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Combined Therapy For Squamous Carcinoma Cells: Application of Porphyrin-Alkaloid Modified Gold Nanoparticles

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1. Introduction

Photodynamic therapy (PDT) is an established and useful modality for the clinical non-invasive treatment of cancer. This therapy requires a photosensitizing agent (photosensitizer) selectively taken up by tumor cells, visible light, and molecular oxygen to generate highly reactive oxygen species (ROS), which ultimately cause tumor destruction. The specificity achieved from drug uptake selectivity combined with light targeting makes PDT an appealing approach.

PDT consists of three phases: excitation of photosensitizers (PS) by light, production of ROS, and induction of cell death (Triesscheijn et al., 2006). In the first phase, irradiated light of a suitable wavelength, typically visible or near-infrared, excites the PS molecules. The light is generally selected to correspond with the maximum absorption wavelength of the PS. The PS molecules then absorb light energy and change to an excited singlet state. These excited molecules can fall back to their native state with emission of fluorescence. Thus, all PS molecules are also examples of fluorescent molecules. On the other hand, the molecules also have the ability to undergo an electron spin conversion to their triplet state followed by the transfer of this energy to oxygen molecules or to other substrate molecules in the surroundings which then react with oxygen.

1.1 History of PDT

The fact that sunlight can be used to treat a variety of diseases such as rickets, psoriasis, and skin cancer is known from ancient civilizations, i.e. Egyptian, Chinese and Indian (Ackroyd et al., 2001; Daniell & Hill, 1991; Fitzpatrick & Pathak, 1959). At the beginning of the 20th century the term “photodynamic action” was used by Tappeiner et al. to explain the oxygen-consuming chemical reactions induced by photosensitization (Moan & Peng, 2003; Szeimies et al., 2001). Tappeiner, in cooperation with Jesionek, successfully treated patients

suffering from stage II syphilis, lupus vulgaris, and superficial skin cancer with topical eosin red solution (Szeimies et al., 2001). In 1942, Auler and Banzer observed specific uptake and retention of hematoporphyrin in tumors followed by higher fluorescence in cancer cells as compared with the surrounding tissue, and induction of necrosis after irradiation (Szeimies et al., 2001). Afterwards, PDT had not been used until Dougherty initiated revitalization by treating a group of patients suffering from cutaneous and subcutaneous tumors with the injection of photosensitizer dihematoporphyrin and red light produced by laser. The majority of the treated tumors showed either complete or partial remission (Dougherty et al., 1975; Dougherty et al., 1978; Szeimies et al., 2001).

Particularly, PDT has grown in reputation in dermatology, mostly due to the simple accessibility of light exposure for the skin and the simplicity of topical use of photosensitizers. In the late 1970s, Thomas Dougherty initiated human clinical trials of PDT with hematoporphyrin derivative (HpD) for the treatment of cutaneous cancer metastases (Blume & Oseroff, 2007; Dougherty, 1996; Zeitouni, 2003). PDT has been revived and has become more applicable to common dermatology since 1990, when Kennedy et al. introduced 5-aminolevulinic acid (ALA) (Fig. 1), a topical porphyrin precursor causing local accumulation of the endogenous photosensitizer protoporphyrin IX (PpIX) (Fig. 2) with no significant prolonged phototoxicity (Kennedy, 1990). Nowadays, PDT is used to treat diseases in a variety of fields, including respiratory medicine (Ost, 2001; Suttedja & Postmus, 1996), urology (Jichlinski, 2006; Juarranz et al., 2008; Pinthus et al., 2006), ophthalmology (Mitra, 2002), and gastroenterology (Barr et al., 2001; Wiedmann & Caca, 2004), as well as dermatology. Mostly porphyrins or phthalocyanines have been studied (Marmur et al., 2004). On the other hand, for dermatological purposes, only hematoporphyrin derivatives such as porfimer sodium, or PpIX-inducing precursors such as ALA or methyl aminolevulinate (MAL) are of useful concern. As systemic photosensitizing drugs caused extended phototoxicity (Marmur et al., 2004), topical photosensitizers are preferred for the use in dermatology. Several drugs containing ALA or MAL are used for treating epithelial cancers and there is an increasing importance in the use of PDT (Braathen, 2001; Dragieva et al., 2004a; Dragieva et al., 2004b).

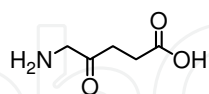


Fig. 1. Structure of 5-aminolevulinic acid

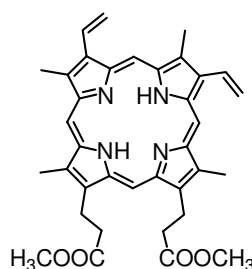


Fig. 2. Structure of protoporphyrin IX

1.2 Mechanism of PDT

PDT requires an interaction of three key elements: light, a photosensitizer, and oxygen. After exposure to particular wavelengths of light, the photosensitizer is excited from a ground state (S_0) to an excited singlet state (S_1) (Fig. 3) followed by intersystem crossing to a longer-living excited triplet state (T_1).

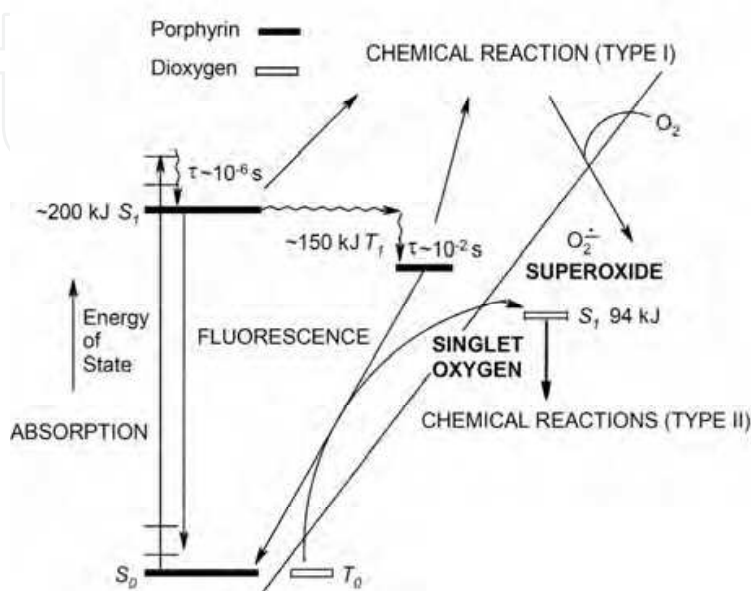


Fig. 3. Mechanism of PDT; State energies are represented by thick lines:

■ porphyrin sensitizer, □ dioxygen; reactive dioxygen intermediates are in bold

After that, the photosensitizer at T_1 state is able to go through two types of reaction with nearby molecules: either a type I reaction through hydrogen or electron transfer generating free radicals, or a type II reaction through energy transfer to oxygen, creating molecular singlet oxygen (1O_2). The type I reaction results in generation of reactive free radicals or radical ions, which then react with ground-state molecular oxygen to produce superoxide anion radicals, hydrogen peroxides and hydroxyl radicals (Foote, 1991). The type II reaction produces singlet oxygen which has an important role in the molecular processes initiated by PDT (Foote, 1991; Niedre et al., 2002). The singlet oxygen has a lifetime approx. $3 \mu s$ and can diffuse no more than $0.07 \mu m$ in cells (Moan, 1990; Hatz et al., 2007). Therefore, the initial damage is limited to the site of the PS molecule. This is usually the mitochondria, Golgi apparatus, plasma membrane, endosomes, lysosomes, and endoplasmic reticulum (Buytaert et al., 2007). Damage to the subcellular organelles and plasma membrane eventually leads to apoptotic, autophagic and/or necrotic cell death. Generally, PS molecules localized to the mitochondria or the endoplasmic reticulum cause apoptosis, while localization either in the plasma membrane or lysosomes is found to delay or block the apoptotic pathway. On the other hand, if the apoptotic route is blocked, damaged cells still die using the autophagic or necrotic pathways (Buytaert et al., 2007; Oleinick et al., 2002). Latest studies support apoptosis as probably the preferred path to cell death (Buytaert et al., 2007). Even though it is considered that 1O_2 is the main cytotoxic species and starts the pathway responsible for the damaging effects of PDT, free radicals formed by type I reactions significantly contribute to cell death as well (Foote, 1991).

1.3 Photosensitizers in PDT

The first generation of PS molecules was represented by HpD or its purified version porfimer sodium (Photofrin) (Fig. 4). Primarily, they were used as general PS and tested for cutaneous malignancies. On the other hand, general intravenous administration and the consequential prolonged phototoxicity, which can last 6–10 weeks, restricted their use (Dragieva et al., 2004; Fritsch et al., 1998).

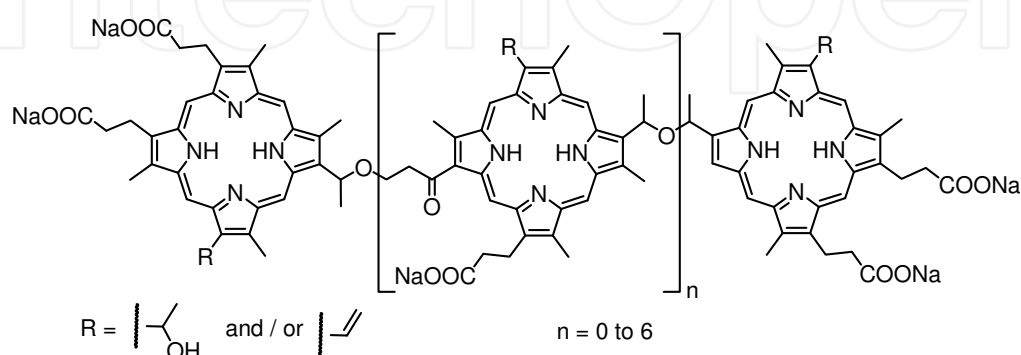


Fig. 4. Structure of Photofrin

Second generation PS molecules such as *m*-tetrahydroxyphenyl-chlorin, tin ethyl etiopurpurin, phthalocyanines, and chlorins (Fig. 5) are pure compounds that can be activated by light wavelengths in the range of 660–690 nm. Most significantly, they all have a lower tendency to cause prolonged photosensitivity compared with the first generation of photosensitizers (Moan & Berg, 1992).

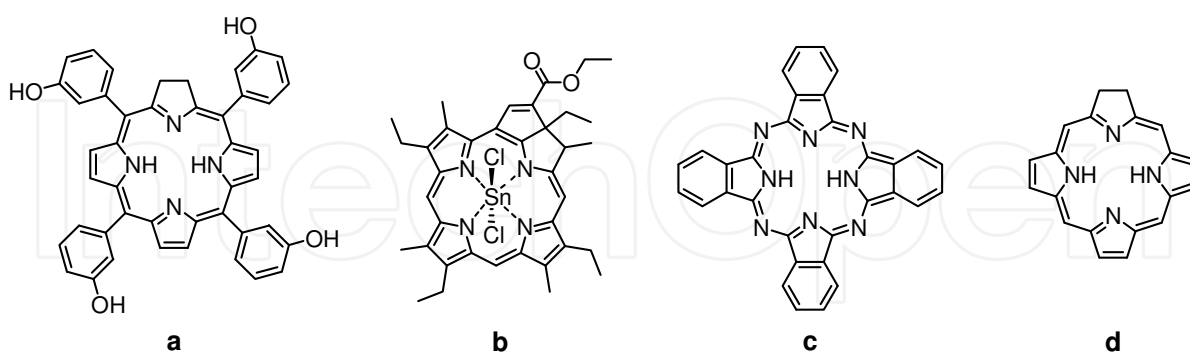


Fig. 5. Structure of *m*-tetrahydroxyphenyl-chlorin (a), tin ethyl etiopurpurin (b), phthalocyanines (c), and chlorins (d)

Third generation PS molecules (not yet approved) consist of antibody-conjugated PS (Josefsen & Boyle, 2008) and lutetium texaphyrin (Fig. 6) (Woodburn et al., 1998; Young et al., 1996). These drugs supporting deeper penetration into tissue with absorptions of 700–800 nm accumulate in tumor tissues with high selectivity.

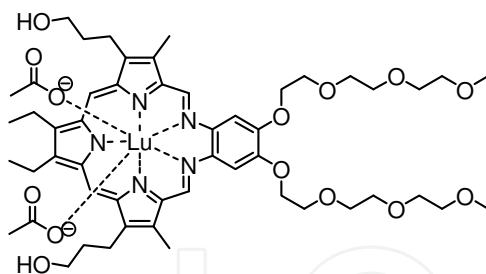


Fig. 6. Structure of lutetium texaphyrin

To avoid the prolonged photosensitivity caused by systemic administration, topically applied photosensitizers have been developed for the treatment of skin cancers. The most successful commercially accessible topical drugs are ALA and its methyl ester MAL. Levulan® using ALA and the Blu-U light source was accepted by the U. S. Food and Drug Administration for the treatment of nonhyperkeratotic actinic keratoses of the face and scalp in 1999 (Babilas et al., 2005; Kormeili et al., 2004). MAL was accepted in Europe for topical PDT of actinic keratosis (AK) and basal cell carcinoma (BCC) in 2001 (Morton, 2003; Morton et al., 2002) and for the treatment of AK in the USA in 2004 (Zeitouni et al., 2003; Garcia-Zuazaga et al., 2005). The endogenous photosensitizer PpIX generated from ALA or MAL can be fully metabolized to photodynamically inactive heme over 24–48 h (Blume & Oseroff, 2007; Morton, 2004), which radically decreases the unpleasant side effect of prolonged cutaneous phototoxicity.

1.4 Nanoparticles in PDT

In 2002 Konan et al. divided methods of PS molecules delivery into passive and active based on the presence or absence of a targeting molecule on the surface (Konan et al., 2002). The methods employed to bring the PS explicitly into diseased tissues using the target tissue receptors or antigens were designated active, whilst others that enable parenteral administration and passive targeting, such as PS conjugates of oil-dispersions, polymeric particles, liposomes, and hydrophilic polymers, were named passive. Active nanoparticles can be subclassified by their mechanism of activation and passive nanoparticles can be subclassified by material composition into (a) non-polymer-based nanoparticles, e.g. ceramic and metallic nanoparticles, and (b) biodegradable polymer-based nanoparticles.

1.4.1 Active nanoparticles

- Photosensitizer nanoparticles

Quantum dots (QDs) have great photostability, intensive fluorescent emission (high quantum yields) and possible use in specific pathological fields. They can be water soluble, and transfer energy to surrounding oxygen with resulting cellular toxicity. Many studies have been devoted to this field (Bakalova et al., 2004). The first report deals with cadmium selenide (CdSe) QDs and was published by Samia et al. in 2002. The authors presented the possibility to use semiconductor QDs alone to generate $^1\text{O}_2$ due to the intercalation of dissolved oxygen at the QD surface (Samia et al., 2003). They predicted a comparable interaction in water-soluble phospholipid-capped QDs. Moreover, they assumed that since the lowest excited state of CdSe QDs is a triplet state, the energy transfer was responsible for

the generation of singlet oxygen ($^1\text{O}_2$) from triplet oxygen ($^3\text{O}_2$). On the other hand, the efficiency of generation of $^1\text{O}_2$ was about 5% (with 65% emission quantum yield of QDs) as compared to 43% for the PS only. It may be due to carrier trapping and nonradiative carrier recombinations occurring on the early picosecond time scale and the very small fraction of QD - $^3\text{O}_2$ pairs created at any moment (Samia et al., 2003). To avoid the ineffectiveness of QDs alone to produce singlet oxygen, several experiments have been carried out to covalently conjugate PSs to CdSe/ZnS via organic bridges (Hsieh et al., 2006; Samia et al., 2003). These experiments have a frequent problem with lower water solubility.

- Self-lighting nanoparticles

Scintillation or persistent luminescence nanoparticles with attached PS molecules such as porphyrins were applied as *in vivo* agents for PDT (Auzel, 2004). After exposure to ionizing radiation such as X-rays, scintillation luminescence is produced from the nanoparticles and stimulates the photosensitizers, followed by production of singlet oxygen that increases the destruction of cancer cells by ionizing radiation. Employment of common radiation therapy with PDT allows application of lower doses of radiation. Using BaFBr:Eu⁺,Mn⁺ nanoparticles displaying luminescence, short X-ray exposures could be applied followed by extended PS excitation. The period of phosphorescent decay is increased *in vivo* due to higher local temperatures (Chen et al., 2006).

- Upconversion nanoparticles

Upconversion and simultaneous two-photon absorption occurs in luminescent materials with triplet excitation states (Auzel, 2004). Upconverting nanoparticles are modified nanometer-sized composites that generate higher energy light from lower energy radiation typically near or middle infrared (anti-Stokes emission) using transition metal ions doped into a solid-state host (Boyer et al., 2006; Pires et al., 2006). For biological use, the desired nanocrystalline core should have morphological and optical features that are appropriate for conjugation with biological molecules and exhibit high intensity emission as well (Pires et al., 2006). Preparation of high-quality nanocrystals is needed, and the surface properties and growth dynamics must be precisely controlled (Wang et al., 2006). Upconversion nanoparticles can be prepared via numerous different ionic materials - typically rare earth ions such as lanthanides and actinides doped in a suitable crystalline matrix (Zijlmans et al., 1999). Micrometer-sized Er³⁺/Yb³⁺ or Tm³⁺/Yb³⁺ co-doped hexagonal NaYF₄ are examples of nanoparticles that exhibit the highest upconversion efficiencies (Heer et al., 2004) and are precursors of upconverting nanoparticles with biological applications (Zhang et al., 2006). In 2006, the NaYF₄ nanocrystals doped with Er and Yb and coated with organic polymers were prepared and strong emission upon activation with 980 nm NIR laser was shown (Feng et al., 2006). One year later, Zhang et al. used upconverting nanoparticles (nanoparticles of NaYF₄:Yb³⁺,Er³⁺ coated with a porous thin layer of silica doped with merocyanine and functionalized with a tumor-targeting antibody) in PDT, but these nanoparticles were not activated in depth in animal tissue and the efficiency in killing cancer cells was very low (Zhang et al., 2007).

Another class of employed upconversion nanoparticles consists of zinc phthalocyanine (ZnPC) (Fig. 7) physically adsorbed to the surface of the nanoparticles with the encapsulation efficiency of 98 % (Ricci-Junior & Marchetti, 2006b). The fluorescence excitation spectrum of ZnPC exhibits an excitation maximum at 670 nm and greatly overlaps

the red emission peak for the upconversion nanoparticles. Creation of $^1\text{O}_2$ by irradiation of the ZnPC-nanoparticle complex with 980 nm light was confirmed through the photobleaching of disodium 9,10-anthracenedipropionic acid (Wieder et al., 2006).

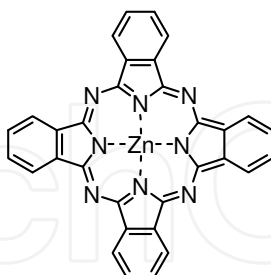


Fig. 7. Structure of zinc phthalocyanine

1.4.2 Passive nanoparticles

- Non-biodegradable nanoparticle carriers

In 2003 Roy et al. first reported ceramic-based nanoparticles used as a new drug-carrier system for PDT. It utilizes 30-nm silica-based spherical particles doped with the anticancer drug 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide (Fig. 8) (Roy et al., 2003).

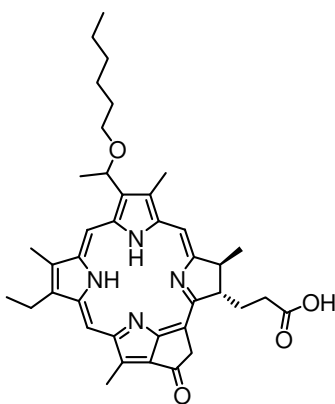


Fig. 8. Structure of 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide

Irradiation of the nanoparticles with light of appropriate wavelength led to efficient creation of singlet oxygen. On the other hand, noncovalent adsorption of PS into porous silica nanoparticles led to drug leakage. Covalent bonding of the PS into organically modified silica nanoparticles produced more stable material (Ohulchanskyy et al., 2007). Organically modified silica nanoparticles were also used for two-photon dye encapsulation (Kim et al., 2007). Cinteza et al. described a combination of magnetism and PDT using micellar polymeric diacylphospholipid-poly(ethylene glycol) capsules for encapsulation of the 2-devinyl-2-(1-hexyloxyethyl)pyropheophorbide PS and magnetic Fe_3O_4 nanoparticles (Cinteza et al., 2006). In contrast to the previous report (Kim et al., 2007), the magnetic nanoparticles were used for targeted delivery of PS to tumor cells and increased imaging (Cinteza et al., 2006). Wieder et al. described a delivery system consisting of gold nanoparticles modified with phthalocyanine (Wieder et al., 2006). Phthalocyanine derivative-modified gold nanoparticles have 2-4 nm in diameter and have a maximum absorption peak at 695 nm. They generated $^1\text{O}_2$ catalytically with high efficiency. Upon irradiation of these nanoparticles, significant improvement in PDT

efficiency was observed, probably thanks to 50% increase of $^1\text{O}_2$ quantum yields as compared to the free PS. In the same year El-Sayed reported efficient conversion of strongly absorbed light by plasmonic gold nanoparticles to heat energy. Easy bioconjugation of nanoparticles used suggests their application as selective photothermal agents in molecular cancer cell targeting (El-Sayed et al., 2006).

Two-photon dyes have received attention lately because of their ability to convert absorbed low-energy radiation to higher energy emissions. Dyes that can direct transfer of the higher energy to molecular oxygen for generation of $^1\text{O}_2$ can be very useful in PDT because they can be activated in deep tissues. The first use of two-photon dyes that are able to convert absorbed low-energy radiation to higher-energy emissions was recently reported using microemulsion to incorporate the two-photon dye porphyrin tetra(*p*-toluenesulfonate) into polyacrylamide nanoparticles (Gao et al., 2006).

- Biodegradable nanoparticle carriers

Biodegradable polymeric nanoparticles allow high drug loading and controlled drug release. They exist in a large variety of materials (Konan et al., 2002). Modifying the surface of nanoparticles with polymers such as poly(ethylene glycol) and poly(ethylene oxide) increases circulation times (McCarthy et al., 2005). Brasseur et al. described hematoporphyrin adsorbed in polyalkylcyanoacrylate nanoparticles (Brasseur et al., 1991), but the resulting materials showed poor carrier capacity and rapid drug release. Encapsulation of tetrasulfonated zinc phthalocyanine or aluminium naphthalocyanine into poly(isobutylcyanoacrylate) or poly(ethylbutylcyanoacrylate) nanocapsules or nanosphere was published in the same year (Labib et al., 1991). Then, second generation phthalocyanine derivatives were used in PEG-poly(lactic acid) nanoparticles (Allemann et al., 1995). The results showed that immobilization in the biodegradable nanoparticle improved PDT response of the tumor in contrast to conventional Cremophor EL emulsion by providing prolonged tumor sensitivity towards PDT (Allemann et al., 1995). After a few years, Konan et al. developed polyester poly(D,L-lactide-coglycolide) and poly(D,L-lactide) doped with PS with much higher loading than ever published before (Konan et al., 2003a; Konan et al., 2003b). In order to further investigate these nanoparticles, the efficacy of the encapsulated drug was assessed on the chick embryo chorioallantoic membrane model (Vargas et al., 2004). In another work the *in vitro* and *in vivo* photodynamic activities of verteporfin-loaded poly(D,L-lactide-coglycolide) nanoparticles were studied. The results showed improved photodynamic activity of PS (Konan-Kouakou et al., 2005).

The problem with side photosensitivity due to non-specific localization of the PS into healthy tissue or skin was studied by McCarthy et al., who developed a new nano-agent that has several desirable properties for use as photodynamic drug including no toxicity in extracellular spaces and time-dependent intracellular release of PS (McCarthy et al., 2005). They demonstrated in cell culture that the phototoxicity caused by non-internalized nanoparticles is minimal (9% cell death) in contrast to the effect of internalized nanoparticles (95% cell death under identical testing conditions) (Dougherty et al., 1978). In another study Ricci-Junior et al. reported the preparation, characterization, and results of the phototoxicity assay of poly(D,L-lactide-coglycolide) nanoparticles containing ZnPC for PDT use (Ricci-Junior & Marchetti, 2006a). Other photosensitizers that have been studied consist of Indocyanine green (Saxena et al., 2006) and Hypericin (Zeisser-Labouebe et al., 2006). These compounds have the potential to be used for both diagnostic and therapeutic purposes.

1.5 Combined therapy

Even if PDT has been used effectively for treating various tumors, it still has several restrictive factors for a target-specific response, such as an observed angiogenic effect and pronounced inflammatory reaction after PDT treatment (Pervaiz & Olivo, 2006). PDT in combination with other types of therapy is an attractive approach to suppress these problematic side effects.

PDT-induced hypoxia has been associated with an increase in the expression of many angiogenic growth factors, such as hypoxia-inducible factor 1 (HIF-1), fibroblast growth factor receptor-1 (FGFR-1), cyclooxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF). Combination therapy using antiangiogenic agents (e.g., COX-2 or VEGF inhibitors) with PDT led to a significant decrease of PDT-induced expression of prostaglandin E2 and VEGF, as well as a marked improvement in tumoricidal response (Akita et al., 2004; Ferrario et al., 2002; Zhou et al., 2005).

In contrast to radiotherapy, surgery or chemotherapy, PDT can lead to a strong acute inflammatory response, generally as tumor-localized edema. This PDT-induced immune activation makes it possible to positively reverse the tumor-host relationship from one that is tumor dominated to one that is oriented against the tumor. The combination with immunotherapy can reinforce the immune response triggered by PDT and thus significantly improve the anti-tumor immune response (Pervaiz & Olivo, 2006). Numerous recent clinical trials conclude that enhanced clinical outcomes can be achieved by a combination of ALA-PDT and immunomodulation therapy for the treatment of premalignant skin diseases, such as Bowen's disease (BD), BCC and AK (Wang et al., 2007; Wang et al., 2008).

In several cases, combination therapy can be done by linking the photosensitizer directly to an anticancer drug or to a specific antibody to target highly tumor-expressed receptors (Palumbo, 2007). It would also be easily accomplished by combining them using nanotechnology.

1.6 Light sources in PDT

A variety of light sources that are used in PDT consist of light-emitting diodes (LEDs), filtered xenon arc and metal halide lamps, fluorescent lamps, and lasers. Lasers and filtered broadband sources provide comparable efficacy in topical PDT (Clark et al., 2003). Non-laser light sources are also important in topical PDT, because in contrast to lasers they are stable, cheap, and offer broad-area illumination fields. Recently, LEDs showed significant progress in design, creating these low-cost sources suitable for broad-area irradiation, and were accepted for patient use. These LEDs are focused on the 630-to-635-nm activation peak of PpIX while excluding the inappropriate wavelengths present in broadband sources, thus allowing shorter irradiation times. Biophysical calculations show that LEDs with peak emission of 631 ± 2 nm can have a deeper PDT action in tissue than filtered halogen lamps with 560–740 nm emission, and hence LEDs may be more successful in treating the deeper parts of tumors (Juzeniene et al., 2004).

PpIX has its main absorption peak in the blue region at 410 nm (Soret band) with smaller absorption peaks at 505, 540, 580 and 630 nm. Most light sources for PDT seek to utilize the 630-nm absorption peak, in order to improve tissue penetration. On the other hand, a blue fluorescent lamp (peak emission 417 nm) is usually used. Nowadays, there are several reports

that blue, green, and red light itself can be efficient in topical PDT of AK; however, the more deeply penetrating red light is better when treating BD and BCC (Morton et al., 2002).

The concept of ambulatory PDT to decrease hospital attendance for PDT was described by Moseley et al. (Moseley et al., 2006). In a study of five patients with BD, PDT was carried out with ALA and a portable LEDs device. Current studies have suggested that pulsed light therapy may be helpful for treatment in topical PDT of acne, AK and photorejuvenation. On the other hand, a recent controlled investigative study carried out in healthy human skin *in vivo* demonstrated that two pulsed light sources formerly reported in PDT brought evidence of minimal activation of photosensitizer, with a significantly smaller photodynamic reaction than observed with a conventional continuous wave broadband source (Strasswimmer & Grande, 2006). These sources deliver intense light in short periods (< 20 ms), which might suppress oxygen consumption (Kawauchi et al., 2004). Unplanned ambient light exposure may have considerably contributed to the clinical effect. However, three studies have recently addressed the possibility of using ambient light for ALA-PDT of AK (Batchelor et al., 2007; Marcus et al., 2007; Strasswimmer & Grande, 2006). Two of them report on therapeutic advantage. Nevertheless, the randomized ambient light-controlled study using ALA demonstrated no significant effect on lesion ablation. A randomized right/left inpatient evaluation of conventional MAL-PDT combined with LEDs device *versus* daylight (for 2.5 h) for the treatment of AK of face and scalp demonstrated corresponding reduction in AK and significantly less pain with daylight (Wiegell et al., 2008).

Total effective light dosage is proposed as a concept for optimizing the accuracy of light dosimetry in PDT considering incident spectral irradiance and optical transmission through tissue and absorption by PS (Moseley, 1996). Actually, light dosimetry is explained as the irradiance rate (mW cm^{-2}) at the skin surface and the total dosage (J cm^{-2}) distributed to the surface, the second being a product of irradiance and time of exposure.

It has been suggested that lower fluence rates and fractionation of light exposure can improve lesional reaction by promotion of the photodynamic reaction (Henderson et al., 2004). A study of superficial BCC illuminated with 45 J cm^{-2} at 4 h and repeated at 6 h with 633-nm laser light at 50 mW cm^{-2} showed a total response of 84 % after a mean of 59 months (Star et al., 2006). Newer studies support advantages of the fractionation approach in BCC, although not in BD (Haas et al., 2006; Haas et al., 2007).

1.7 Synthetic *meso*-tetraphenylporphyrins in PDT

Extensive information about the application of various porphyrins and their derivatives in PDT has been published (Král et al., 2006). Accordingly, our laboratory synthesized porphyrin conjugates with glycol (Králová et al., 2008a), bile acid (Králová et al., 2008b), and cyclodextrins (Králová et al., 2006) and their *in vitro* and *in vivo* PDT activity has been tested. It was shown that these porphyrin conjugates are taken up preferentially by tumor cells and have the potential to be used for PDT to selectively ablate tumors (Králová et al., 2006; Králová et al., 2008a; Králová et al., 2008b).

Our contemporary strategy is to combine favorable features of gold nanoparticles mediating the photothermal effect with a photosensitizing compound mediating the photodynamic effect into one combined modality and thus introduce a therapeutic protocol efficient against SCC.

The key steps in our strategy are: i) generation of a synthetic ligand with photosensitizing properties, ii) ligand immobilization on the surface of modified gold nanoparticles to enable

combination of PDT and thermo-effect, and iii) verification of the biological activity by *in vitro* and *in vivo* studies.

2. Experimental

2.1 Preparation of modified gold nanoparticles

Porphyrin-brucine conjugates **1** and **2** (Fig. 9) were prepared according to the procedure described previously (Král et al., 2005). Gold nanoparticles (14.7 nm) were prepared by citrate reduction of potassium tetrachloroaurate(III) (**Au-citr**). After modification with 3- mercaptopropanoic acid, derivatives **1** and **2** were immobilized as described elsewhere (Řezanka et al., 2008). Here, a solution of **1** or **2** (5 mg) in methanol was added to 50 ml of **Au-citr**. Modified nanoparticles (**Au-1** and **Au-2**, respectively) were isolated by centrifugation after three days of incubation. Using redispersion in methanol, methanol-water, water and dimethylsulfoxide, unbound porphyrin derivatives were removed and **Au-1** and **Au-2** molecules were concentrated to a volume of 1 ml. According to the spectral analysis of supernatants, 0.8 mg of **1** or **2** was present in the final 1 ml solution of **Au-1** and **Au-2** nanoparticles. The core of modified nanoparticles was characterized by transmission electron microscopy and photon cross-correlation spectroscopy (Nanophox). The chemical modification, ligand, was analyzed by absorption and fluorescence spectrometry. Fluorescence spectra were recorded using a Fluoromax spectrometer (Jobin-Yvon, Japan). A volume of 1 ml of sample was placed into 1 cm plastic cuvettes. The excitation wavelength was 520 nm.

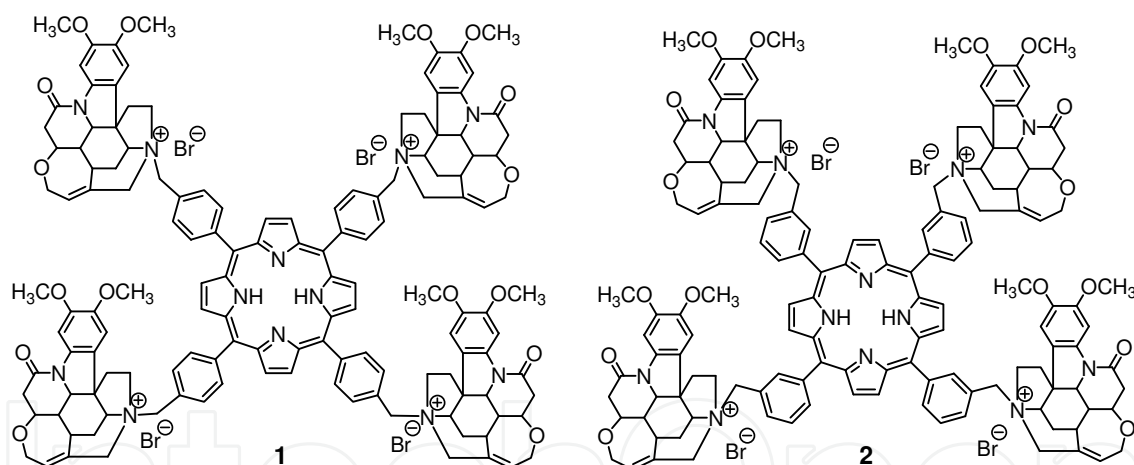


Fig. 9. The structure of **1** and **2**

2.2 Cell culture and *in vitro* experiments

4T1 (mouse mammary carcinoma) and A431 (epidermal squamous carcinoma) cells were purchased from ATCC and PE/CA-PJ34 (human basaloid squamous cell carcinoma) cells were purchased from ETCC. As described before (Králová et al., 2006), all cells were grown exponentially in RPMI 1640 medium with 10% fetal calf serum. For photodynamic experiments, $1-1.5 \times 10^5$ cells were seeded into 1.8 cm^2 wells and incubated overnight with the porphyrin-brucine conjugates or their counterparts immobilized on gold nanoparticles (1 and $2.5 \mu\text{M}$). After incubation, cells were rinsed with PBS, cultured for 1 h in fresh medium without phenol red and illuminated with a 75 W halogen lamp with a band-pass filter (Andover, Salem, NH) that emitted light at wavelengths between 500–520 nm. The

fluence rate at the level of the cell monolayer was 1 mW cm^{-2} , and the total light dose was 7.2 J cm^{-2} . Twenty-four hours post irradiation, the viability of PDT-treated cultures was determined by the Trypan blue exclusion method. In parallel, control “dark” experiments (without illumination) were performed.

2.3 Microscopic studies

Cells grown on coverslips in 35 mm Petri dishes were incubated with $2.5 \mu\text{M}$ porphyrin-brucine conjugates in culture medium for 16 h. After washing, porphyrin fluorescence was observed with a DM IRB Leica microscope equipped with a DFC 480 camera using a x63 oil immersion objective and Leica filter cube N2.1 (excitation filter BP 515–560 nm and long pass filter LP 590 nm for emission). To label lysosomes, 500 nM LysoTracker Green (Molecular Probes) was added to the culture media for 30 min. Cells were washed and examined by fluorescence microscopy using the Leica filter cube I3 (excitation filter BP 450–490 nm and long pass filter LP 515 nm for emission).

2.4 *In vivo* experiments

For *in vivo* experiments, the immuno-compromised nude mice with subcutaneously implanted human SCC tumors were used. When the tumor mass reached a volume of 100 mm^3 (10–14 days after injection), mice were intravenously injected with porphyrin-brucine conjugates (5 mg kg^{-1}) resuspended in a volume of 0.1 ml per 20 g mice and six hours later the tumor area (2 cm^2) was irradiated with a 500–700 nm xenon lamp ONL051 (maximum at 635 nm, Preciosa Crytur, Turnov, Czech Republic) with a total impact energy of 100 J cm^{-2} and fluence rate of 200 mW cm^{-2} . Each experimental group consisted of five or eight mice. The tumor size was measured repeatedly and the tumor volume was determined (Králová et al., 2006). All aspects of animal experimentation and husbandry were carried out in compliance with national and European regulations and were approved by the institutional committee.

3. Results and discussion

3.1 Modification by gold nanoparticles

Gold nanoparticles (14.7 nm) prepared by citrate reduction of potassium tetrachloroaurate(III) (**Au-citr**) were modified with 3-mercaptopropionic acid, and the derivatives **1** and **2** were immobilized. Gold nanoparticles modified with **1** and **2** are designated **Au-1** and **Au-2**, respectively.

3.2 Fluorescence spectra

The fluorescence intensity of **1** and **2** was strongly dependent on the solvent used. The influence of additional compounds on the intensity of emitted fluorescence wavelengths was tested by measuring the emission spectra (excitation of the first Q-band of porphyrins at 520 nm) of **1** and **2** in water, an inorganic salt solution (corresponding to the cell culture media) supplemented with a 50 mg ml^{-1} solution of human serum albumin (HSA) (Fig. 10A). In comparison with water, the emission bands of **1** and **2** measured in the media were red-shifted (for **1**, from 638 and 700 nm to 644 and 709 nm, and for **2**, from 643 and 707 nm to 647 and 710 nm) and the fluorescence intensity of **1** increased slightly whilst that of **2** decreased. After immobilizing the porphyrin conjugates on nanoparticles, the intensity of fluorescence emission

spectra significantly decreased (Fig. 10B) despite the concentration of porphyrins remained the same. The weak quantum yield may be attributed to that: (1) both porphyrins and nanoparticles absorb light at approximately 520 nm, (2) fluorescence quenching by porphyrin-to-metal energy transfer, (3) partial aggregation of the modified nanoparticles. In the case of **Au-1**, aggregation seems to be the cause (Fig. 10B, compare traces “**Au-1/water**” and “**Au-1/medium**”), as the intensity of emitted fluorescence was several times higher in cell culture medium compared to water only. These results demonstrate that both para- (**1**) and meta- (**2**) derivatives aggregate in a solution-dependent manner that is not affected by the presence of PS or immobilization on gold nanoparticles. Importantly, the presence of model plasma proteins present in the cell medium dramatically reduced the aggregation of modified nanoparticles. This observation led us to further test these compounds for *in vivo* PDT efficacy.

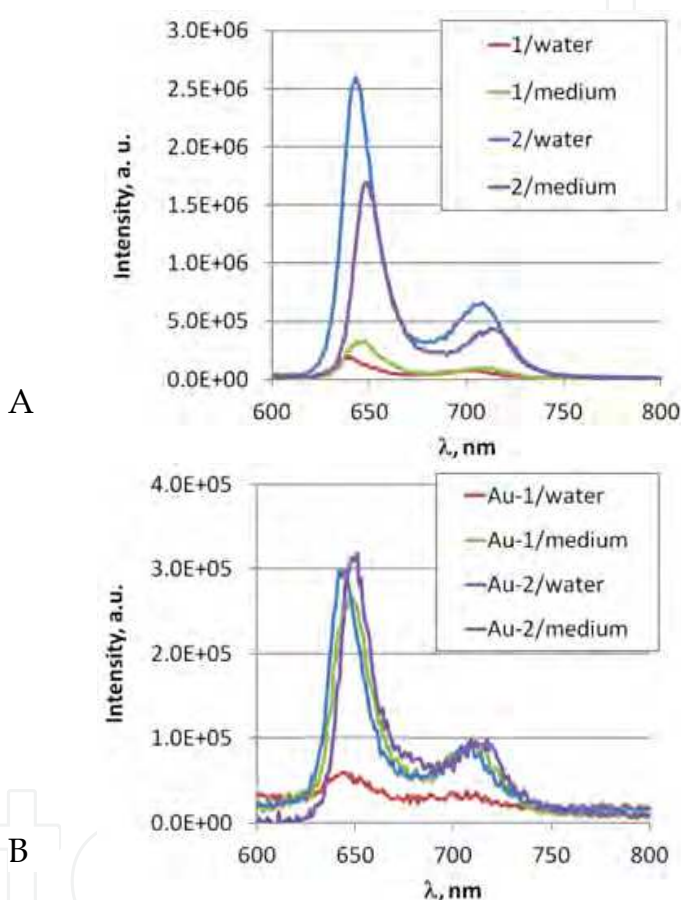


Fig. 10. The fluorescence emission spectra of porphyrins **1** and **2** (left) and porphyrin-modified nanoparticles **Au-1** and **Au-2** (right) in water and cell culture media. Excitation was performed at 520 nm. Porphyrin-brucine conjugates were used at a concentration of 3.5 μM . The concentration of human serum albumin used in growth medium was 50 mg ml⁻¹.

3.3 Intracellular localization

The porphyrin-brucine conjugates (**1** and **2**) were next analyzed for tumor cell uptake and intracellular distribution. The mammary carcinoma cell line, 4T1 was cultivated in the presence of the conjugates for 16 h, during which time the cells were well-dispersed and growing mostly as planar sheets, enabling focused images of fluorescence to be recorded. These cells exhibited punctate red fluorescence (Fig. 11).

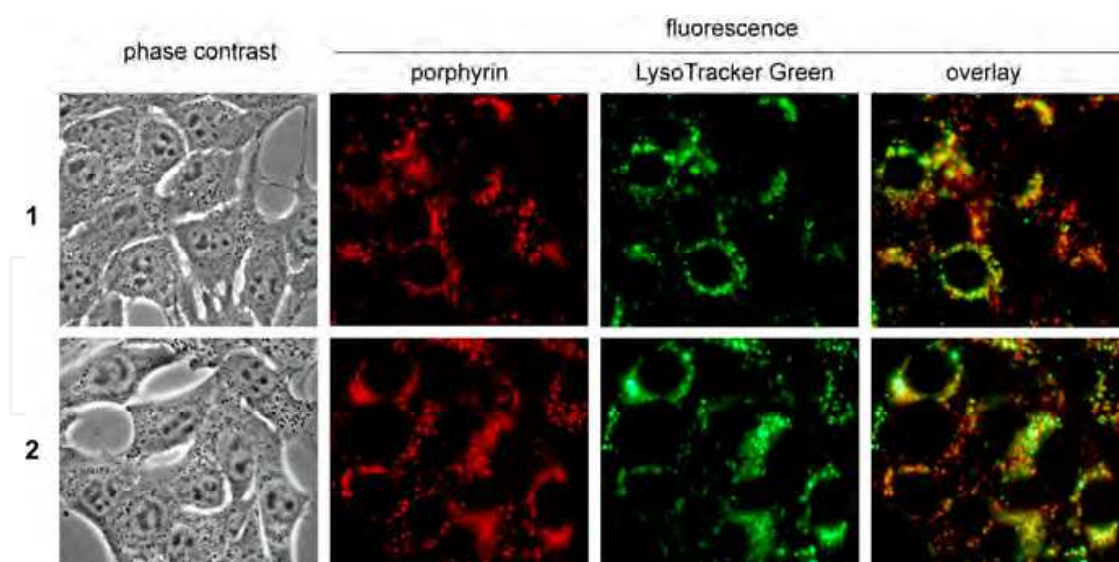


Fig. 11. The intracellular localization of porphyrin-brucine conjugates in 4T1 cells. The middle panels show the red fluorescence of **1** and **2** and co-staining with the lysosomal specific probe (LysoTracker Green); right panels represent an overlay of the green and red images and demonstrate co-localization (shown in orange/yellow). Porphyrin-brucine conjugates were used at a concentration of 2.5 μM .

To identify the intracellular compartment where **1** and **2** accumulate, co-staining with the LysoTracker Green fluorescence probe was performed. The merged images revealed that **1** and **2** colocalized to a subset of LysoTracker-stained structures that represent lysosomes. Similar localization was also observed in PE/CA-PJ34 basaloid squamous cell carcinoma cells and A431 epidermal squamous carcinoma, cell lines that were predominantly used in our study (data not shown). Upon addition of gold nanoparticle-conjugated **1** and **2** to cell culture media, aggregates formed, which were visible as a reddish precipitate that covered parts of the cell. These were particularly abundant in the case of **Au-1** (Fig. 12).

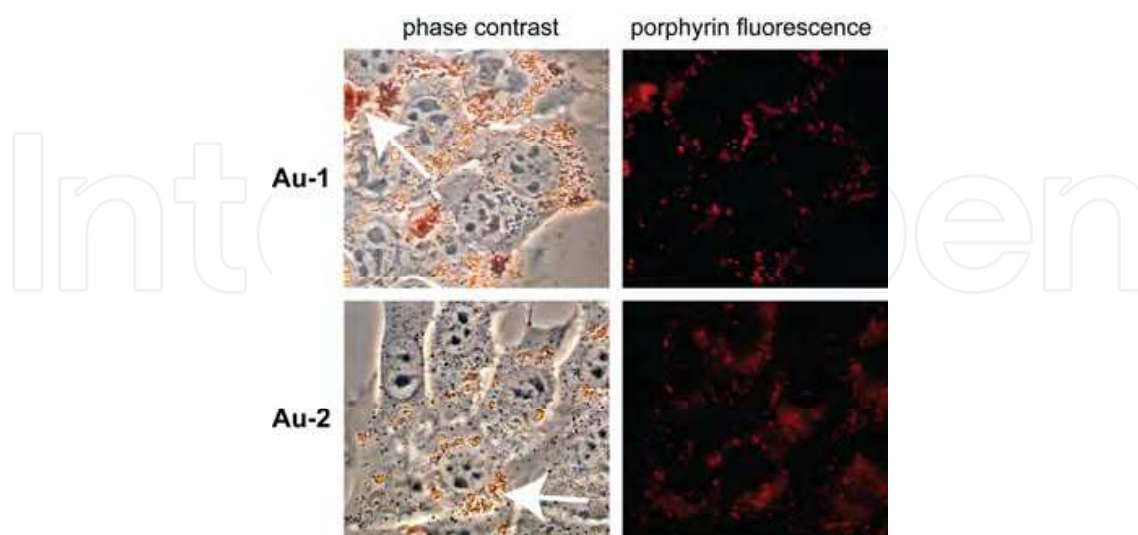


Fig. 12. Difference in aggregation behavior of porphyrin-brucine conjugates immobilized on gold nanoparticles (left panels). 4T1 cells were incubated with **Au-1** and **Au-2** at a concentration of 2.5 μM for 4 h before pictures were taken. Aggregates are highlighted by arrows.

3.4 *In vitro* phototoxicity

To investigate the photodynamic potential of the free porphyrin-brucine conjugates or those immobilized on gold nanoparticles, we incubated PE/CA-PJ34 cells in the presence of the conjugates for 16 h and subjected them to PDT. In parallel, cells were incubated with porphyrins without illumination to serve as dark controls. Twenty-four hours following the illumination of cells with filtered light, the mortality of post-PDT cultures was determined (Fig. 13).

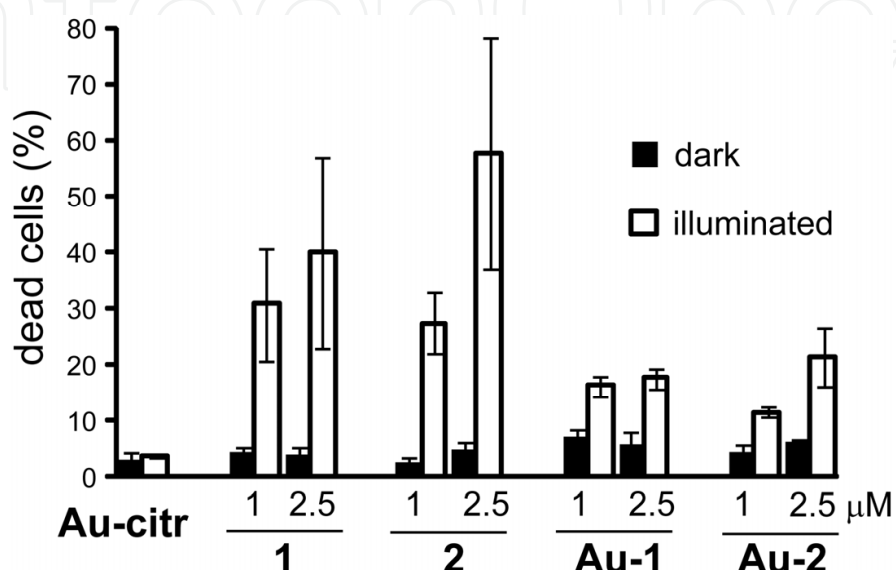


Fig. 13. The effect of free or immobilized porphyrin-brucine conjugates on the induction of cell death via PDT. PE/CA-PJ34 cells were incubated with either 1 or 2.5 μM of **1** and **2** or their modified Au-nanoparticles for 16 h. Cells were then illuminated with filtered light (500–520 nm, 7.2 J cm⁻²). The percentage of dead cells was established the following day by using the Trypan blue exclusion method. The average and standard deviation for three independent experiments is shown.

Satisfyingly, the induction of cell death was both light and drug-dose dependent. Control cells incubated with unconjugated gold nanoparticles (**Au-citr**) did not display any increase in cell death after illumination. Thus, under these *in vitro* conditions we can exclude the possibility that any case of cell death is due to the photothermal activity of the gold nanoparticles. Interestingly, the phototoxicities of unbound porphyrin-brucine conjugates **1** and **2** were higher than those immobilized on gold nanoparticles. This reduction of photodynamic efficacy is likely to be a consequence of **Au-1** and **Au-2** aggregation that occurs in the aqueous cell growth media (Fig. 12).

3.5 *In vivo* PDT efficacy

Using an *in vivo* mouse cancer model, the PDT effectiveness of the unbound porphyrin-brucine conjugates **1** and **2** was compared with those immobilized on gold nanoparticles (**Au-1**, **Au-2**). Nude mice (NuNu) bearing basaloid squamous cell carcinoma PE/CA-PJ34 cells received by intravenous injection either unmodified porphyrins or their gold nanoparticle-modified counterparts. Six hours post injection, tumors were illuminated

with light at a dose of 100 J cm^{-2} . Mice not injected with unmodified porphyrins or nanoparticles served as controls. Tumor size was measured after PDT at regular intervals (Fig. 14).

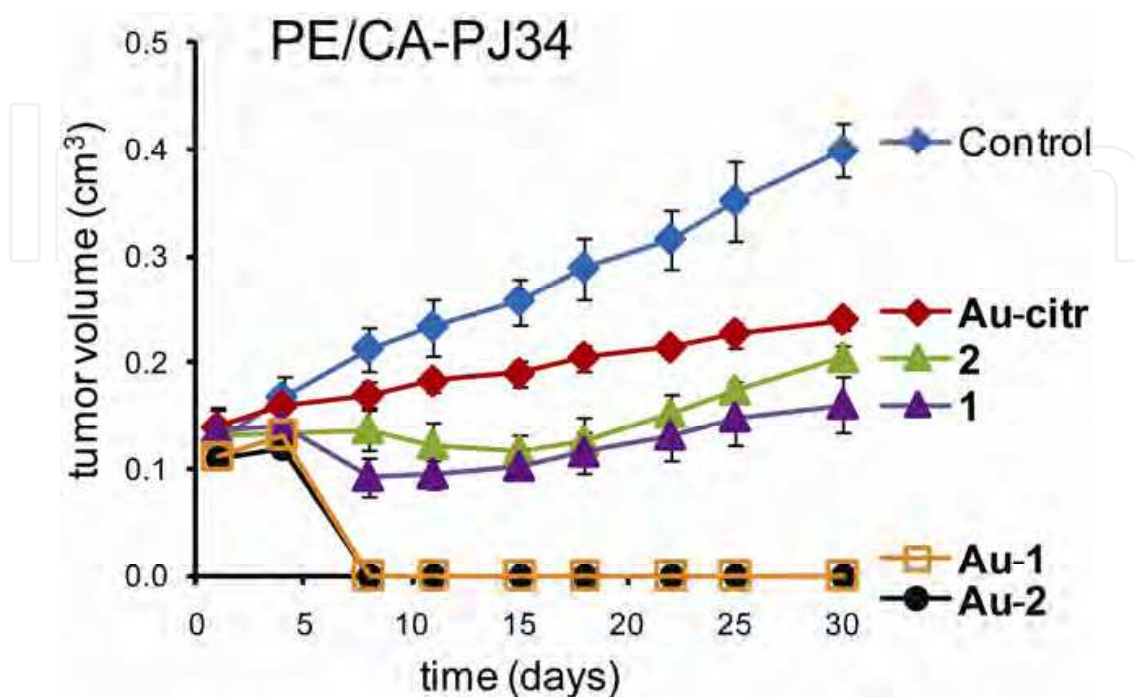


Fig. 14. The PDT effectiveness of **1** and **2** and their respective Au-immobilized nanoparticle counterparts to eradicate mouse tumors. Nude mice (NuNu) bearing subcutaneous PE/CA-PJ34 tumors ($n = 8$ per each group) received an intravenous dose of the drug (5 mg kg^{-1}). Tumors were illuminated with light (100 J cm^{-2}) six hours after injection. The tumor size was measured repeatedly and the tumor volume was determined. Control mice were exposed to illumination but did not receive the porphyrin drug. The **Au-citr** group represents mice injected with Au nanoparticles, **1** and **2** groups received porphyrin conjugates, **Au-1** and **Au-2** groups received porphyrin-modified Au nanoparticles.

We observed the greatest reduction in tumor growth in mice treated with **Au-1** and **Au-2**. All tumors were eliminated in animals that received these conjugated porphyrins and importantly, no detectable relapse of the primary tumor was observed. In contrast, animals treated with unbound **1** and **2** exhibited only a transient regression in tumor size that lasted until day 18, when the primary tumors began to gradually regrow. Presumably, this relapse in tumor growth comes from the small population of tumor cells that survived the PDT. Interestingly, mice treated with unconjugated gold nanoparticles exhibited slight tumor retardation in growth, which is most likely due to the photothermal effect described in other systems (Gamaleia et al., 2010; O'Neal et al., 2010; Řezanka et al., 2008).

These results clearly show that porphyrin alkaloid-modified gold nanoparticles are very effective against basaloid SCC *in vivo*. To verify more general applicability of porphyrin alkaloid-modified gold nanoparticles, the same approach was used against epidermal SCC tumors (Fig. 15). A431 cells formed fast progressing subcutaneous tumors, which were completely eradicated after **Au-2**-mediated PDT treatment in 60% mice or their growth was substantially reduced. These results demonstrate a high potential of porphyrin alkaloid-modified gold nanoparticles to fight SCC.

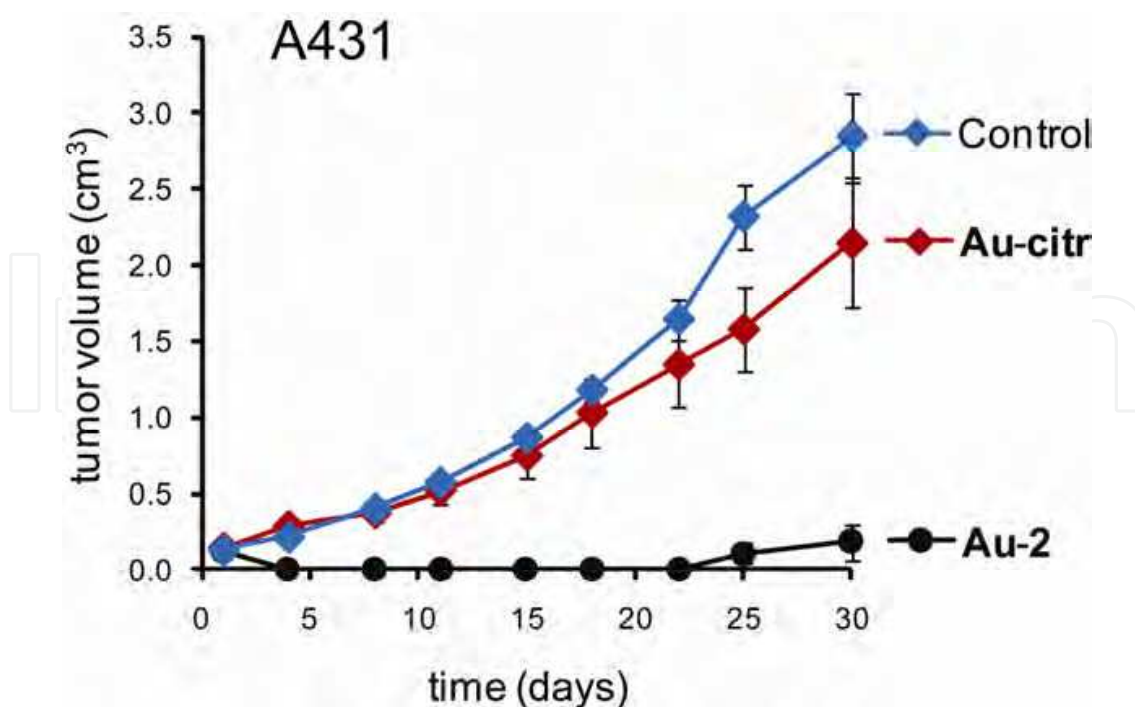


Fig. 15. The PDT effectiveness of Au-2 against fast progressing epidermal squamous carcinoma A431. Nude mice (NuNu) bearing subcutaneous A431 tumors ($n = 5$ per each group) received an intravenous dose of the drug (5 mg kg^{-1}). Tumors were illuminated with light (100 J cm^{-2}) six hours after injection. The tumor size was measured repeatedly and the tumor volume was determined. Control mice were exposed to illumination but did not receive the porphyrin drug. The **Au-citr** group represents mice injected with Au nanoparticles without porphyrin.

The apparent discrepancy in the *in vitro* and *in vivo* performance of unbound porphyrin-brucine conjugates **1** and **2** and those immobilized on gold nanoparticles (**Au-1** and **Au-2**) is likely to be due to the differing environmental conditions to which the porphyrin conjugates were exposed. The fluorescence data revealed that conjugates **1** and **2** were efficiently taken up by cells under the *in vitro* conditions tested. However, in culture media, **Au-1** and **Au-2** tended to aggregate, which resulted in their lower intracellular availability (Fig. 12) and lower PDT efficacy (Fig. 13). Under the *in vivo* conditions tested, the gold nanoparticle-immobilized conjugates were more effective than free conjugates alone. Both spectroscopic and ECD studies demonstrated that conjugated nanoparticles exhibited a strong interaction with plasma proteins (mainly HSA), which led to their self-assembly and to generation of supramolecular complexes. Subsequently, thanks to the enhanced permeability and retention (EPR) effect resulting in potent accumulation of **Au-1** and **Au-2** in the tumors, their PDT efficacy was increased. Moreover, the direct lethal effect of PDT on tumor cells combines well with the nanoscale size of gold-immobilized porphyrins that may limit the local blood supply (vascular impairment). This hypothesis of vascular damage after PDT with nanoparticles will be the subject of future work.

4. Conclusion

The spectroscopic studies demonstrated that fluorescence intensity of free and immobilized conjugates were strongly dependent on the solvent used. After immobilizing the porphyrin

conjugates 1 and 2 on nanoparticles, the intensity of fluorescence emission spectra significantly decreased. The weak quantum yield may be attributed to that: (1) both porphyrins and nanoparticles absorb light at approximately 520 nm, (2) fluorescence quenching by porphyrin-to-metal energy transfer, (3) partial aggregation of the modified nanoparticles. Importantly, the presence of model plasma proteins in the cell medium dramatically reduced the aggregation of modified nanoparticles and prompted their use *in vivo*.

The evaluation of the biological activity of porphyrin-brucine conjugates, either free or immobilized to gold nanoparticles, started with determination of their intracellular uptake. It was shown that both forms were effectively taken into the cell, although a lower level was observed for immobilized forms. To investigate the photodynamic potential of the conjugates, SCC were exposed *in vitro* to photodynamic treatment and cell mortality of post-PDT cultures was determined. The phototoxicities of unbound porphyrin-brucine conjugates were higher than those of conjugates immobilized on gold nanoparticles. This reduction of photodynamic efficacy is likely to be a consequence of nanoparticle aggregation that occurs in the aqueous cell growth media.

In contrast, when the PDT effectiveness was tested *in vivo*, the greatest reduction in tumor growth was observed in mice treated with porphyrin conjugates immobilized on gold nanoparticles. All tumors were eliminated and no detectable relapse of the primary tumor was observed. When animals were treated with unbound conjugates, they exhibited only a transient regression in tumor size that lasted until day 18, and then the primary tumors began to gradually re-grow. Importantly, mice treated with gold nanoparticles without porphyrin exhibited slight tumor retardation in growth that is most likely attributed to the photothermal effect described in other systems. Thus, under the *in vivo* conditions tested, the gold nanoparticle-immobilized conjugates were more effective than free conjugates alone. In addition, both spectroscopic and ECD studies demonstrated that conjugated nanoparticles exhibited a strong interaction with plasma proteins (mainly serum albumin), which led to their self-assembly and generation of supramolecular complexes, and thereby to the enhanced permeability and retention effect. It further contributed to potent accumulation of immobilized conjugates in tumors leading to increased PDT efficacy. Moreover, the direct lethal effect of PDT on tumor cells combines well with the nanoscale size of gold-immobilized porphyrins that may limit the local blood supply (vascular impairment).

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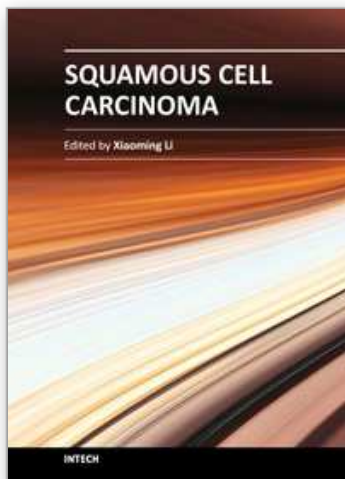
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This book points to some new areas for investigation on squamous cell carcinoma (SCC). Firstly, the features and management of some specific SCC is discussed to give the readers the general principles in dealing with these uncommon and sophisticated conditions. Some new concepts in adjuvant therapy including neoadjuvant therapy and gold nanoparticle-based photo dynamic therapy are introduced. Secondly, a detailed discussion of molecular aspects of tumor invasion and progression in SCC is provided with the emphasis on the roles of some important factors. The role of tumor microenvironment in head and neck SCC is specifically discussed. Thirdly, the roles of cancer stem cells (CSC) in cancer therapy of SCC are described. Molecular mechanisms involving therapeutic resistance and new therapeutic strategies targeting CSC are discussed in detail. Finally, other aspects concerning SCC are included, which involve the assessment, genetic manipulation and its possible clinical implications for the treatment of SCC.

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