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FAST TRACK

Photodynamic Cell-Kill Analysis of Breast Tumor Cells With a Tamoxifen-Pyropheophorbide Conjugate

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Abstract We hypothesized that estrogen receptor (ER) in hormone-sensitive breast cancer cells could be targeted for selective photodynamic killing of tumor cell with antiestrogen-porphyrin conjugates by combining the over-expression of ER in hormone-sensitive breast cancer cells and tumor-retention property of porphyrin photosensitizers. In this study we describe that a tamoxifen (TAM)-pyropheophorbide conjugate that specifically binds to ERα, caused selective cell-kill in MCF-7 breast cancer cells upon light exposure. Therefore, it is a potential candidate for ER-targeted photodynamic therapy of cancers (PDT) of tissues and organs that respond to estrogens/antiestrogens. J. Cell. Biochem. 99: 665–670, 2006. © 2006 Wiley-Liss, Inc.

Key words: estrogen receptor targeted delivery of phototoxins; tamoxifen-porphyrin conjugate; photodynamic cell-kill; breast cancer

Breast cancer continues to be a major threat towards women's health, and a leading cause of fatality. Extensive research has emphasized the critical role of endogenous estrogen in the development and progression of breast cancer; and stressed the interaction between estrogen and its cellular receptor, estrogen receptor (ER) in these processes. 'Double-headed' molecules containing estradiol and toxins (geldanamycin, chlorambucil, diynes) have been synthesized to target endogenous ER in hormone-sensitive breast tumor for tumor-selective delivery and toxicity as well as radioimaging of tumor, potentially taking advantage of the over-expression of ER in tumor cells relative to healthy tissues [Kuduk et al., 1999; Skaddan et al., 1999; Essigman et al., 2001; Purohit et al., 2001; Sharma et al., 2004]. These conjugates, however, contain toxins that do not have any particular tendency to be retained by tumor cells. As a result the toxin part of the linked drug do not contribute towards tumor-accumulation of the conjugate. Considering that the ER-content of estrogen-responsive cells is roughly 100,000 copies per cell [Webb et al., 1992], ER binding affinities of majority of these compounds are not high enough for their selective accumulation into the tumor.

Porphyrins are photosensitizers. Therefore, when they are exposed to visible light they catalyze the formation of singlet oxygen, that is, cytotoxic. In addition, porphyrins have a useful property of being retained somewhat preferentially by malignant tissues, possibly due to their unique chemical structures. This is the basis of photodynamic therapy of cancer (PDT) [Sibata et al., 2001].

We hypothesized that chemical coupling of estradiol with a porphyrin might diminish the sole dependency of the conjugate on ER binding. Recently we synthesized several estrogen-porphyrin conjugates to harness the tumor-retention property of porphyrins. We showed that

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these conjugates accumulated into ER-positive breast tumor cells, despite low ER binding affinities [James et al., 1999; Swamy et al., 2002; Swamy et al., in press] and selectively killed ER-positive breast tumor cells [Swamy et al., in press].

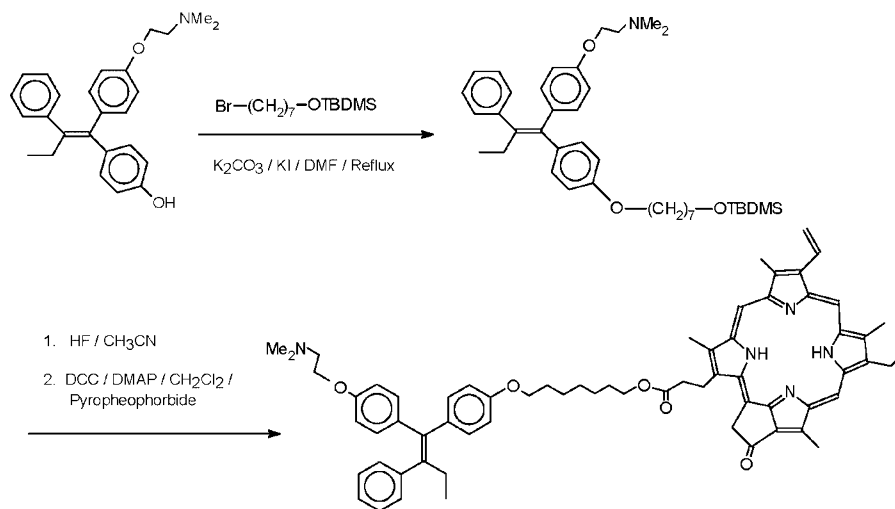
As noted earlier, estradiol is implicated in the development and progression of breast cancer. As a result antiestrogens that disrupt the interaction between ER and estrogen (specific estrogen receptor modulators, SERMs) have been developed. Tamoxifen (TAM), a SERM, has enjoyed considerable success in the hormone treatment of breast tumor [Dardes et al., 2002; Park and Jordan, 2002]. Several other SERMS are currently under various phases of clinical trials with strongly encouraging results. We hypothesize that an antiestrogen-porphyrin conjugate might produce selective phototoxicity in breast tumor without any untoward systemic effect. In this communication we describe results of our initial effort to demonstrate photodynamic cell-kill of MCF-7 breast cancer cells with a TAM-porphyrin conjugate.

EXPERIMENTAL METHODS

Synthesis of the TAM-pyropheophorbide conjugate (TAM-Pyro) (Scheme 1), included in this communication, was reported earlier in a scientific meeting [Swamy et al., 2001]. Detailed description of the synthesis will be published elsewhere.

Competitive Binding Assay of TAM-Pyro With ERα

Competitive ER binding analysis was carried out by incubating baculovirus-expressed recombinant ER-α (Panvera, Madison, WI) with 0.125 nM of $[^3H]$-17β-estradiol (sp. activity 3 Ci/mmol) in the presence of increasing concentrations of estradiol or TAM-Pyro (as denoted in Fig. 1), dissolved in 10 μl of ethanol, in an assay buffer (10 mM Tris, pH 7.5, 10% glycerol, 2 mM of monothioglycerol, and 1 mg/ml BSA, total volume 0.5 ml) for 15 h at 4°C. This was followed by the addition of hydroxylapatite (HAP) slurry to remove protein-bound to $[^3H]$-17β-estradiol from unbound $[^3H]$-17β-estradiol. After centrifugation and three washes with a wash buffer (40mM Tris, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA) the HAP pellet was transferred to a scintillation vial and resuspended in 200 μl of ethanol. Radioactivity, bound to the HAP-pellet was determined in a liquid scintillation counter after the addition of scintillation cocktail. Total binding was determined by treating ER samples with $[^3H]$-17β-estradiol only, while non-specific binding was determined by incubating ER samples with $[^3H]$-17β-estradiol and 1 μg of estradiol. Maximum specific binding (B_0) was calculated by subtracting non-specific binding from total binding, while specific binding (B) at each concentration was calculated by subtracting non-specific binding from binding at each concentration. Each concentration was run in triplicate.



Scheme 1. Synthesis of TAM-PYRO.

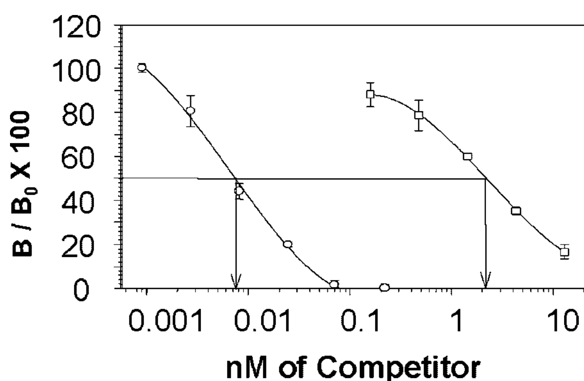


Fig. 1. Competitive ERα binding assays of TAM-Pyro (—□—) and estradiol (—○—).

Photodynaimc Cell-Kill Analysis of MCF-7 Cells Treated With TAM-Pyro or Pyropheophorbide

MCF-7 cells (ATCC, Manasas, VA) were grown in 0.5 ml of DMEM media containing 1% antibiotics and 5% fetal bovine serum to approximately 60% confluence in 24-well cell culture plates. Then the cells were dosed with ethanol, or 5.3 μM of pyropheophorbide or 5.3 μM of TAM-Pyro for 60 min (pyropheophorbide and TAM-Pyro were dissolved in ethanol, and required amounts were diluted with DMEM media so that amount of ethanol was 0.1%). At the end of the incubation one plate was exposed to red light for 10 min and the other was not. Light exposure was carried out by placing the cell culture plate on a slide-viewing box whose lighted surface was covered with a red plastic sheet. [The lamp was equilibrated for 15 min prior to placing the cell culture dishes. Heat was dissipated with a cooling fan. Transmittance of the red filter was determined in a UV-VIS spectrophotometer (Hewlet-Packard, Model 8453). Fluence was determined by a Coherent Lasermate detector with a 2.54 cm^2 detection area (total fluence was 3.5 J/cm^2)].

After the irradiation step, media were removed from both the plates and replaced with DMEM containing 5% FBS and 1% antibiotics, and the cells were allowed to recover for 16 h. Then the wells were washed twice with PBS (1.0 ml), and fixed by adding 1.0 ml of methanol (−20°C), and incubating on ice for 20 min. Then methanol was aspirated off and the plates were dried in air for 30 min. One milliliter of methylene blue solution (1% in 10 mM borate buffer, pH 8.5) was added to each well and incubated at 25°C for 30 min. The plates were washed three times with 10 mM borate buffer, pH 8.5, and the cells were photographed with an inverted microscope fitted with digital imaging system (Twin-Cam Digital imaging system, Camdek Precision instruments, Boston, MA). The entire assay was carried out three times and the photograph shown in Figure 2 is a representative one.

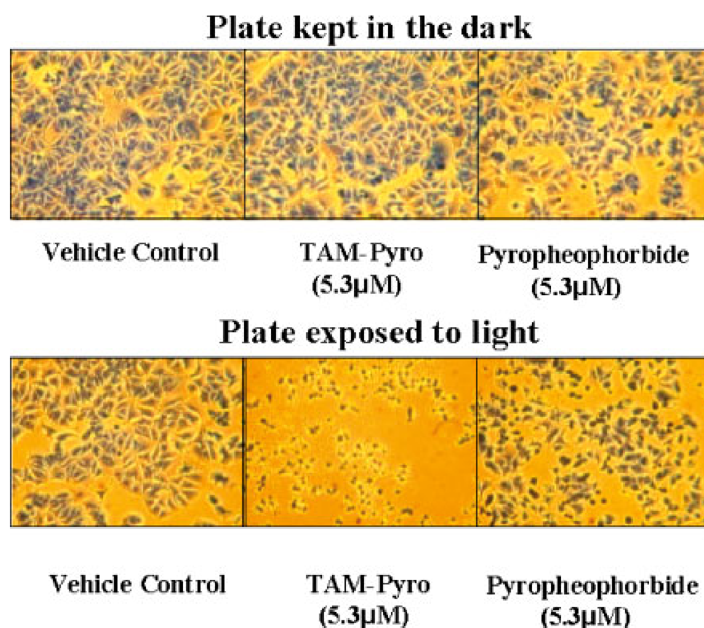


Fig. 2. Cell-killing assays of MCF-7cells treated with TAM-Pyro or pyropheophorbide, and either exposed to red light or kept in the dark. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS AND DISCUSSION

PDT is a localized therapy for the treatment of early stage malignancy, palliative therapy for late stage disease, and for tumor bed sterilization to destroy any residual tumor cells detached during resection or any metastasized cells in the area of light illumination. In the US, PDT has been approved for early or late stage lung cancer that are not amenable to surgery, obstructive esophageal cancer, actinic keratoses of the skin, as well as for age-related macular degeneration of the eye [Fisher et al., 1995; Axer-Siegel et al., 2004; Marmur et al., 2004]. PDT was investigated for palliative treatment for the cutaneous recurrence of breast cancer [Mang et al., 1998; Allison et al., 2001]. Recently Dolmans et al. [2002] reported delay of tumor growth in the PDT of a murine orthotopic breast tumor model.

A limiting factor in PDT involves insufficient localization of the PDT dyes into tumor leading to significant damage to surrounding normal tissue. Development of PDT dyes that localize into tumors with high degree of selectivity has been a major challenge. Several methods for the enhanced delivery of PDT dyes to tumors by chemical conjugation or association with LDL, liposomes, and microspheres have been attempted with limited success [Derycke and Witte, 2004; Sharman et al., 2004]. Recently unique immune signals on the surface of certain cancer cells have been harnessed by chemically conjugating PDT dyes to antibodies to these signals [Goff et al., 1996; Vrouenraets et al., 2001]. However, paucity of active mechanism for the internalization of these immunotoxins has limited their applicability.

On the other hand, TAM, clinically the most widely used antiestrogen, was shown to have cytostatic effects in ER-positive and ER-negative breast cancer cells in vitro [Goldenberg and Froses, 1982]. Paradoxically, TAM was found to stimulate cellular growth in the endometrium, putting the women taking TAM into small but significant risk of endometrial cancer [Dardes et al., 2002]. This puzzle was deciphered after the discovery of ERβ phenotype [Peach et al., 1997]. It was realized that TAM acts as an AP-1 site antagonist in ERα and AP-1 site agonist in ERβ. It was also discovered that endometrial tissues predominantly contains ERβ [Peach et al., 1997]. Therefore, duality of action of TAM is ascribed to its undesired migration into endometrium and the subsequent side effect.

We hypothesized that by chemically conjugating TAM with a porphyrin it might be possible to reduce the dependence on ER binding, and direct the conjugate selectively to the tumor cells. To provide a proof of this hypothesis we synthesized a TAM-porphyrin conjugate (Scheme 1). In this synthetic scheme (*Z*)-4-hydroxytamoxifen (Sigma Chemical Co., St. Louis, MO), a naturally occurring metabolite of TAM and a strong ER binder, was used as the starting material. Pyropheophorbide (Frontier Science, Logan, UT), a porphyrin, was attached to TAM via a seven-carbon long tether.

Results of the ER binding assays showed that the half-maximal concentrations of TAM-Pyro and E_2 were 2.2 and 0.0075 nM, respectively, suggesting a significantly lower ER binding affinity of TAM-Pyro compared with E_2 (Fig. 1). In a recent study we observed that low ER binding affinity of an estradiol-porphyrin conjugate did not prevent the conjugate to be taken up at a significantly higher concentration by ER-positive MCF-7 human breast cancer cells compared with ER-negative Hs578t human breast cancer cells; as well as demonstrating selective phototoxicity in MCF-7 cells [Swamy et al., 2002; Swamy et al., in press]. These results suggested that low ER binding of the estradiol-porphyrin conjugate might be compensated for, at least in part, by the natural tumor-retaining property of the porphyrin part of the conjugate. In the same token we anticipated that TAM-Pyro, despite low ER binding affinity might be taken up by MCF-7 cells, and display enhanced phototoxicity relative to an equivalent amount of pyropheophorbide, the unconjugated porphyrin.

Targeting a nuclear component (i.e., ER, a nuclear receptor) of tumor cells for phototoxicity has certain advantage. For example, Akhlynina et al. [1997] recently demonstrated that targeting a nuclear signal in glioma cells with a chlorin e6 conjugate dramatically increased the photodynamic cell-kill relative to the un-conjugated porphyrin (chlorin e6).

We incubated MCF-7 cells with 5.3 μM of pyropheophorbide or 5.3 μM of TAM-Pyro for 60 min in the dark followed by exposure to red light. In this preliminary study we used this dose based on our experience with estrogen-porphyrin conjugates [Swamy et al., in press] as well as literature procedure. For example, Yamamoto et al. [2005] recently carried out an in vitro PDT study of glial cells with a dose of

3.5–20 μg/ml of the porphyrin. In our case, 5.3 μM of TAM-Pyro used for our study translates into approximately 5.4 μg/ml of TAM-Pyro. After the light exposure the cells were allowed to recover for 16 h and methylene blue assay was performed. This assay is routinely used for cell viability, because only the live cells are stained by methylene blue, providing an index for cell viability.

As shown in Figure 2, upper panel, when the cells were not exposed to red light, there was no significant cell-kill by pyropheophorbide or TAM-Pyro. In contrast, when the cells were exposed to red light, strong cell-kill (reduced number of viable cells after 16 h of recovery period) was observed with TAM-Pyro (Fig. 2, lower panel, middle figure), but there was very little cell death in pyropheophorbide and light-treated cells (Fig. 2, lower panel, right figure). We have carried out this assay three times and Figure 2 is a representation of a typical case. We counted the live cells after methylene blue treatment under a microscope. There were approximately 10–15% of live cells (average of three experiments) in TAM-Pyro and light-treated cells (Fig. 2, middle figure of the bottom panel) compared with 100% (live cells) with vehicle-treated cells. In all other cases there was no significant difference between vehicle-treated cells and cells treated with TAM-Pyro (no light) or pyropheophorbide

The above results strongly suggest that the interaction between the endogenous ER in the cells and the TAM part of the TAM-Pyro conjugate might have caused a selective accumulation of the conjugate into the cells, which resulted in a higher cell-kill upon exposure to red light. It is to be noted that we did not use a ER-negative cell as control, because TAM has been shown to be effective in killing ER-negative cells also by an ER-independent pathway [Goldenberg and Froses, 1982]. Such a phenomenon might confound our photodynamic cell-kill data.

On the other hand, lack of cell-death in pyropheophorbide light-treated cells indicated that either an insignificant amount of the dye was taken up by the cells to cause any cell-death or pyropheophorbide light treatment caused minor damage to the cells that recovered quickly. In the former case, majority of pyropheophorbide probably stayed dissolved in a large volume of the media. Although exposure to light produced cytotoxic singlet oxygen in the media (as well as in the cells), these molecules (singlet oxygen) are very short-lived and travel very short distance to result any cell-kill. In the latter case considerably higher dose of pyropheophorbide would have been required to impart significant cell death. These results also suggest that in a clinical set up considerably less amount of the conjugate (TAM-Pyro) would be required to cause tumor cell death, thus avoiding side effects.

In summary, TAM-Pyro, a TAM-pyropheophorbide conjugate showed specific binding affinity for ERα and displayed stronger cell-killing property in MCF-7 breast cancer cells compared with un-conjugated pyropheophorbide upon exposure to red light. Therefore, this conjugate is potentially a reagent for ER-targeted PDT of hormone-sensitive cancers of breast and other estrogen-sensitive organs and tissues. In addition this compound might be devoid of systemic adverse effects of the corresponding estrogen compounds. However, it should be noted that this report includes data that are preliminary in nature to basically provide the proof of the concept.

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