal tissue damage was observed between PDT combined with TNF-α a few hours before the irradiation regimen and PDT alone. In contrast, normal cells exhibited an increased photosensitization when TNF-α was given immediately before light administration, but not as high as that displayed by cancer cells. Although the mechanism of interaction during this combination treatment remains unclear, the authors suggest that the interaction may occur directly on the tumor cells or in the tumor parenchyma. While PDT induces the formation of singlet oxygen and superoxide anion radicals, TNF-α may involve the production of reactive oxygen species produced by neutrophils, tumor cells, or macrophages, both resulting in cellular damage. Alternatively, the effect may take place at the tumor vasculature, which both modalities damage severely.

According to several studies, neutrophils are essential for anti-tumor efficacy PDT [121] and therefore, administration of macrophage activating factors might improve such effectiveness. In a study carried out by Krosl and co-workers [122], genetically modified cells were engineered to produce murine granulocyte-colony-stimulating factor (GM-CSF). Then, mice transplanted with murine squamous cell carcinoma, a poorly immunogenic tumor, were treated with these modified cells and either Photofrin®-PDT or BPD-MA-PDT. For both combined regimens, cells were injected 48 hours before, immediately after, or 48 hours after irradiation. Complete tumor ablation was observed with all regimens. The combined Photofrin®-PDT combined with GM-CSF doubled the tumor-free period and increased survival of tumor-bearing mice compared to PDT alone. Although PDT regimens with BPD-MA seemed to be less effective than with Photofrin®, similar results were obtained when comparing monotherapy and combination therapy. Approximately 50% of animals receiving BPD-MA-PDT combined with GM-CSF remained tumor-free, while tumor regrowth was observed for all animals without GM-CSF treatment.

Later, Golab *et al*. [123] evaluated the cytotoxicity of PDT with Photofrin® in association with an intensive treatment with G-CSF in colon (C-26) and Lewis lung (3LL) carcinoma *in vivo*. The results showed a significant reduction of tumor growth and prolongation of the survival time of tumor-bearing mice using the associated regimen. About 33% of C-26-bearing mice treated with PDT in combination with G-CSF showed complete remission. Interestingly, tumors were rejected when mice were rechallenged with C-26 cells, but not other tumor cells suggesting the development of specific immunity against the treated tumor. Histopathology, immunohisto-

chemical and TUNEL staining of tumors demonstrated more intensive neutrophil infiltration and multiple apoptotic cells in the combined regimen compared to PDT alone. Co-administration of G-CSF and PDT synergistically stimulated bone marrow and spleen myelopoiesis increasing the number of active neutrophils. Furthermore, PDT increased vascular permeability facilitating tumor infiltration.

Preparations of microbial stimulators have been shown to improve the immune response to particular antigens, but are not always effective in reversing tumor progression. Therefore, microbial stimulators of innate immunity have been proposed as adjunctive therapy to other treatment modalities such as PDT. Myers *et al.* [124] investigated the interaction of HpD-PDT and *Corynebacterium parvum* in a murine transitional cell carcinoma (MBT-2) model. A low dose of *C. parvum* significantly improved the cancer cells sensitization to PDT, while PDT reduced the benefit obtained with high doses of the microbial adjuvant. However, high doses of the *C. parvum* given post-PDT resulted in a significant greater effect than the low dose of *C. parvum* before PDT. Similarly, Cho *et al.* [125] assessed the interaction of PDT and intravesical drugs, including some cytostatic agents and Bacillus Calmette-Guerin (BCG) in the same model. The associations were well tolerated and improved the cytotoxic effect of individual treatments. Korbelik and co-workers [126,127], evaluated combined regimens of either BCG or mycobacterium cell-wall extract (MCWE) treatment combined with PDT mediated by six clinically relevant photosensitizers: Photofrin®, BPD-MA, *m*-THPC, Mce_6, lutetium texaphyrin, and zinc phthalocyanine (ZnPC). The PDT doses used in all cases were chosen to achieve strong tumor reduction response, but low levels of permanent tumor cures. Irrespective of the photosensitizer, beneficial effects on PDT-mediated cures were obtained using both bacterial strains. According to flow cytometry-based analysis of cellular populations found in excised EMT6 tumor, the association of PDT and MCWE enhanced the infiltration of neutrophils when given after PDT. Deeper investigations of the effect of BCG adjuvant to PDT showed that treatments with BCG pre- and post-*m*-THPC-PDT effectively reduced the incidence of tumor recurrence and tumor cures. Again, irrespective of the photosensitizer, the use of BCG as adjuvant therapy enhanced the cure rate of PDT-treated tumors, but not tumor recurrence after the tumor-free period, except ZnPC which resulted in no tumor recurrence. In contrast to MCWE, BCG administration did not affect significantly PDT-induced accumulation of activated myeloid cells, but apparently increased immune memory T cells in tumor-draining lymph nodes indicating that the interaction may take place in later events involved in preventing tumor recurrence.

Among other immunostimulants investigated by the group of Korbelik [128] as adjuvant therapies to PDT, sonifilan, a β-D-glucan obtained from the Aphyllophoral fungus *Schizophyllum commune*, has been proven to be of particular interest. The administration of sonifilan before PDT to tumor-bearing mice resulted in an increased Photofrin® retention in cancer cells and a three times higher PDT response. In contrast, the administration of sonifilan after PDT was not beneficial with respect to tumor growth. Glycated chitosan was another immunostimulant that was evaluated as adjuvant to PDT. This agent, administered immediately after PDT with Photofrin® to EMT6 tumor-bearing mice, provided significant improvement in the long-term survival of these animals [129].

Other microbial preparations have been evaluated as adjuvant treatments to PDT. Uehara and co-workers [130] investigated the anti-tumor effect of PDT when combined with a streptococcal preparation (OK-432) in a murine model. The PDT activity against NR-S1 mouse squamous cell carcinoma was improved by OK-432 in particular when administered a few hours before PDT, while OK-432 alone did not produce a significant effect on the neoplastic cells *in vivo*. The combined regimen resulted in an important immune reaction evidenced as intense hemorrhage and marked infiltration of inflammatory cells into the necrotic area. However, the number of neutrophils was not increased by the treatment. Moreover, mice treated with the PDT combined with OK-432 tended to survive longer than those treated with any of the two therapies alone.

Adoptive cellular therapies constitute another interesting combination for PDT. Mature dendritic cells are professional antigen-presenting cells and the most effective inducers of adaptative immunity which is not the case of immature dendritic cells [131]. Jalili *et al.* [132] evaluated the tumor sensitization to Photofrin®-based-PDT in association with the administration of immature dendritic cells in C-26 bearing BALB/c mice. Dendritic cells co-cultured with PDT-treated C-26 cells resulted in efficient endocytosis (50%) of tumor cells and/or remnants tumor cells as compared to dendritic cells co-cultured with non-PDT-treated cancer cells (3%). In addition, the increased secretion of IL-2 by dendritic cells co-cultured with PDT-treated C-26 cells unequivocally showed that immature dendritic cells became functional after their interaction with damaged tumor cells. *In vivo*, dendritic cells administered to C-26 tumor were capable to reach local and, to a lesser extent, distant lymph nodes. Cytotoxicity of lymph node cells towards tumor cells was only detectable with tumors treated with dendritic cells combined with PDT. Moreover, spontaneous cytotoxicity (attributed to NK cells) was present in all of the treated groups, whereas specific cytotoxicity (attributed to $CD8^+$ T cells) was markedly increased (5-fold) by the administration of dendritic cells alone or in combination with PDT. Altogether, these findings support the strong reduction of tumor growth in mice receiving PDT in association with dendritic cells.

Korbelik and Sun [133] reported a study of *m*-THPC-PDT in combination with a biological therapy involving a genetically altered NK92MI cell line to produce IL-2. The interaction was investigated in NOD-scid mice bearing SiHa and HT-29 tumors. Low doses of NK92MI cells were effective enhancers of the anti-tumor response to PDT when administered pertumorally, while no obvious benefit was observed when administered intravenously. In contrast, at higher concentrations NK92MI cells given immediately after PDT substantially increased the cure rates of PDT-treated tumors, irrespective to the administration route. Although similar cure rates were observed in both SiHa and HT-29 tumors, i*n vitro* cytotoxicity assays showed that NK92MI cells were more aggressive against SiHa cells than HT-29 cells. Treatment with NK92MI alone did not lead to cures of subcutaneous tumors under comparable experimental conditions. Furthermore, immunocompatibility studies in immunocompetent BALB/c recipients bearing PDT-treated EMT6 tumors showed a clear therapeutic benefit, which indicates that the adoptively transferred cells were not destroyed before exerting their anti-tumor action. The enhancement produced by adjunctive NK92MI may be simply due its cytotoxic activity on the remaining foci of viable malignant cells not destroyed by PDT. However, the interaction between pathways stimulated by PDT and NK92MI cell activity may also have a synergistic character that requires further elucidation.

A further biological therapy involves the administration of antibodies against cancer cells or cancer-associated targets. However, to the best of our knowledge, few experiments combining this modality with PDT have been reported. Del Carmen and colleagues [134] studied C225, a monoclonal antibody against the epidermal growth factor receptor (EGFR), as an adjunctive therapy to BPD-MA-PDT. A challenging model, NIH:OVCAR-5 bearing mice, was chosen for this study. Although the overexpression of EGFR is associated with the development of ovarian cancer resistant to chemotherapy and immunotherapy, the combinatory regimen showed increased anti-tumoral activity. C225 adjuvant to PDT resulted in a 7- or 4-fold reduction of tumor burden compared to C225 or PDT alone as well as improvements in survival time. *A priori*, this synergistic response may reflect the fact that the individual monotherapies target non-overlapping molecular pathways. C225 blockade of EGFR activity prevents cancer cells that

EGFR activity prevents cancer cells that overexpress EGFR from aberrantly entering S phase, which in turn makes them more vulnerable to PDT. An alternative explanation is that C225 mitigates the enhanced activation of EGFR after PDT and suppresses downstream survival signals. Because recurrent epithelial ovarian cancer is rarely curable, this approach may offer a new possibility to women suffering from this disease.

Although promising results of PDT-combined regimens with immunotherapy have been observed, so far, few studies have been performed in humans. Szygula *et al.* [135] reported a pilot study of 14 patients with Transitional cell carcinoma (TCC) of the urinary bladder. Patients were subjected to 5-ALA-mediated-PDT with subsequent BCG-therapy after the previous transurethral resection of bladder tumor (TUR-BT). In 10 cases, neither macroscopic recurrence nor cytologic signs of cancer or pre-cancerous lesions has been noted in a 2-year follow-up. From this group, total response was observed in eight patients (normal urothelium or urocystitis were found during control examinations) and partial response in two (diagnosed with low or high-grade dysplasia in microscopic examination). Tumor recurrence was observed in four patients (cancer cells in excised specimens). Although the number of patients included in this trial was small, these preliminary results show that the association of TUR-BT, 5-ALA-mediated-PDT and BCG might improve the efficacy of urinary bladder cancer therapy.

6. CONCLUSIONS

Chemotherapy as an option for the treatment of cancer represents some inconveniences including dose-limiting toxicity and treatment resistance. Furthermore, other therapies such as immunotherapy may not be totally effective as single treatment modality. This has led to the search for more effective therapeutic agents or mechanism-based combination therapies that overcome these drawbacks and, thus offer cancer patients more safe and effective treatments.

So far, PDT has been shown to be a useful and well-tolerated therapy for different diseases. Moreover, a large body of *in vitro* and *in vivo* evidence has been accumulated demonstrating the potential benefits of combination therapies including PDT in association with other pharmacological therapies over monotherapy for the treatment of several types of cancer. These results are mainly supported on the multiple mechanisms of action of PDT which usually single drugs do not exhibit. PDT in combination with chemotherapeutic agents has shown not only to increase the direct damage to the targeted cells by cytotoxic agents, but also to affect the tumor microvasculature and, in some cases, induce a host immune response against cancer cells. On the other hand, the final PDT outcome could be reduced by PDT-induction of potent angiogenic factors. The use of anti-angiogenic agents has been shown to counteract the PDT-induced angiogenesis and cell proliferation that otherwise could lead to disease recurrence. Although PDT can stimulate the host immune response, it can also induce the expression of some immune suppressor factors. Some immunologic agents have resulted in an enhancement of the PDT-induced host immune response against cancer cells or overcome PDT-induced immune suppression.

Despite the increased number of preclinical reports, there are still considerable efforts to undertake in terms of clinical trials for assessing the risk-benefit of such PDT-combined treatments in order to establish them as alternative or first choice treatments for malignant disorders.

ACKNOWLEDGEMENTS

The authors would like to thank the Swiss National Science Foundation (Grant # 205320-112234 / 1) and Oncosuisse (Grant # OCS – 01948 – 08 – 2006) for their financial support. Furthermore, we are grateful to Prof. Robert Gurny for carefully reading the present review and giving his much valued input.

LIST OF ABBREVIATIONS

5-ALA	=	5-aminolaevulinic acid
AlS_2Pc	=	aluminum phthalocyanine
BCA	=	bacteriochlorin a
BCG	=	bacillus Calmette-Guerin
BHA	=	butyl hydroxyanisole
BPD-MA	=	benzoporphyrin derivative monoacid ring A
BSO	=	buthionine sulfoximine
COX-2	=	cyclooxygenase-2
DMXAA	=	5,6 Dimethylxanthenone-4-acetic acid
EAC	=	Ehrlich ascites carcinoma
EGFR	=	epidermal growth factor receptor
EMMPRIN	=	extracellular matrix metalloproteinase inducer
G-CSF	=	granulocyte-colony-stimulating factor
GM-CSF	=	murine granulocyte-colony-stimulating factor
GSH	=	glutathione
HeLa	=	human cervix carcinoma
HIF	=	hypoxia inducible factor
HpD	=	hematoporphyrin derivative
HPMA	=	N-(2-hydroxypropyl) methacrylamide
HPPH	=	hexylether pyropheophorbide-a
IFN	=	interferon
ICG	=	indocyanine green
IL	=	interleukin
MAPK	=	mitogen-activated protein kinase
MC540	=	merocyanine 540
Mce_6	=	meso-chlorin e_6 monoethylene diamine
MCWE	=	mycobacterium cell-wall extract
MMP(s)	=	metalloproteinase(s)
MPM	=	malignant pleural mesothelioma
m-THPC	=	meso-tetrahydroxyphenylchlorin
NF	=	nuclear transcription factor
NK	=	natural killer cells
NPe_6	=	mono-L-aspartyl chlorine e6
OVCAR	=	human epithelial ovarian carcinoma
PDT	=	photodynamic therapy
PG	=	prostaglandin
PPC	=	porphyrin platinum conjugates
PPCII	=	porphyrin platinum conjugates second generation
RTK	=	tyrosine kinase
TCC	=	Transitional cell carcinoma
TIMP	=	tissue inhibitor of metalloproteinase
TNF	=	tumor necrosis factor
TUR-BT	=	transurethral resection of bladder tumor
VEGF(s)	=	vascular endothelial growth factor(s)
ZnPC	=	zinc phthalocyanine

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Received: February 17, 2008 **Revised: May 02, 2008** **Accepted: May 12, 2008**