Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 3237–3243

BIOORGANIC & MEDICINAL CHEMISTRY

An Estradiol-Porphyrin Conjugate Selectively Localizes Into Estrogen Receptor-Positive Breast Cancer Cells

Narasimha Swamy,[a,†] David A. James,[a] Scott C. Mohr,[b] Robert N. Hanson[c] and Rahul Ray[a,*]

[a]*Bioorganic Chemistry and Structural Biology, Section in Endocrinology, Diabetes and Metabolism, Department of Medicine, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA*
[b]*Department of Chemistry, Boston University, Boston, MA, USA*
[c]*Department of Chemistry, Northeastern University, Boston, MA, USA*

Received 25 February 2002; accepted 16 May 2002

Abstract—A conjugate of a C_{11}-β-derivative of estradiol and an asymmetric tetraphenylporphyrin was synthesized to study its potential selective uptake by breast cancer cells naturally over-expressing the nuclear receptor for estrogen (ER). Competitive radioligand binding assays of this conjugate with recombinant ER showed that the conjugate bound to ER in a dose-dependent manner with an EC_{50} of 274 nM, compared with 1 nM for estradiol, the natural ligand. Cellular uptake studies with ER-positive MCF-7 and ER-negative HS578t human breast cancer cells revealed that, the conjugate was taken up by MCF-7 cells in a dose-dependent manner, which was obliterated by co-incubation with a large excess of estradiol. On the other hand there was very little uptake of the un-conjugated porphyrin by MCF-7 and Hs578t cells. HS578t cells also showed insignificant uptake of the conjugate under the conditions of our experiment. These results strongly suggested that specific interaction between the endogenous ER in MCF-7 cells and the estrogen part of the conjugate enabled these cells to selectively internalize the conjugate over the un-conjugated porphyrin. Therefore, ER-binding conjugates of estradiol and porphyrins could potentially be used for ER-targeted photodynamic therapy of hormone-sensitive cancers of breast, ovary, gonads etc.

Introduction

Selective delivery of cytotoxins to tumors is highly desirable in treating carcinomas. Yet such a feat often remains unattainable due to the lack of targets in cancer cells that are uniquely recognized by these toxins. Unique immune-signals on the surface of certain cancer cells have been harnessed by chemically conjugating toxins to antibodies to these signals. This method has enjoyed limited applicability due mostly to the paucity of active mechanisms for the internalization of the immuno-toxins into cancer cells.

Nuclear receptors, due to their high-affinity binding to their cognate ligands, act as beacons to attract and selectively localize their ligands and ligand-mimics into cells where these receptors are expressed. For example, estrogen receptor (ER), the primary modulator in the manifestation of biological effects of estrogens and anti-estrogens, is abundantly expressed in breast, ovary and gonads that are primary target organs for estrogen-action.[1–4] Thus, ER provides a viable target for the selective delivery of toxins, by chemically conjugating toxins/drugs to estrogens/anti-estrogens; and such a process might constitute a potentially attractive method for delivering drugs/toxins to hormone sensitive breast, ovarian and cervical cancers.

The concept of ER-targeting has been previously investigated with phenylindole-aniline mustards, exploiting the ER-binding and DNA-cleaving properties of phenylindoles and aniline mustards respectively.[5,6] These conjugates showed affinity for calf-uterine ER and selective toxicity for the ER-positive cell line MCF-7, compared to the ER-negative cell line MDA-MB-231. However, it was suggested that the selective toxicity was not due to nuclear ER delivery, as similar levels of inter-strand cross-linking was observed in both cells lines. Recently preliminary reports on the synthesis and

*Corresponding author. Tel.: +1-617-638-8199; fax: +1-617-638-8149; e-mail: bapi@bu.edu
†Present address: Department of Pediatrics, Women's and Infants Hospital, Providence, R.I., USA.

0968-0896/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(02)00242-0

cytotoxicity studies of estradiol-enediyne,[7,8] and estradiol-geldanamycin conjugates have been published.[9]

Breast tumor cells are known to over-express estrogen receptor (ER) in high levels particularly in their earlier stages and under hormone-treatment.[10,11] Therefore, we hypothesized that a synthetic conjugate of estradiol and a cytotoxin with high-affinity binding for indigenous ER in the cancer cells might selectively localize the conjugate into the tumor. To test this hypothesis we chose a porphyrin as the cytotoxin partner of the estradiol-toxin conjugate

Porphyrins occupy a central position in photodynamic therapy of cancer (PDT), which relies upon the selective accumulation of a photosensitizer (e.g., psoralens, pthalocyanins, acridine orange, merocyanines and porphyrins) into cancerous tissue followed by irradiation of the diseased tissue. Upon irradiation with red light, the excited state of the photosensitizer generates singlet oxygen, which damages cellular components and ultimately leads to cell death.[12,13]

We hypothesized that the tumor-localizing property of a porphyrin in an estradiol-porphyrin conjugate could be significantly enhanced by the strong interaction between the estradiol part of the conjugate and over-expressed ER in breast tumor cells. The resultant effect would be reflected in the enhanced uptake of the conjugate by hormone-sensitive (ER positive) tumor cells opposed to unconjugated porphyrin.

Based on the above hypothesis we published a preliminary report on the synthesis of an estradiol–porphyrin conjugate (**E_2–Por**).[14] In the present study we chose breast cancer cells with or without indigenous ER as model systems to validate our hypothesis. We compared the cellular uptake of a porphyrin (**Por**) and its correspondng estradiol-porpyrin conjugate (**E_2–Por**) in ER-positive MCF-7 breast cancer cells to determine the role of the estradiol part of **E_2–Por** in the cellular uptake. We also carried out uptake assays with ER-negative HS578T cells to determine the role of indigenous ER in the localization process. The present communication includes detailed accounts of the synthesis of **E_2–Por** and its ER-binding and cellular uptake studies.

Results and Discussion

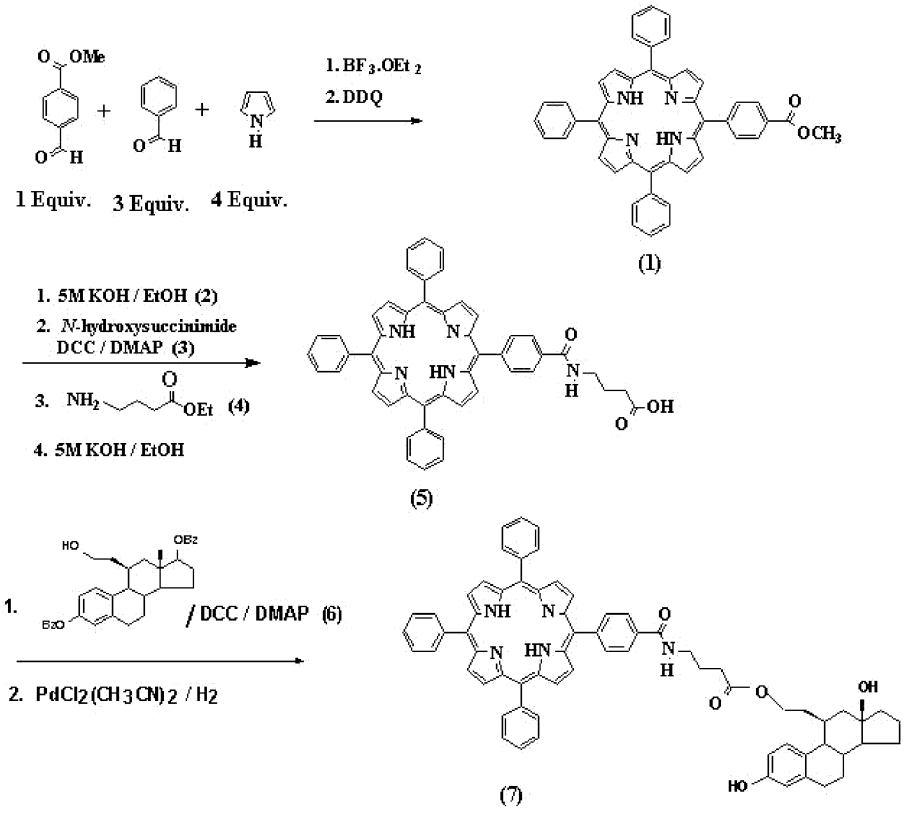
An essential part in designing estradiol-porphyrin conjugates to target nuclear ER was to identify positions of the parent estradiol molecule where an appendage (porphyrin) could be attached without seriously compromising ER-binding activity. During the past decade numerous structure–activity studies have been carried out, and three such positions, 7α, 11β and 17α have been identified.[15,16] It has be shown that modifications at the $C_{11\beta}$ and $C_{17\alpha}$ positions of estradiol, particularly with hydrophobic groups, are well tolerated towards ER-binding. The recently determined crystal structures of the hormone-binding domain of ER, bound to estradiol, has substantiated the earlier structure–activity data in demonstrating that those positions are indeed present in areas with little interference from the peptide backbone structure of ER.[17,18]

Synthesis of estradiol–porphyrin conjugates in which porphyrin moieties are attached to estradiol via the 3-hydroxyl group was reported by Chen et al.[19,20] However, it has been amply demonstrated that modification of the 3-hydroxyl group (of estradiol) leads to serious impairment of ER-binding.[20] Another report on the synthesis of several asymmetric porphyrins, one of them containing a 17α-ethynylestradiol, was published in 1995.[21]

We embarked upon the synthesis of an estradiol porphyrin conjugate in which the porphyrin moiety is attached to 11-β-position of estradiol via a long tether (Scheme 1). We reasoned that placing the porphyrin away from the 11β-position of estradiol via a long tether might not impair ER-binding seriously. Huang et al.[22] has previously reported the synthesis of the carboxymethyl-porphyrin (**Por 1**). We decided to adopt a more convenient procedure based on the popular method of Lindsey et al.[23] for the synthesis of unsymmetrical tetraphenylporphyrins. This procedure involved the $BF_3.OEt_2$-catalyzed condensation of 4-carboxymethylbenzaldehyde, benzaldehyde and pyrrole followed by oxidation with DDQ. Formation of the mono-(carboxymethylphenyl)porphyrin was favored by adjusting the stoichiometry of the reaction as indicated in Scheme 1 and high dilution. A mixture of porphyrins resulted and (**1**) was obtained after multiple flash column chromatographic purification steps.

Following the hydrolysis of (**1**), a DCC-mediated coupling of (**2**) with *N*-hydroxy succinimide produced the activated ester (**3**). This ester readily reacted with the carboxy-protected amino acid, ethyl 4-aminobuyrate, resulting in the protected porphyrin-amino acid (**4**). Hydrolysis of the ester group produced the free carboxylic acid (**5**). The carboxy-porphyrin (**5**) reacted with the protected 11β-substituted hydroxy-estradiol[24,25] via DCC-coupling reaction to produce the porphyrin conjugate (**6**). Removal of the benzyl-protecting groups was readily achieved with $H_2/PdCl_2(CH_3CN)_2$ resulting in the porphyrin-estradiol conjugate (**7**, **E_2–Por**).

A competitive radio-ligand binding assay was used to determine the ability of the porphyrin-estradiol conjugate (**E_2–Por**) to bind specifically to ER. The results of this binding assay, shown in Figure 1, demonstrated that **E_2–Por** displaced [^{3}H]-estradiol, bound to the estradiol-binding pocket of ER, in a dose-dependant manner, similar to estradiol. Therefore, these results strongly suggested that the conjugate (**E_2–Por**) bound to ER in a specific manner. However, the ER-binding affinity (EC_{50}) of **E_2–Por** was 274 nM compared to one nM for estradiol, the natural ligand, indicating a significant reduction in the ER-binding affinity. This could be a reflection of the modification of the 11β-position of estradiol and introduction of a large and polar porphyrin group. It is also probable that an eight (8) atom tether between estradiol and the porphyrin was not sufficient



Scheme 1. Synthesis of C11-β-estradiol-porphyrin conjugate (**E_2–Por**).

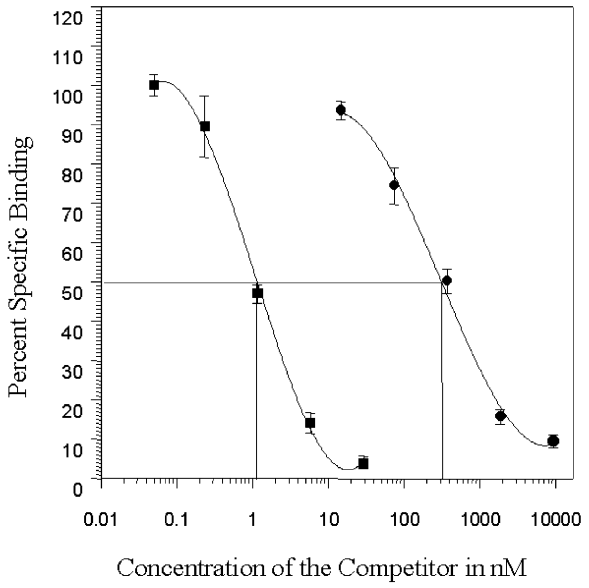


Figure 1. Competitive binding assays of estardiol and C11-β-estradiol-porphyrin conjugate (**E_2–Por**) with baculovirus expressed recombinant ER. Concentrations of estradiol or the conjugate at 50% specific binding (EC_{50}) are denoted in the X-axis.

to mitigate the significant loss in ER-binding by the conjugate, **E_2–Por**. However, we surmised that the relatively low binding affinity of **E_2–Por** might be sufficient to target nuclear ER with this conjugate.

We hypothesized that the estradiol part of the estrogen-porphyrin conjugate might bind to ER in cells that express ER (e.g., breast tumor cells). Such an interaction would enhance the natural tumor-localizing property of the porphyrin part (of the conjugate). As a result, there would be higher uptake of the conjugate than the unconjugated porphyrin. However, cells, which do not express ER, will not display such selectivity. Our hypothesis and the related possibilities are graphically described in Figure 2.

We chose two cell lines, MCF-7 (hormone-sensitive breast cancer cells, ER-positive) and Hs578T (hormone-resistant breast cancer cells, ER-negative) to test our hypothesis about targeting ER for the selective delivery of **E_2–Por**. As shown in Figure 3, when ER-negative HS578t cells were incubated with the un-conjugated porphyrin (**Por 1**) or estradiol-conjugated porphyrin, **E_2–Por**, there was little, if any, uptake of either porphyrin derivative at various concentrations under the condition of these assays. Similarly there was very little uptake of the un-conjugated porphyrin (**Por 1**) by MCF-7 cells containing indigenous ER. In contrast, there was a strong and dose-dependent uptake of the estradiol–porphyrn conjugate (**E_2–Por**) by the MCF-7 cells. These results strongly suggested that the uptake of the conjugate (**E_2–Por**) might be ER-mediated, and presence of ER (in MCF-7 cells) significantly increased the uptake.

In order to unequivocally establish the role of ER in the preferential uptake of **E_2–Por** by MCF-7 cells we incubated MCF-7 cells with various concentrations (2.5, 5, 10, 20, and 40 nM) of either **E_2–Por** or **Por 1**, in the

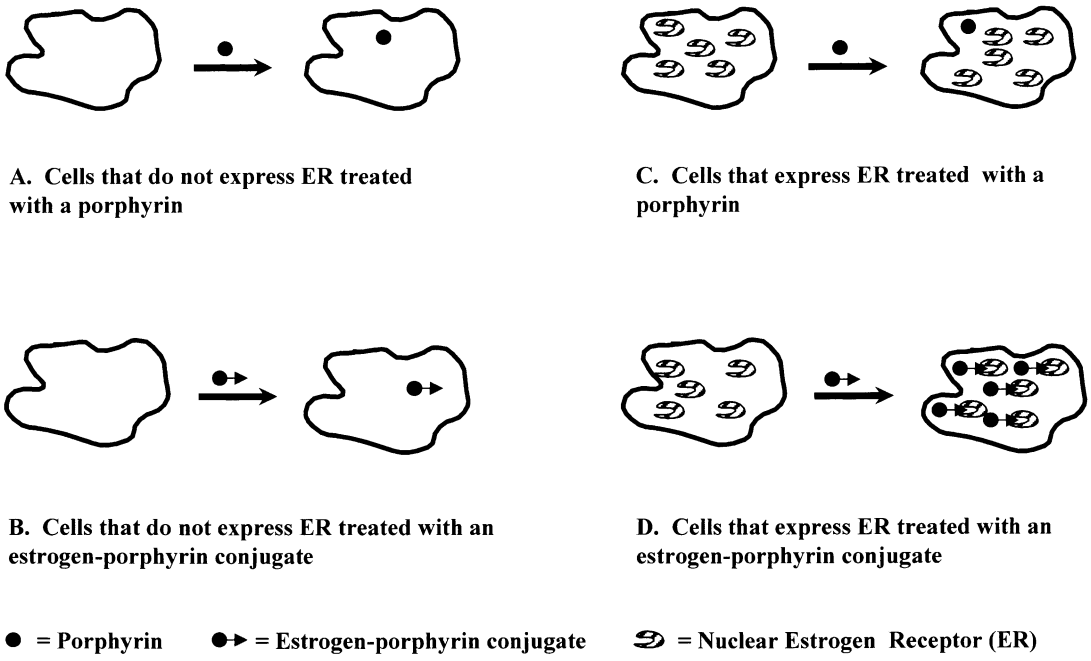


Figure 2. Graphic description of probable outcome of the internalization process of a porphyrin or a prophyrin-estradiol conjugate in cells that do or do not express nuclear ER.

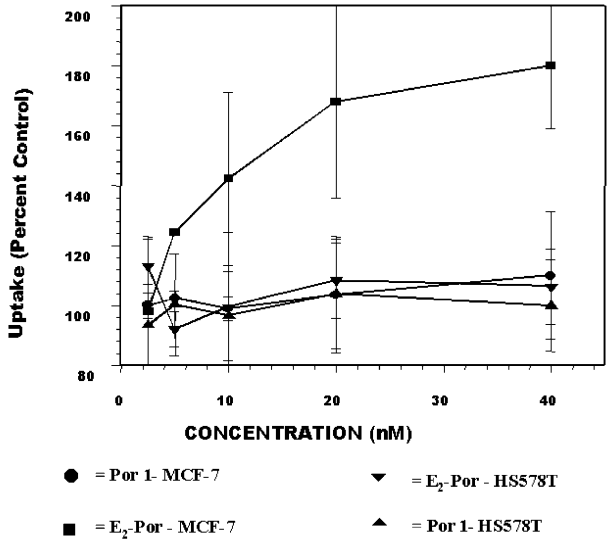


Figure 3. Uptake assays of un-conjugated porphyrin (**Por 1**) or the conjugate (**E_2–Por**) by ER +ve MCF-7 and ER -ve HS578T cells.

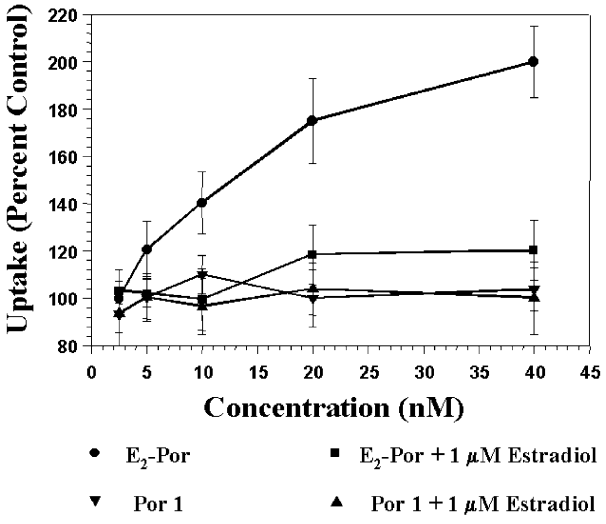


Figure 4. Uptake assays of un-conjugated porphyrin (**Por 1**) or the conjugate (**E_2–Por**) by ER +ve MCF-7 cells in the presence or in the absence of one μM of estradiol.

presence or in the absence of an excess (1 μM) of estradiol, the natural ligand for ER. As shown in Figure 4, presence of an excess of estradiol in the incubate almost completely obliterated the cellular uptake of **E_2–Por**. There was no significant difference in the uptake of **Por 1** in the presence or in the absence of estradiol, as expected. These results conclusively proved that selective uptake of **E_2–Por** by MCF-7 cells was mediated by endogenous ER.

It should be noted that the differential uptake of (**E_2–Por**) by the MCF-7 and Hs578t was substantial despite relatively low ER-binding affinity of **E_2–Por** compared to estradiol. It is also noteworthy that the uptake of **Por 1** by MCF-7 and Hs578t or **E_2–Por** by Hs578t was quite low, contrasting the natural tumor-localizing property of porphyrins. Tumor accumulation of porphyrins is known to be dependent on the chemical nature porphyrin as well as the dose. Therefore, we believe that this apparent anomaly was due to the low concentrations of **Por 1** and **E_2–Por** used in our assays.

Our observation emphasizes a classic problem in PDT involving low tumor-accumulation of PDT dyes leading to photo-toxicity of large body-areas. Clinically these problems are often countered with administration of high dose of the dye, which causes killing of healthy cells surrounding the malignant tissues, and prolonged toxicity of large body areas towards light-exposure. Attempts to enhance the low tumor-selective accumulation of the

PDT-dyes have been made by conjugating porphyrins with monoclonal antibodies[26–28] with limited success. On the other Akhlynina et al. recently demonstrated that coupling chlorin e_6 to a nuclear localization signal and targeting nuclear insulin receptor in PLC/PRF/5 and rat glioma C6 cells resulted in a more than 2000-fold reduction of EC_{50} (opposed to chlorin e_6 alone) and increase of its photosensitizing activity.[29]

In conclusion, our results strongly suggested that hormone-responsive breast tumors, naturally over-expressing ER, could be targeted for the selective delivery of estrogen-porphyrin conjugates for targeted PDT. However, we noted that photosensitization by Por 1 or **E_2–Por** was not sufficient to kill the cells. Therefore, we are currently in the process of attaching porphyrins with high fluorescence yield and cell-killing properties, such as chlorin e6,[29] to C-11β and C-17α positions of estradiol. Results of these studies will be reported elsewhere.

Experimental

Synthesis

All reagents were purchased from Aldrich Chemical Co., Milwaukee, WI, unless otherwise stated. Hexane, methanol and CH_2Cl_2 were obtained from American Bioanalytical, Natick, MA. Pyrrole and CH_2Cl_2 were distilled from CaH_2 immediately before use. [^{3}H]-estradiol (sp. activity 72 Ci/mmol) was obtained from New England Nuclear, Boston, MA. NMR spectra of the samples were obtained in $CDCl_3$-solution with TMS as internal standard on either a 270 MHz ((Jeol GSX 270, JEOL USA, Peabody, MA) or a 400 MHz (Jeol GSX 400) spectrometer. Matrix-assisted laser desorption–time of flight (MALDI–TOF) mass spectra of the synthetic compounds were obtained at Analytical Biotech Services, Watertown, MA. Fluorescence spectra were obtained in a Hitachi F2000 spectrofluorimeter.

5-(4-Carboxymethylphenyl)-10,15,20-triphenylporphyrin (1). Benzaldehyde (1.86 mL, 18.2 mmol), methyl 4-formylbenzoate (1.0 g, 6.09 mmol) and pyrrole (1.69 mL, 24.4 mmol) were dissolved in CH_2Cl_2 (1 lit), ethanol (10 mL) was added and the reaction flask was protected from light. Argon gas was bubbled through this solution for 10 min. $BF_3.OEt_2$ (0.5 mL) was added and the reaction was stirred under argon for 1 h. DDQ (4.14 g, 18.2 mmol) was added and the reaction was stirred for another hour exposed to the air. NEt_3 (10 mL) was added to the reaction mixture and it was evaporated to dryness to produce a black tarry solid. This crude product was adsorbed onto silica gel and purified by flash column chromatography (silica gel, 230–400 mesh, 25% CH_2Cl_2/hexane for the elution of tetraphenylporphyrin, and 50% CH_2Cl_2/hexane for the removal of (**1**). Fractions containing the desired product were combined and recrystallized from CH_2Cl_2/methanol to produce (**1**) as a purple solid (yield 0.57 g, 14%). ^{1}H NMR δ −2.86 (s, 2H, pyrrole–NH), 4.09 (s, 3H,–$PhCO_2CH_3$, 7.73 (m, 9H, Ph-H), 8.20 (d, J = 7.5 Hz, 6H, Ph-H), 8.29 (d, J = 7.5 Hz, 2H, Ph-H), 8.42 (d, J = 7.5 Hz, 2H, Ph-H), 8.76 (s, 2H, β-pyrrole H), 8.84 (s, 6H, β-pyrrole H). MALDI-TOF MS: 673.82 (calcd 672.78).

5-(4-Carboxyphenyl)-10,15,20-triphenylporphyrin (2). To a suspension of (**1**) (160 mg, 0.236 mmol) in EtOH (50 mL) was added KOH (100 mL, 5 M aqueous solution). This suspension was heated to reflux for 18 h. The reaction was allowed to cool to room temperature, then the solid product was collected by vacuum filtration, washed with water, then 1 M HCl. The product was dissolved in CH_2Cl_2/MeOH, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness to produce (**2**) as a green solid (yield 120 mg, 77%). MS: 660.082 (calcd 658.76).

5′-(4-Succinimidylcarboxy)phenyl,10′,15′,20′-triphenylporphyrin (3). To a solution of (**2**) and *N*-hydroxysuccinimide (41 mg, 0.355 mmol) in dry DMF (0.5 mL) and CH_2Cl_2 (5 mL) was added DCC (73 mg, 0.355 mmol) and DMAP (1 mg). The reaction was stirred under argon for 30 min. The solution was filtered, then evaporated to dryness. The crude product was re-dissolved in CH_2Cl_2, washed with water, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The product was purified further by flash column chromatography (silica gel, 230–400 mesh, 25% CH_2Cl_2/hexane). Fraction containing the desired product were combined and evaporated to dryness to produce (**3**) as a purple solid (yield 105 mg, 78%). ^{1}H NMR0 δ −2.82 (s, 2H, pyrrole-NH), 3.00 (m, 4H, succinyl–CH_2–), 7.77 (d, J = 7.7 Hz, 9H, Ph-H), 8.21 (d, J = 7.7 Hz, 6H, Ph-H), 8.35 (d, J = 7.7 Hz, 2H, Ph-H), 8.37 (d, J = 7.7 Hz, 2H, Ph-H), 8.77 (s, 2H, β-pyrrole H), 8.86 (s, 6H, β-pyrrole H).

5″-[{4′-(3-Carbethoxypropyl)carboxamido}]phenyl,10″,15″,20″-triphenylporphyrin (4). Ethyl 4-aminobutyrate hydrochloride (0.22 g, 1.32 mmol) was dissolved in water (2 mL). NEt_3 (1 mL) was added and extracted with CH_2Cl_2 (5 mL). The CH_2Cl_2 extract was dried over anhydrous MgSO, filtered and evaporated to dryness, then re-dissolved in CH_2Cl_2. This solution was added to a solution of (**3**) (100 mg, 0.132 mmol) in dry CH_2Cl_2 (5 mL) and NEt_3 (0.5 mL). The reaction was stirred under argon for 15 min then evaporated to dryness. The crude product was purified by flash column chromatography [silica gel 230–400 mesh, CH_2Cl_2 to remove minor impurities then EtOAc to elute (**4**)]. Fraction containing the desired product were combined and recrystallized from CH_2Cl_2/hexane to produce (**4**) as a purple solid (yield 98 mg, 95%). MS 773.077 (calcd 771.918).

5″-[{4′-(3-Carboxypropyl)carboxamido}]phenyl,10″,15″,20″-triphenylporphyrin (5). To a suspension of (**4**) (42 mg, 53.4 μmol) in EtOH (10 mL) was added KOH (20 mL, 5 M solution). This suspension was heated to reflux for 4 h. The reaction was allowed to cool to room temperature, then the solid product was collected by vacuum filtration, washed with water and then with 1 M HCl. The product was dissolved in CH_2Cl_2/MeOH, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness to produce (**5**) as a green solid (yield 35 mg, 88%).

Porphyrin-dibenzyl-estradiol conjugate (6). To a solution of (**5**) (10 mg, 13.2 μmol) in dry DMF (0.1 mL) and CH_2Cl_2 (2 mL) was added 3,17-dibenzyl, 11β-(2-hydroxyethyl)estradiol (50 μL, 0.35 M solution in CH_2Cl_2, 17.5 μmol) under argon. DCC (14 mg, 66.0 μmol) and DMAP (1 mg, 8.20 μmol) were added and the reaction was stirred under argon for 30 min. The reaction mixture was evaporated to dryness and the crude product was purified by preparative TLC (25% CH_2Cl_2/hexane) to produce (**6**) (yield 11 mg, 69%). ^{1}H NMR δ −2.82 (s, 2H, pyrrole–NH), 0.99 (s, 3H, C(18)-CH_3), 1.0–2.96 (overlapping m, 18H), 3.31 (t, J = 8.0 Hz, 1H, C18-H), 3.63 (m, 2H), 4.10 (m, 1H,–C(20)H_2O–), 4.25 (m, 1H,–C(20)H_2O–), 4.43 (s, 2H, C(17)OCH_2Ph), 4.96 (s, 2H, C(3)OCH_2Ph), 6.64 (d, J_1 = 2.0 Hz, 1H, C(4)H), 6.76 (dd, J_1 = 2.0 Hz, J_2 = 8.5 Hz, 1H, C(2)H), 7.01 (d, J_2 = 8.5 Hz, 1H, C(1)H), 7.26 (t, J = 8.0 Hz, C(3)Ph-H), 7.32 (d, J = 8.0 Hz, C(3)Ph-H), 7.72 (m, 9H, C(3′) & C(4′) Ph-H), 8.12 (d, J = 7.5 Hz, 2H, C(2″) Ph-H), 8.17 (d, J = 7.5 Hz, 6H, C(2′) Ph-H), 8.23 (d, J = 7.5 Hz, 2H, C(3′) Ph-H), 8.74 (s, 2H, β-pyrrole H), 8.81 (s, 6H, β-pyrrole H).

Porphyrin-estradiol conjugate (7, E_2–Por). $PdCl_2(CH_3CN)_2$ (1 mg) was added to a solution of (**6**) (11 mg, 9.0 μmol) in 50% EtOAc/EtOH (5 mL) under H_2 and the reaction was stirred for 30 min. The solution was filtered, and then evaporated to dryness. The crude product was purified by preparative TLC (CH_2Cl_2) to produce (**7**, **E_2–Por**), as a purple solid (yield 7 mg, 78%). ^{1}H NMR δ −2.84 (s, 2H, pyrrole–NH), 0.72–3.21 (overlapping m, 22H), 3.95 (m, 1H), 4.18 (m, 1H) 6.52 (s, 1H, C(4)H), 6.69 (d, J = 8.5 Hz, 1H, C(1)H), 6.85 (d, J = 8.5 Hz, 1H, C(2)H), 7.73 (m, 9H, Ph-H), 8.15 (overlapping m, 10H, Ph-H), 8.69 (d, J = Hz, 1H), 8.78 (overlapping m, 7H, C(2″)); MALDI-TOF MS 1043.26 (calcd 1043.298).

Competitive binding assays of estradiol and estradiol-porphyrin conjugate (E_2–Por) with ER

Baculovirus expressed recombinant ER (2 nM, PanVera, Madison, WI) was incubated with 0.125 nM of [^{3}H]17-β-estradiol in the presence of increasing concentrations of estradiol or the conjugate (**E_2–Por**) in an assay buffer (10 mM Tris, pH 7.5, 10% glycerol, 2 mM of monothioglycerol, and 1 mg/mL BSA) for 15 h at 4 °C. A 50% hydroxylapatite (HAP) slurry was added to remove protein-bound to [^{3}H]17-β-estradiol from unbound [^{3}H]17-β-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 ethanol. Radioactivity, bound to the HAP-pellet was determined in a liquid scintillation counter after the addition of scintillation cocktail.

Cellular uptake of estradiol-porphyrin conjugate (E_2–Por) and un-conjugated porphyrin (Por, 1) in ER-positive MCF-7 and ER-negative Hs578t cells

Incubation. MCF-7 and Hs578T cells (ATCC, Rockville, MD) were grown in DMEM containing 5% charcoal-stripped fetal bovine serum (FBS) in the absence of phenol red (100,000 cells/well in a 24-well plate). They were serum depleted for one day, and then were treated with 0.5 mL of media containing increasing concentrations of un-conjugated prophyrin (**Por 1**) or the estradiol-porphyrin conjugate (**E_2–Por**) (2.5, 5, 10, 20 and 40 nM) for 3 h in dark. After the incubation the wells were washed twice with in DMEM containing 5% charcoal-stripped FBS in the absence of phenol red followed by DMEM without phenol red.

In another experiment MCF-7 cells were incubated with **E_2–Por** or **Por 1** (2.5, 5, 10, 20, and 40 nm) in the absence or in the presence of one μM of estradiol. Rest of the procedure was same as described earlier.

Uptake-measurement. We observed that fluorescence-yields of **Por 1** and **E_2–Por** was not sufficient enough to be detected directly in a spectrofluorimeter. Therefore, we applied an indirect method, which exploited high fluorescence-yield of the dihydrofluorescien diacetate-fluorescein diacetate redox system. However, this system required activation of dihydrofluorescein diacetate with singlet oxygen, which was provided by exposing the cells to red light in the presence of porphyrins as the photosensitizers. It should be noted that there was no observable cell-kill by this process.

Therefore, the cells, after DMEM-wash, were incubated with dihydrofluorescein diacetate (2 mM final) in 0.5 mL of DMEM without phenol red for 30 min. The wells were washed thrice with ice-cold PBS, and dissociated from the plate using enzyme free cell dissociation solution (Speciality Media, Lavallette, NJ). The plates were exposed to visible light using a slide viewer with a red filter for 20 min at 25 °C (to photoactivate the porphyrin and generate oxygen which oxidized the non-fluorescent dihydrofluorescein diacetate to fluorescein diacetate, a fluorescent molecule). After the light-exposure the cells were lysed by the two additions of 0.5 mL of PBS containing 0.4% Triton X100 to each well. The contents of each well were transferred to microcentrifuge tubes and centrifuged at 10,000×g for 5 min. The supernatants were used to determine the fluorescence at 530 nm (excitation wavelength at 485 nm).

Acknowledgements

Part of the work was conducted by a grant from the Community Technology Development Fund, Boston University.

References and Notes

1. Jordan, V. C. *Breast Cancer Res. Treatment* **1995**, *36*, 267.
2. Hansen, N.; Morrow, M. *Med. Clin. N.A.* **1998**, *82*, 203.
3. Lemieux, P.; Fuqua, S. *J. Steroid Biochem. Mol. Biol.* **1996**, *56*, 87.
4. Park, W.-C.; Jordan, V. C. *Trends Mol. Med.* **2002**, *8*, 82.
5. Rink, S. M.; Yarema, K. J.; Soloman, M. S.; Paige, L. A.; Mitra Tadayoni-Rebek, B.; Essigmann, J. M.; Croy, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 15063.

6. Essigmann, J. M.; Rink, S. M.; Park, H. J.; Croy, R. G. *Adv. Exp. Med. Biol.* **2001**, *500*, 301.
7. Jones, G. B.; Huber, R. S.; Mathews, J. E.; Li, A. *Tetrahedron Lett.* **1996**, *37*, 3643.
8. Purohit, A.; Wyatt, J.; Hynd, G.; Wright, J.; El Shafey, A.; Swamy, N.; Ray, R.; Jones, G. B. *Tetrahedron Lett.* **2001**, *42*, 8579.
9. Kuduk, S. D.; Zheng, F. F.; Sepp-Lorenzino, L.; Rosen, N.; Danishefsky, S. *J. Bioorg. Med. Chem. Lett.* **1999**, *9*, 1233.
10. Gotteland, M.; May, E.; May-Levin, F.; Contesso, G.; Delarue, J. C.; Mouriesse, H. *Cancer* **1994**, *74*, 864.
11. Soubeyran, I.; Quenel, N.; Mauriac, L.; Durand, M.; Bonichon, F.; Coindre, J.-M. *Br. J. Cancer* **1996**, *73*, 735.
12. Sibata, C. H.; Colussi, V. C.; Oleinick, N. L.; Kinsella, T. J. *Expert Opin. Pharmacother.* **2001**, *6*, 917.
13. Dalla Via, L.; Marciani Magno, S. *Curr. Med. Chem.* **2001**, *12*, 1405.
14. James, D. A.; Swamy, N.; Hanson, R. N.; Ray, R. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2379.
15. Katzenellenbogen, J. A.; O'Malley, B. W.; Katzenellenbogen, B. S. *Mol. Endocrin.* **1996**, *51*, 267.
16. Anstead, G. M.; Carlson, K. E.; Katzenellenbogen, J. A. *Steroids* **1997**, *62*, 268.
17. Brzozowski, A. M.; Pike, A. C. W.; Dauter, Z.; Hubbard, R.; Bonn, T.; Engstrom, O.; Ohman, L.; Greene, G. L.; Gustafsson, J.-A.; Carlquist, M. *Nature* **1997**, *389*, 753.
18. Tannenbaum, D. M.; Wang, Y.; Williams, S. P.; Sigler, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5998.
19. Chen, S.-H.; Zhu, H.-J.; Liu, B.; Zhao, H.-M. *Youji Huaxue* **1988**, *8*, 34.
20. Chen, S.-H.; Li, D.-H.; Chen, Y. *Youji Huaxue* **1992**, *12*, 102.
21. Boyle, R. W.; Johnson, C. K.; Dolphin, D. *J. Chem. Soc., Chem. Commun.* **1995**, *5*, 527.
22. Huang, D.; Matile, S.; Berova, N.; Nakanishi, K. *Heterocycles* **1996**, *42*, 723.
23. Lindsey, J. S.; Prathapan, S.; Johnson, T. E.; Wagner, R. W. *Tetrahedron* **1994**, *50*, 8941.
24. Hanson, R. N.; Napolitano, E.; Fiaschi, R.; Onan, K. D. *J. Med. Chem.* **1990**, *33*, 3155.
25. Napolitano, E.; Fiaschi, R.; Hanson, R. N. *Gazz. Chim. Italiana* **1990**, *120*, 1.
26. Hasan, T. In *Photodynamic Therapy. Basic Principles and Clinical Applications;* Henderson, B. W., Dougherty, T. J., Eds, New York: Marcel Dekker, 1992; pp 187–200.
27. Hamblin, M. R.; Del Governator, M.; Rizvi, I.; Hasan, T. *Br. J. Cancer* **2000**, *11*, 1544.
28. Goff, B. A.; Blake, J.; Bamberg, M. P.; Hasan, T. *Br. J. Cancer* **1996**, *8*, 1194.
29. Akhlynina, T. V.; Jans, D. A.; Rosenkranz, A. A.; Statsyuk, N. V.; Balashova, I. Y.; Toth, G.; Pavo, I.; Rubin, A. B.; Sobolev, A. S. *J. Biol. Chem.* **1997**, *272*, 20328.