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Nuclear Estrogen Receptor Targeted Photodynamic Therapy: Selective Uptake and Killing of MCF-7 Breast Cancer Cells by a $C_{17\alpha}$-Alkynylestradiol-Porphyrin Conjugate

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Abstract We hypothesized that over-expression of estrogen receptor (ER) in hormone-sensitive breast cancer could be harnessed synergistically with the tumor-migrating effect of porphyrins to selectively deliver estrogen-porphyrin conjugates into breast tumor cells, and preferentially kill the tumor cells upon exposure to red light. In the present work we synthesized four (4) conjugates of C_{17}-α-alkynylestradiol and chlorin e6-dimethyl ester with varying tether lengths, and showed that all these conjugates specifically bound to recombinant ERα. In a cellular uptake assay with ER-positive MCF-7 and ER-negative MDA-MB 231 human breast cancer cell-lines, we observed that one such conjugate (E_{17}-POR, XIV) was selectively taken up in a dose-dependent and saturable manner by MCF-7 cells, but not by MDA-MB 231 cells. Furthermore, MCF-7 cells, but not MDA-MB 231 cells, were selectively and efficiently killed by exposure to red light after incubation with E_{17}-POR. Therefore, the combination approach, including drug- and process modalities has the potential to be applied clinically for hormone-sensitive cancers in organs where ER is significantly expressed. This could potentially be carried out either as monotherapy involving a photo-induced selective destruction of tumor cells and/or adjuvant therapy in post-surgical treatment for the destruction of residual cancer cells in tissues surrounding the tumor. J. Cell. Biochem. 99: 966–977, 2006.

Key words: estrogen receptor targeted delivery of phototoxins; targeted photodynamic therapy; estrogen-porphyrin conjugates; cellular assays for uptake and cell-kill; breast cancer

Porphyrins are photosensitizers; and they have a useful property of being retained somewhat preferentially by malignant tissues, possibly due to their unique chemical structure. Porphyrins absorb in the visible region of electromagnetic radiation. Therefore, upon activation with visible light (often red light), porphyrins produce singlet oxygen that kills tumor cells (Photodynamic therapy, PDT). In general, PDT is a localized therapy for the treatment of early stage malignancy, palliative therapy for late-stage disease and for tumor bed sterilization by destroying any residual tumor cells after surgery or any metastasized cells in the area of light-illumination [Dougherty et al., 1998; Dalla Via and Marciani, 2001; Sibata et al., 2001; Dougherty, 2002; Moan and Peng, 2003; Axer-Siegel et al., 2004; Marmur et al., 2004]. Recently two PDT dyes, namely Visudyne and Foscan have been approved by the Food and Drug Administration for the treatment of age-related macular degeneration, and palliative treatment of head and neck cancer respectively. In the case of breast cancer, PDT was investigated as a palliative treatment for the cutaneous recurrence [Khan et al., 1993; Mang et al., 1998; Allison et al., 2001], and was suggested as a probable treatment. Recently

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Dolmans et al. [2002] reported that PDT delayed tumor-growth in a murine orthotopic breast tumor model.

Accumulation of most PDT dyes in malignant cells is, however, modest, and 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 [Hasan, 1992; Kramer et al., 1996; 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., 1994, 1996; Vrouenraets et al., 2000, 2001, 2002]. However, paucity of active mechanism for the internalization of these immunotoxins has limited their applicability.

Nuclear receptors, by virtue of their high-affinity binding to their cognate ligands, have been employed as molecular targets to deliver ligand-mimics as drugs. For example, estrogen receptor (ER), the primary modulator of the biological effects of estrogens and anti-estrogens, has been targeted with estrogens as carriers of cytotoxins [nitrogen mustards, genotoxins, geldanamycin, enediynes [Rink et al., 1996; Kuduk et al., 1999, 2000; Essigman et al., 2001; Purohit et al., 2001; Sharma et al., 2004], and radioisotopes (for radioimaging [Skaddan et al., 1999, 2000]), taking advantage of the over-expression of ER in cancerous cells relative to healthy tissues [Gotteland et al., 1994; Traish et al., 1995; Soubeyran et al., 1996; Struse et al., 2000]. However, these "double-headed" conjugates in general have low ER-binding affinities due in parts to modification of the parent estradiol molecule, and addition of appendages of varying chemical nature and steric bulk. As a result desired degree of accumulation of the conjugate selectively into tumor often remains unachieved.

We hypothesized that tumor-accumulation (of the conjugates) could be enhanced significantly if we couple estrogen with a toxin that has propensity for accumulation into tumor cells. This way it might be possible to diminish the sole dependency of these conjugates on ER-binding. Such a strategy will have the benefit of providing significantly higher efficiency over traditional PDT, and might constitute a potential tumor-specific therapeutic modality for hormone-sensitive cancer of organs where ER is expressed in significant degree.

Based on the above hypothesis we synthesized a conjugate of $C_{11\beta}$-estradiol and tetraphenylporphyrin, and showed that this compound selectively accumulated in MCF-7 breast tumor cells [James et al., 1999; Swamy et al., 2002]. However, we noted that the photosensitizing capability of neither the un-conjugated porphyrin nor the conjugate was sufficiently high to kill the cells under the conditions of our experiment [Swamy et al., 2002].

In the present study we anchored chlorin e6-dimethyl ester, a known photo-toxin to C_{17}-α-alkynylestradiol via tethers of various lengths and determined their ER-binding capabilities. In addition, we carried out cellular uptake and light-induced cell-killing studies of one of these conjugates (E_{17}-POR, n = 3, XIV, Fig. 1) with ER positive MCF-7 and ER-negative MDA-MB 231 human breast cancer cells. These experiments demonstrated that this conjugate selectively accumulated into MCF-7 cells; and viable cells were significantly reduced by exposure to red light. Results of these studies and their implications are discussed in this communication.

MATERIALS AND EXPERIMENTAL METHODS

MCF-7 and MDA-MB 231 human breast cancer cells were purchased from ATCC (Manasas, VA). Baculovirus expressed recombinant ERα was obtained from PanVera, Madison, WI. All the chemicals, except chlorin e6-dimethyl ester (Frontier Science, Logan, UT), were purchased from Sigma-Aldrich Chemical Co., Milwaukee, WI. Solvents were obtained from American Bioanalytical, Natick, MA. [^{3}H]17-β-estradiol (sp. activity 3 Ci/mmol) was synthesized in-house by reducing 3-*t*-butyldimethylsilyl estrone with NaB^3H_4 (Amersham Corpn., Springfield, IL, sp. activity 12 mCi/mmol) followed by removal of the tert-butyldimethylsilyl protecting group. Synthesis of the compounds (Fig. 1), included in this communication, was reported earlier in a scientific meeting abstract [Swamy et al., 2001]. Detailed description of the synthesis will be published elsewhere.

Competitive Binding Assays of C_{17}-α-alkynylestradiol-chlorin e6 Conjugates (XII–XV) with ERα

Baculovirus-expressed recombinant ERα (2 nM) was incubated with 0.13 nmol of [^{3}H]17-β-estradiol in the presence of increasing concentrations of estradiol or the conjugates (as

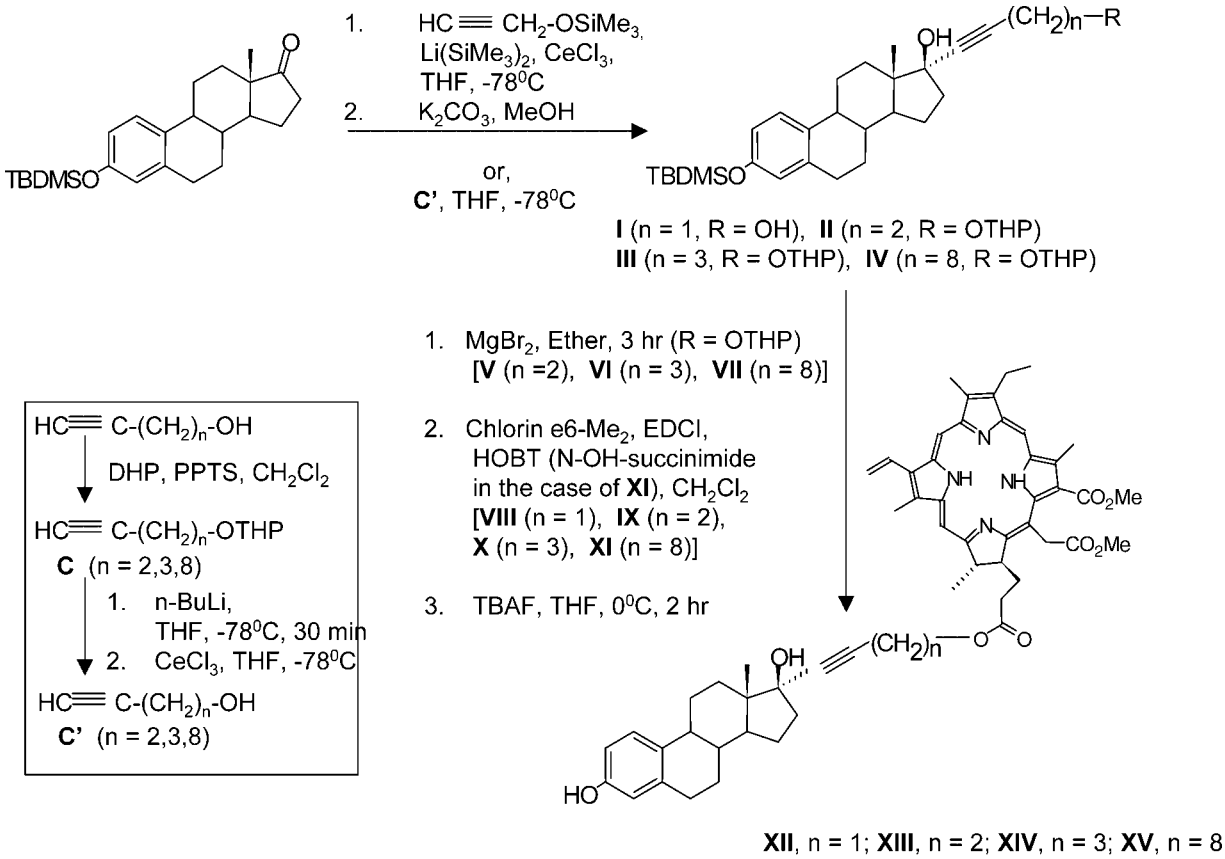

Fig. 1. Scheme for the synthesis of C_{17}-α-alkynylestradiol-chlorin e6 conjugates.

denoted in (Fig. 2), 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. A 50% hydroxylapatite (HAP) slurry was added to remove [^{3}H]-17β-estradiol, bound to the protein from unbound [^{3}H]17β-estradiol. After centrifugation and three washes in the ER wash buffer (40 mM Tris, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM EGTA) the HAP pellet was transferred to a scintillation vial and re-suspended 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 [^{3}H]-17β-estradiol only, while non-specific binding was determined by incubating ER samples with [^{3}H]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.

Cell-Culture

MCF-7 and MDA MB 231 cells (approximately 10^6 cells/well) were seeded into 24-well plates and grown in DMEM media containing 1% Penn/Strep and 5% fetal bovine serum (FBS) till approximately 70% confluence, followed by treatment with various reagents. E_{17}-POR (estradiol porphyrin conjugate, XIV) or chlorin e6 dimethyl ester (Ce6-Me_2, the un-conjugated porphyrin) were dissolved in ethanol to desired concentration, and the cells were dosed with these solutions.

MCF-7 and MDA MB 231 Cell-Uptake Assay

MCF-7 or MDA MB 231 cells were treated with various concentrations of either E_{17}-POR, XIV or Ce6-Me_2 in cell culture media without FBS for 30 min. After the incubation media was withdrawn and the cells were washed 3 times with PBS, and 1 ml of methanol was added to each plate, and cells were allowed to lyse for

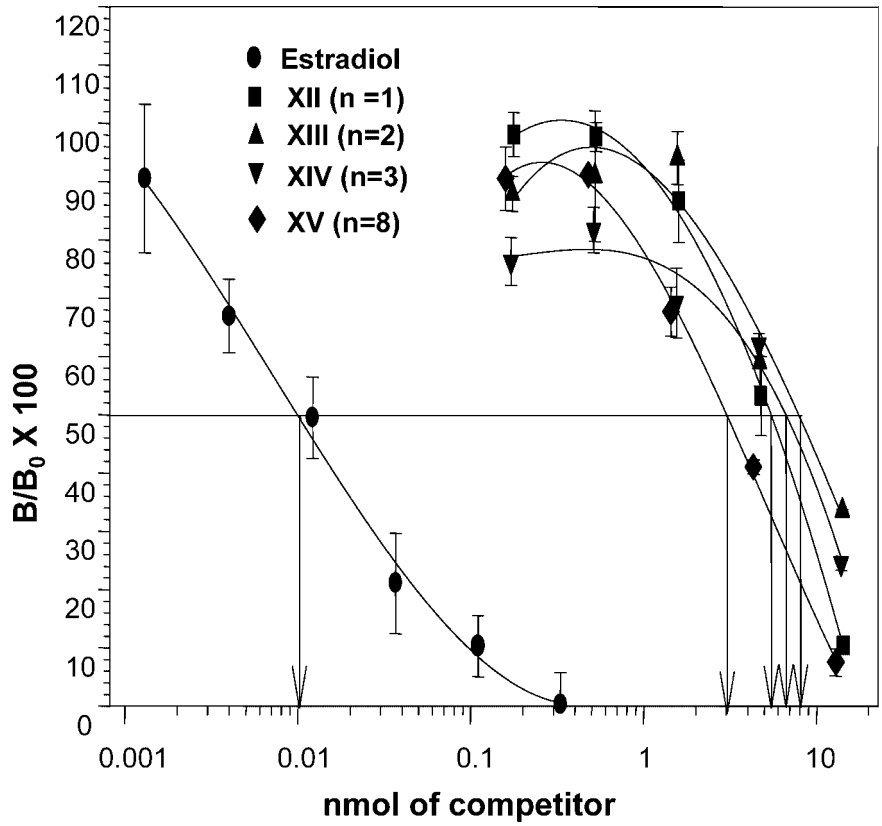

Fig. 2. Competitive binding assays of estardiol and C_{17}-α-alkynylestradiol-chlorin e6 conjugates with baculovirus expressed recombinant ER. Briefly ERα (2 nM) was incubated with 0.125 nmol of [^{3}H]17-β-estradiol in the presence of increasing concentrations of estradiol or the conjugates for 15 h at 4°C, followed by treatment with a 50% hydroxylapatite (HAP) slurry to remove [^{3}H]17β-estradiol, bound to the protein from unbound [^{3}H]17β-estradiol. After centrifugation and three washes in the ER wash buffer the HAP pellet was transferred to a scintillation vial, re-suspended in 200 μl of ethanol, and radioactivity was measured in scintillation counter. At each concentration specific binding divided by maximum specific binding (B/B_0) in percent was plotted against concentration. 50% specific binding (EC_{50}) for each compound is denoted in the X-axis. Each concentration was run in triplicate.

15 min. The cells were scraped with a rubber policeman and the mixture was transferred to a test tube. This step was repeated twice. The combined mixture was centrifuged and supernatants were concentrated under nitrogen, dissolved in 1 ml of methanol, and fluorescence in the extracts was determined in a Hitachi U2000 spectrofluorimeter ($E_x = 405$ nm and $E_m = 670$ nm). To determine the extraction-efficiency, known amounts of the conjugate (XIV) and chlorin e6-dimethylester were added to MCF-7 cells and they were extracted with methanol in the same fashion as described before; and the methanol-extracts were assayed fluorimetrically. The extraction-efficiency was >95% (results not shown). In general each concentration was run in six (6) replicates. Statistics was done by student's *t*-test. Although cellular uptake assays are usually carried out by dispersing the cells in a detergent (e.g., 1% SDS) after the incubation with a porphyrin, and measuring the fluorescence of the mixture [Momma et al., 1998], we found that addition of methanol to the cells (after removing the media and washing the cells with PBS) lysed the cells completely and allowed a near complete extraction of the porphyrins inside the cells. A similar procedure for the extraction of tri(glucosyloxyphenyl)chlorin with an organic solvent was described recently [Laville et al., 2004].

Cell-Viability Assays of MCF-7 and MDA-MB 231 Cells Treated With Various Doses of E_{17}-POR (XIV) or Ce6-Me_2

MCF-7 and MDA-MB 231 cells were treated with E_{17}-POR (XIV, 0.02, 0.03, 0.07, 0.13, 0.27, 0.54, or 1.07 μM) or chlorin e6 dimethyl ester (Ce6-Me_2, 0.01, 0.02, 0.05, 0.09, 0.18, 0.36, or 0.73 μM) in DMEM in the absence of FBS for 1 h in the cell culture incubator. Then the plates

were exposed to red light for 10 min at 25°C (heat was dissipated with a cooling fan). Illumination of the cells was carried out by placing the cell-culture dishes on the top of a light box covered in the top with a red plastic sheet. The lamp was equilibrated for 15 min prior to placing the cell culture dishes. 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). A control plate was set up in parallel, but the plate was not exposed to light. At the end, the media was replaced with fresh media containing FBS and the cells were allowed to recover and grow for an additional 14 h. This was followed by Methylene Blue cell-viability assay (vide infra). We also carried out an assay where cells were exposed to light for 10, 20, 30, and 90 min; and observed that a 10-min exposure was sufficient for significant and consistent reduction in the number of viable cells (results not shown). Furthermore, a shorter exposure-time was deemed desirable to avoid "heating" related to longer exposures.

Methylene Blue Cell-Viability Assay

After the experiment the cells were washed with ice-cold PBS (0.5 ml), followed by the addition of methanol (chilled at −20°C) and the cells were allowed to incubate on ice for 10 min. Methanol was removed by suction and the cells were air-dried for 20 min followed by the addition of 0.25 ml of Methylene Blue solution to each well. The cells were allowed to incubate at 25°C for 30 min. Methylene Blue solution was aspirated off, and the cells were washed four (4) times with 10 mM borate buffer, pH 8.5 (1.0 ml at a time). Then the cell-bound dye was released by adding 1.0 ml of ethanol-0.1 M HCl (1:1 v/v) mixture. The absorbance of the solution from each well was determined at 650 nm against ethanol-HCl solvent. The cell viability was expressed as percent of the control (which did not receive any compound, but received only plain DMEM).

Imaging of MCF-7 or MDA-MB 231 Cells after Incubation With E_{17}-POR or Ce6-Me_2 and Either Exposed to Red Light or Kept in the Dark

MCF-7 or MDA-MB 231 cells (~200,000) were seeded in 30 mm dishes and grown overnight in DMEM containing Penn/Strep and 5% FBS. The cells were treated with 1.07 μM of E_{17}-POR or Ce6-Me_2 in DMEM in the absence of FBS for 1 h. Then the plates were exposed to red light (light box fitted with a red filter, as described before) for 10 min at 25°C. A control plate was set up in parallel but the plate was not exposed to light. At the end, the media was replaced with fresh media containing FBS and the cells were allowed to recover and grow for an additional 14 h. After this period the wells were washed twice with PBS (1.0 ml), and fixed by adding 1.0 ml of methanol (−20°C) to each well and incubating on ice for 20 min. Methanol was aspirated off and the plates were dried in air for 30 min. One ml of 1% Methylene Blue solution was added to each well and cells were incubated at 25°C for 30 min. The plates were washed three times with 10 mM borate buffer pH 8.5, and photographed using an inverted microscope fitted with digital imaging system (Twin-Cam Digital imaging system by Camdek Precision instruments, Boston, MA).

RESULTS AND DISCUSSION

Targeting ER in the nucleus of breast cancer cells with an estrogen-toxin conjugate has certain advantages. For example, it has been shown that the nucleus of cancer cells contains higher number of copies of ER than the non-cancerous tissues where ER is expressed [Gotteland et al., 1994; Traish et al., 1995; Soubeyran et al., 1996; Struse et al., 2000]. Therefore, it is expected that a larger quantity of an estrogen-conjugate would accumulate in the cancer cells than the non-cancerous ones. Furthermore, nucleus contains the genomic DNA; and its damage is most desired in cancer therapy. Additionally, cancer cells divide rapidly and the chromosomal DNA remains in a bare form instead of being surrounded by histones and thus protected from damage. Therefore maximum damage to cells could be expected if the toxins are targeted to the nucleus of the cancer cells.

Support for the benefit of nuclear targeting was provided in a recent article where Akhlynina et al. demonstrated that coupling chlorin e_6, a PDT dye, to a nuclear localization signal and targeting nuclear insulin receptor in PLC/PRF/5 and rat glioma C6 cells resulted in a more than 2,000-fold reduction of EC_{50} (opposed to chlorin e_6 alone) and significantly increased the photosensitizing activity of chlorin e6 [Akhlynina et al., 1997].

In a previous report we delineated the synthesis of a C_{11}-estradiol-tetraphenyl porphyrin conjugate and showed specific binding of this conjugate to ER [James et al., 1999]. Furthermore, we demonstrated that this compound selectively accumulated in ER-positive MCF-human breast cancer cells opposed to ER-negative HS578t cells [Swamy et al., 2002].

Although the above results provided the "proof-of-the principle" of our hypothesis about targeting ER in cancer cells with a "double-headed molecule" in which one half has ER-localizing property while the other has tumor-localizing property, this compound showed very low photosensiting capability under the conditions of our experiment [Swamy et al., 2002]. This prompted us to consider chlorin e6 as the photosensitizer, particularly in conjugation with estrogen-mimics. Hamblin et al. recently described that conjugation of polyethylene glycol to chlorin e6 significantly enhanced the phototoxicity of chlorin e6 in ovarian cancer cells [Hamblin et al., 2001]. Furthermore, as described earlier, coupling chlorin e_6 to a nuclear localization signal significantly increased the photosensitizing activity of chlorin e6 [Akhlynina et al., 1997]. These data provided potential support for our hypothesis involving estrogen-porphyrin conjugates for targeting ER in breast cancer cells and killing them in a selective fashion upon light-exposure.

An important consideration in the tumor-selective delivery of estrogen-conjugates is high binding affinity between these compounds and ER. This is necessary for the selective accumulation of these compounds in the desired ER-targeted tissues, and not in other healthy tissues where is ER is expressed, that is, brain, ovary etc. C_{17}-α-alkynylestradiol and its derivatives are known to bind ER with high affinity [Anstead et al., 1997]. Therefore, we postulated that C_{17}-α-alkynylestradiol-porphyrin conjugates might be endowed with high ER-binding and enhanced tumor-localizing properties.

In the present study, we synthesized four (4) conjugates of C_{17}-α-alkynylestradiol with various tether lengths and chlorin e6-dimethyl ester (Fig. 1; $n = 1–3$, 8). Introduction of the tethers at the C_{17}-α position of estradiol was carried out by nucleophilic addition of suitably derivatized alkynyl carbanions to protected estrone followed by standard synthetic manipulations.

It is known that alkyne group and its derivatives at C_{17}-α position of estradiol are tolerated well by ER [Anstead et al., 1997], but the effect of a large porphyrin group at the end of the alkyne on ER-binding is not known. Competitive binding assays of these conjugates (XII–XV) with recombinant ER demonstrated that all of them specifically bound to ERα in a dose-dependent manner, however, with significantly less affinity than estradiol (Fig. 2). Concentration at half-maximal binding of XII-XV ($n = 1–3$ and 8) were 5.6, 8.1, 6.8 and 3.0 nM respectively compared with 0.01 nM for estradiol. Although these compounds showed low ER-binding properties, we hypothesized that such deficiency might be mitigated, at least to some extent by the tendency of the porphyrin part of the conjugates to be retained by the tumor cells.

We continued our biochemical studies with one of the conjugates (E_{17}-POR, XIV, $n = 3$), because we had maximum amount of this compound available to us. Since ER-binding affinities of these compounds (XII–XV) were very similar, we argued that XIV would be a valid representative of the four conjugates. Purity of this compound (E_{17}-POR, XIV) was determined by HPLC analysis, which showed that XIV was not contaminated with chlorin e_6 dimethyl ester (results not shown).

We observed that when MCF-7 or MDA-MB 231 cells were incubated with various doses of either E_{17}-POR or Ce_6-Me_2, the conjugate was taken up by ER-positive MCF-7 cells in a dose-dependent and saturable manner, while Ce_6-Me_2 was not (Fig. 3). Both E_{17}-POR and Ce_6-Me_2 showed a low-level and dose-independent uptake by ER-negative MDA-MB 231 cells. These results strongly suggested that binding of E_{17}-POR by endogenous ER in MCF-7 cells might be responsible for dose-dependent and saturable uptake of this compound.

In the next study, MCF-7 cells were incubated with various doses of either E_{17}-POR or Ce_6-Me_2 followed by exposure to red light, under conditions described in the Materials and Methods section. Following the light-exposure the cells were allowed to grow back, and cell-viability was determined by Methylene Blue assay. We used this assay in our experiment because it has been used traditionally for cell survivability/viability. In this assay only the live cells are stained by Methylene Blue, providing an index for cell viability. Recently this assay was used to

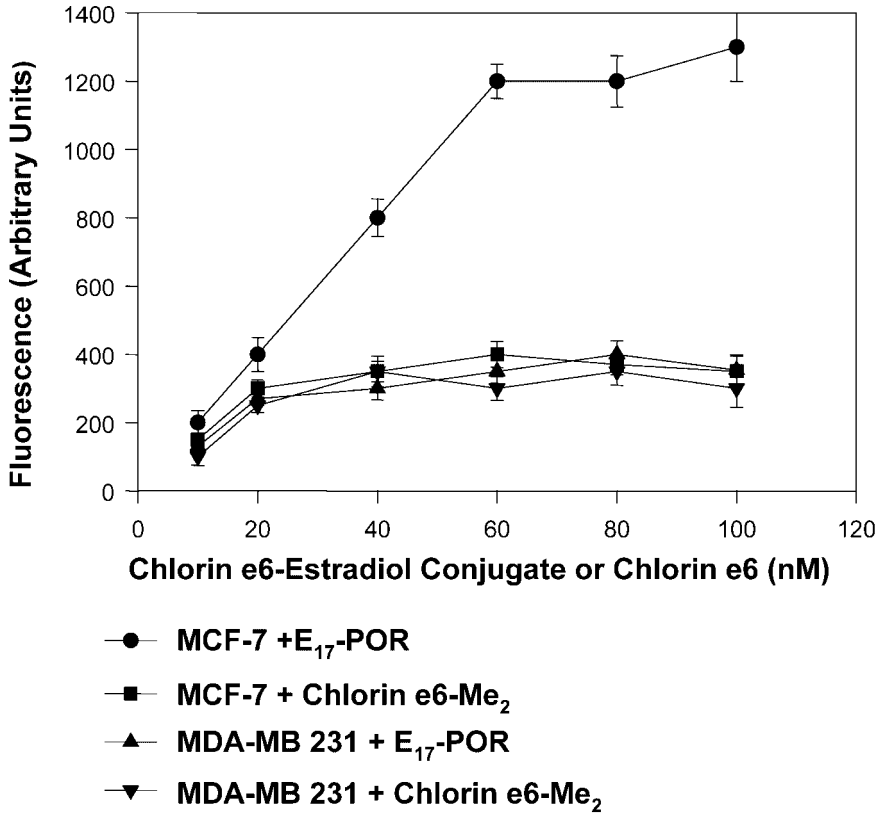

Fig. 3. Cellular uptake assay of E_{17}-POR and Ce6-Me_2 in MCF-7 and MDA-MB 231 cells. Cells were treated with increasing concentrations of either E_{17}-POR or Ce6-Me_2 in cell culture media without FBS for 30 min. Then the cells were washed three times with PBS, and were extracted with 1 ml of methanol. The fluorescence in methanol extracts was determined ($E_x = 405$ nm and $E_m = 670$ nm). Each point in the graph represents an average of six (6) replicates.

determine cell-survivability after PDT with a photoproduct of protoporphyrin IX induced by 5-aminolevulinic acid [Ma et al., 2000]. We observed that there was a dose-dependent decrease of viable cells in cells treated with E_{17}-POR and red light; and 0.18 μmol of the conjugate was required for 50% cell-viability/cell-kill (Fig. 4A). On the other hand, there was almost 100% cell-viability with E_{17}-POR (no light-exposure) and Ce_6-Me_2 (no light-exposure). The Ce_6-Me_2-control that was exposed to light showed some cell-killing properties at higher concentrations reflecting low-efficiency tumor cell-retaining tendency of porphyrins.

The above results strongly suggested that presence of ER in MCF-7 cells might be responsible for the enhanced accumulation of the conjugate into cells that led to significantly higher degree of cell-kill upon light-exposure compared to un-exposed sample. Another important observation was that conjugation of Ce_6-Me_2 to estrogen strongly reduced the amount of porphyrin required for cell-kill. For example, at a concentration of 0.18 μM there was 50% cell-viability with the conjugate (light-exposed), while there was almost 100% viability with Ce_6-Me_2 (light-exposed) at this concentration.

In the case of ER-negative MDA-MB 231 cells there was no significant cell-kill with Ce_6-Me_2 in the presence or absence of light (Fig. 4B). This is in contrast with MCF-7 cells where low but significant cell-kill was observed at high doses of Ce_6-Me_2 (Fig. 4A). This might be a reflection of the inherent difference between these cell lines towards photo-sensitivity. On the other hand, almost equal level of cell-kill was observed in the absence or in the presence of light when the cells were treated with high concentrations of E_{17}-POR. This phenomenon may be related to "dark toxicity" involving low-level toxicity of porphyrins that are not exposed to light, that has been shown in several systems, particularly when the core porphyrin moiety is modified [Stilts

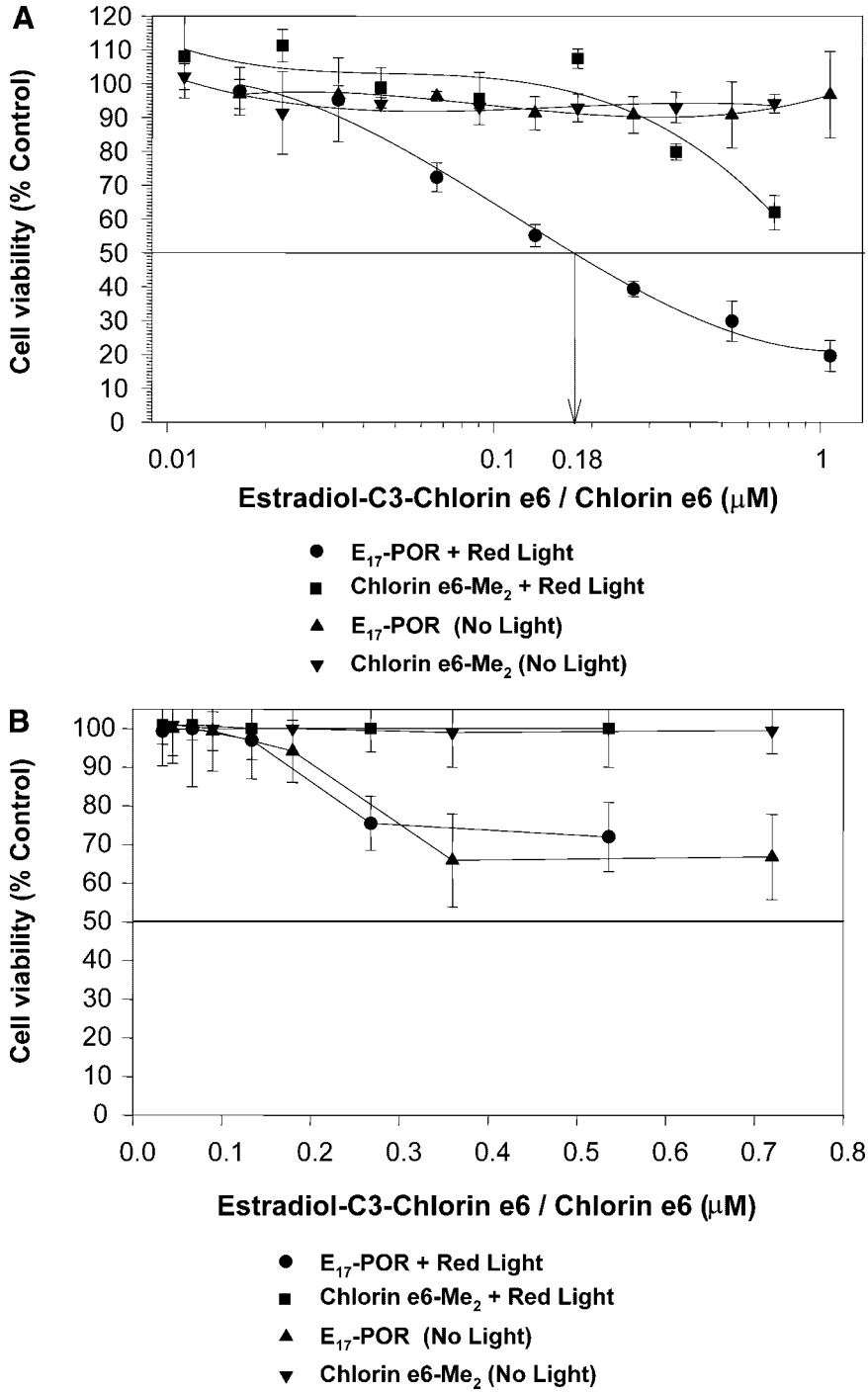

Fig. 4. **A**: Methylene Blue cell-viability assays of MCF-7 cells treated with various concentrations of E_{17}-POR and Ce6-Me_2 followed by exposure to red light. Briefly MCF-7 cells were treated with E_{17}-POR (0.02, 0.03, 0.07, 0.13, 0.27, 0.54, or 1.07 μM) or Ce6-Me_2 (0.01, 0.02, 0.05, 0.09, 0.18, 0.36, or 0.73 μM) in DMEM in the absence of FBS for 1 h, followed by exposure of the plates to red light for 10 min. A control plate was not exposed to light. At the end, the media was replaced with fresh media containing FBS and grown for 14 h followed by Methylene Blue cell-viability assay. Each position in the graph represents an average of six (6) replicates. **B**: Methylene Blue cell-viability assays of MDA-MB 231 cells treated with various concentrations of E_{17}-POR and Ce6-Me_2 followed by exposure to red light. Briefly MDA-MB 231 cells were treated with 0.02, 0.03, 0.07, 0.13, 0.27, and 0.54 μM of E_{17}-POR or Ce6-Me_2 in DMEM in the absence of FBS for 1 h, followed by exposure of the plates to red light for 10 min. At the end, the media was replaced with fresh media containing FBS and grown for 14 h followed by Methylene Blue cell-viability assay. Another set of cells, incubated with 0.01, 0.02, 0.05, 0.07, 0.18, 0.36, and 0.73 μM of E_{17}-POR or Ce6-Me_2 was treated exactly the same way, except they were not exposed to red light. Each point in the graph represents an average of six (6) replicates.

et al., 2000; Vicente et al., 2002]. This is exemplified by the "leveling off" of toxicity at 60%–70% cell viability (Fig. 4B). Furthermore, it should be appreciated that such an effect was observed at high concentrations. For example, with MCF-7 cells 50% cell-kill was observed at a concentration of 0.18 μmol of E_{17}-POR (Fig. 4A). But at this concentration cell-kill in MDA-MB-231 cells was only approximately 5% in "light-exposed" and "dark" samples (Fig. 4B) (please note that different scaling methods in the abscissa were used in Fig. 4A,B).

Collectively, the above results showed that the presence of ER significantly increased the accumulation of the conjugate and strongly reduced the concentration of the porphyrin required for effective cell-kill. Observations in Figure 4A,B were visualized by incubating MCF-7 and MDA-MB 231 cells with a fixed concentration of either XIV or Ce6-Me_2 and then exposing them to red light for 10 min, or keeping the cells in the dark. After the treatment the cells were allowed to grow back and Methylene Blue was added to stain viable cells followed by photographic imaging of the cells. Results of these assays are shown in Figures 5 and 6.

With MCF-7 cells there was no significant difference in viable cells between E_{17}-POR and Ce6-Me_2-treated samples when the cells were not exposed to light (Fig. 5, upper half, middle, and right panels respectively). Although number of viable cells in untreated dark control (Fig. 5, upper half, left panel) appeared to be less than the treated samples, this could be due to photographing of an area with less density in

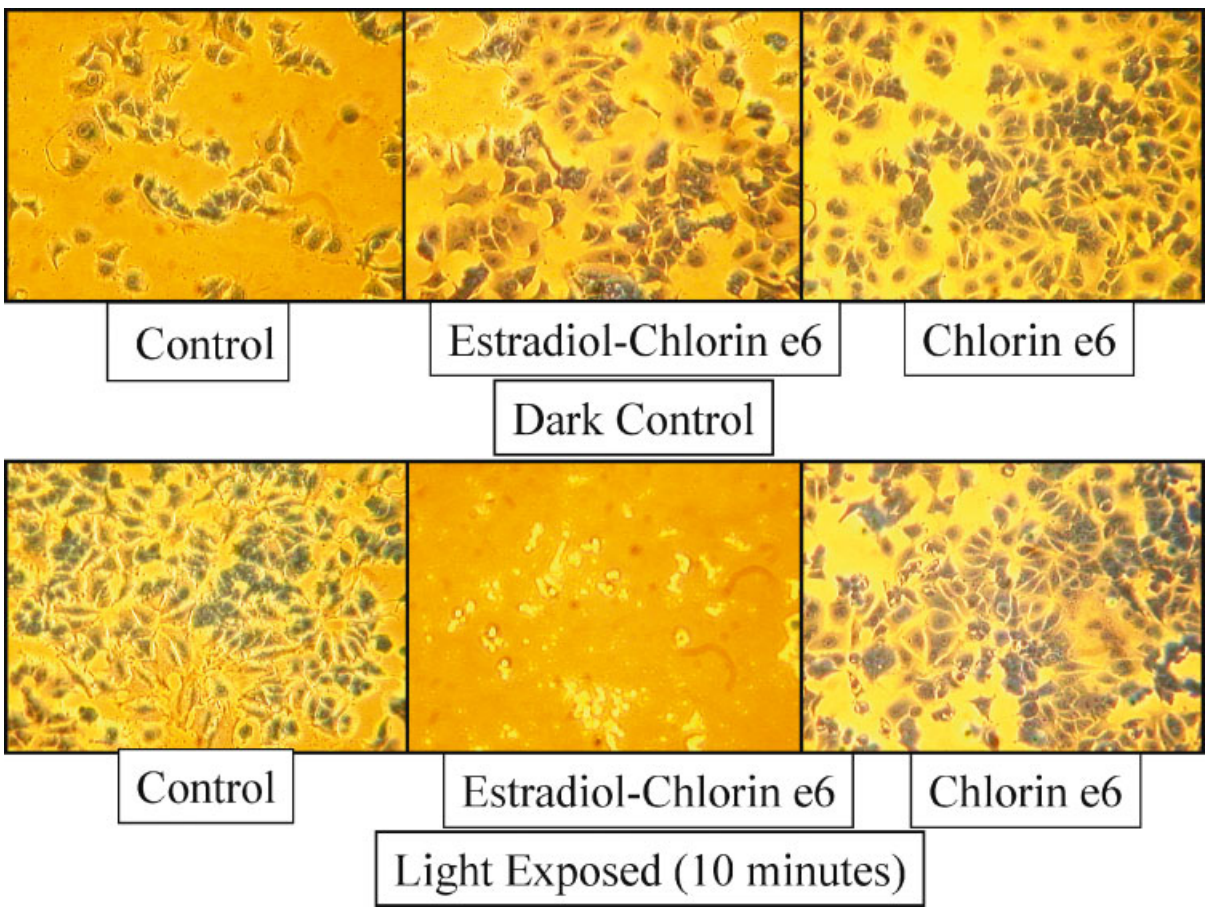

Fig. 5. Imaging of MCF-7 cells after incubation with E_{17}-POR or Ce6-Me_2 and either exposed to red light or kept in the dark. MCF-7 cells were treated with 1.07 μM of E_{17}-POR or Ce6-Me_2 in DMEM in the absence of FBS for 1 h. Then the plates were exposed to red light for 10 min at 25°C. A control plate was set up in parallel that was not exposed to light. At the end, the media was replaced with fresh media containing FBS and grown for 14 h. After this period the wells were washed twice with PBS (1.0 ml) followed by Methylene Blue cell-viability assay. The cells were photographed using an inverted microscope fitted with digital imaging system. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

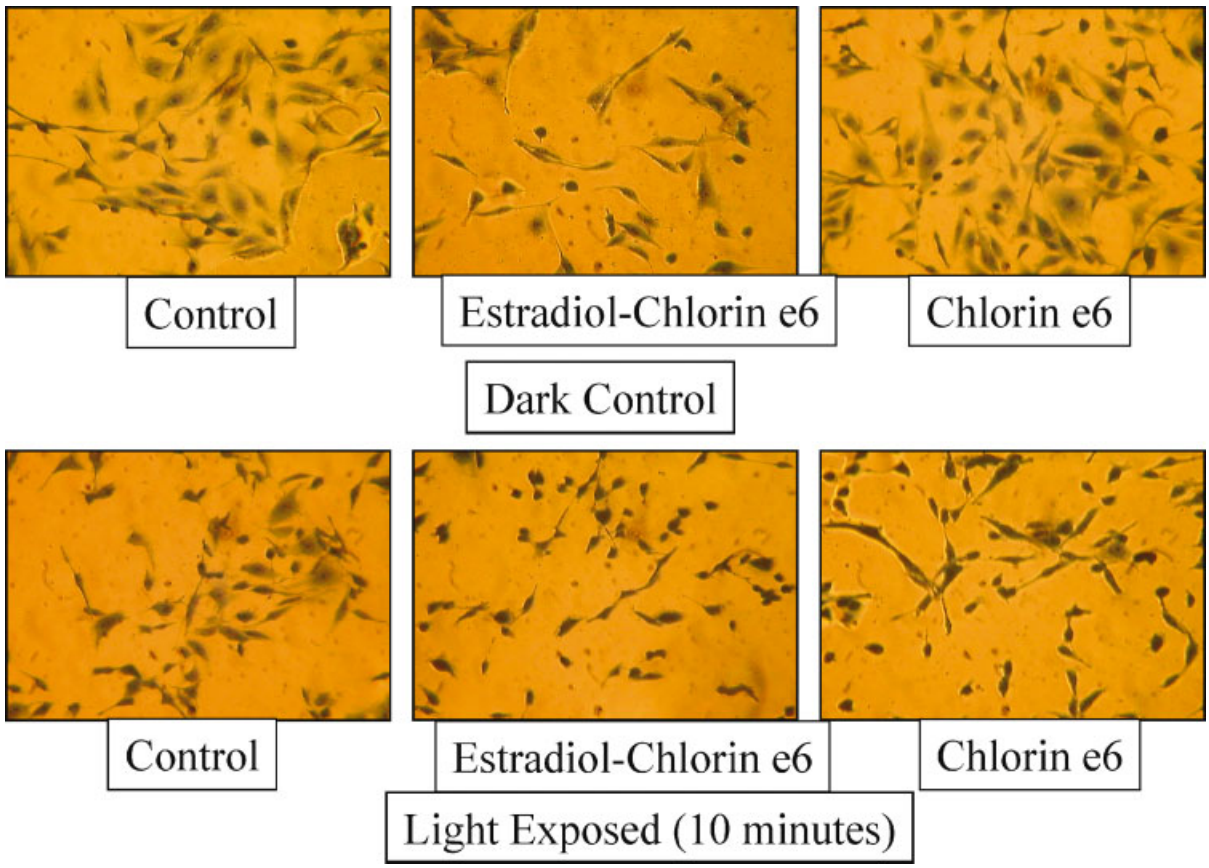

Fig. 6. Imaging of MDA-MB 231 cells after incubation with E_{17}-POR or Ce6-Me_2 and either exposed to red light or kept in the dark. The cells were treated with 1.07 μM of E_{17}-POR or Ce6-Me_2 in DMEM in the absence of FBS for 1 h. Then the plates were exposed to red light for 10 min at 25°C. A control plate was set up in parallel that was not exposed to light. At the end, the media was replaced with fresh media containing FBS and grown for 14 h. After this period the wells were washed twice with PBS (1.0 ml) followed by Methylene Blue cell-viability assay. The cells were photographed using an inverted microscope fitted with digital imaging system. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cell-population. In the light-exposed samples, there was strong cell-kill in the case of E_{17}-POR-treated sample (Fig. 5, lower half, middle panel). But there was no significant difference between untreated and Ce6-Me_2-treated cells (Fig. 5, lower half, left and right panels respectively).

In contrast, MDA-MB 231 cells appeared to be practically unchanged when exposed to light or kept in the dark in the presence of the conjugate (E_{17}-POR) or un-conjugated porphyrin (Ce6-Me_2) (Fig. 6, all the panels).

Collectively above results demonstrated that presence of ER in tumor cells significantly increased the uptake of the conjugate, despite relatively low ER-binding efficiency of the latter. This observation supported our hypothesis that poor ER-binding affinity (of the conjugates) might be mitigated by the tendency of the porphyrin part of the conjugate to be retained by tumor cells. Thus, higher accumulation of the conjugate in the ER-positive tumor cells lead to significantly higher cell-kill upon light-exposure. This phenomenon might also be further augmented by increased photosensitivity of the unconjugated porphyrin (chlorin e6-dimethyl ester) upon chemical conjugation with a hydrophobic molecule (a derivative of estradiol in this case) as noted by others [Hamblin et al., 2001].

Furthermore, these results strongly suggested that conjugation of chlorin e6 to estrogen sharply lowered the amount of the dye to obtain cell-kill in ER-positive breast cancer cells. Therefore, collectively these results underscored the strong potential of targeting ER in ER-expressing breast, ovarian and cervical tumors for selective and efficient delivery of photo-toxins to allow selective tumor cell-kill sparing surrounding healthy tissues. Therefore, this

approach could potentially alleviate certain drawbacks in traditional photodynamic therapy of cancer involving less-than desirable accumulation of PDT dyes into cancer cells.

However, it should be noted that the results described in this communication were generated strictly in cellular assays. Therefore, translation of this data into an animal model or into a clinical situation requires further work. In all the assays, cells were dosed for an hour in a media that was free of serum. This was done to enhance the sensitivity of the assays, although it represents a non-physiological situation. Furthermore, tumors are often heterogeneous, and some ER positive tumors might express ER at significantly higher amounts than others, and vice versa. As a result there will be differential accumulation of the conjugate into ER-positive tumors depending on their ER-content. However, it should also be appreciated that low ER-content in some tumors would still allow a low dose of the conjugate to accumulate into tumor and preserve its phototoxic nature. In such cases the tumor-retaining property of the conjugate will probably be governed predominantly by the porphyrin part of the conjugate. Therefore, in a clinical set up, low ER-containing tumors could still be treated with these conjugates by intratumoral injection of the conjugate, opposed to systemic administration. Such a delivery route might be beneficial for the desired accumulation of the conjugate in the tumor.

It should also be noted that in an in vivo system an estrogen-porphyrin conjugate is bound to accumulate into organs, including breast, where ER is significantly expressed. However, for breast tumor, for example, light will strictly be focused on the breast. Thus other ER-containing organs will be spared from toxicity (due to the photoactivating nature of the conjugate). Therefore, by harnessing the higher expression of ER in hormone-sensitive breast tumor and focusing the light only on the tumor it might be possible to induce phototoxicity and resultant cell-death in the tumor selectively.

In conclusion, the combination approach, involving a "double-headed drug" with dual mechanism of action has the potential to be applied clinically for hormone-sensitive cancers in organs where ER is significantly expressed, either as monotherapy involving a photo-induced selective destruction of tumor cells and/or adjuvant therapy in post-surgical treatment for the destruction of residual cancer cells in tissues surrounding the tumor. However, much further studies will be required to bring this method to the realm of treating breast tumor.

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