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Hydrogen peroxide overproduced in breast cancer cells can serve as an anticancer prodrug generating apoptosis-stimulating hydroxyl radicals under the effect of tamoxifen–ferrocene conjugate

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Abstract

A new approach to the treatment of cancer is suggested, based on the innate overproduction of hydrogen peroxide in cancer cells. Hydrogen peroxide serves as a prodrug in the presence of transition metal ions, such as iron delivered by ferrocene. Under the effect of ferrocene, hydrogen peroxide is split into hydroxyl anions and highly reactive hydroxyl radicals. The latter cause oxidative DNA damage, which induces apoptosis, leading to elimination of cancer cells. Tamoxifen, a drug that interacts with oestrogen receptors, was used as a carrier to deliver ferrocene to breast cancer cells. For this aim tamoxifen conjugated to ferrocene (Tam-Fer) was synthesized. We have shown that the frequency of apoptotic events in MCF-7 breast cancer cells treated with Tam-Fer is significantly higher than in cells treated with tamoxifen or ferrocene separately. The increase of apoptosis correlates well with the rise in generation of reactive oxygen species in cancer cells. These results show that the hydrogen peroxide overproduced in tumour cells can serve as a prodrug for the treatment of cancer.

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Introduction

Apoptosis has an important protective role since it selectively kills abnormal cells, particularly pre-cancerous and cancerous cells (Kerr et al 1994). Despite the existence of this natural anticancer barrier, cancer continues to be a leading cause of human mortality (Edwards et al 2002). Enhancement of apoptosis selectively in cancer cells might serve as an effective way to treat cancer (Salganik et al 2000; Salganik 2001; Albright et al 2004). However, means for the selective induction of apoptosis in cancer cells are limited. The accumulation of high concentrations of hydrogen peroxide is one of the characteristic biochemical features of cancer cells (Szatrowski & Nathan 1991; Burdon 1995; Toyokuni et al 1995; Okamoto et al 1996; Benhar et al 2002). Hydrogen peroxide accumulates because of the high level of superoxide dismutase that transforms superoxides ions generated by mitochondria into hydrogen peroxide (Oberley & Oberley 1997). Low levels of catalase and glutathione peroxidase also act to increase concentrations of hydrogen peroxide in cancer cells (Punnonen et al 1994).

Hydrogen peroxide that is accumulated in cancer cells might play the role of a prodrug. Use of prodrugs is a promising approach to the treatment of cancer that may reduce the adverse effects associated with conventional chemotherapy. These adverse events arise primarily from delivery of cell-damaging compounds to non-cancerous cells. In contrast to these systemically delivered compounds, administered prodrugs are converted into the active form of drugs by enzymatic or chemical entities that are characteristic of tumours (Denny 2004). However, in some cases a natural compound acting as a prodrug might pre-exist in cancer cells, and could theoretically be transformed by exogenous agents into an active cancer-targeted drug. Here we suggest a new approach to prodrug activation, which exploits the innate overproduction of hydrogen peroxide specifically in cancer cells which might act as a prodrug when administration of a targeted catalyst transforms hydrogen peroxide into hydroxyl radicals, the therapeutically active entities.

Materials and Methods

Chemical methods

^{1}H NMR spectra were obtained with a Bruker AC200 spectrometer (Billerica, MA, USA). UV spectra were recorded using a UV-VIS 1201 spectrophotometer (Shimadzu, Tokyo, Japan). HPLC was performed using Dynamax instrumentation (Rainin Instrument Co., Woburn, MA, USA). Chromatography refers to column chromatography on silica gel 60 Geduran (40–63 μm, EM Science, Gibbstown, NJ, USA) with the eluant indicated. Anhydrous tetrahydrofuran (THF) was obtained by distillation from sodium and benzophenone; 3-trifluoroacetamido-1-propene was obtained by reacting allylamine with trifluoroacetic anhydride in the presence of triethylamine, followed by evaporation and distillation in vacuo (bp 45°C, 0.7 mmHg).

(E)-1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene (4)

To a stirred solution of 1,4-diiodobenzene (3.4 g, 10.3 mmol) in anhydrous THF (10 mL) was added n-butyllithium (1.6 M, 6.5 mL, 10.3 mmol) in hexanes at −78°C, and stirring was continued for 15 min. A solution of 1-[4-[2-(dimethylamino) ethoxy]phenyl]-2-phenyl-1-butanone (3.2 g, 10.3 mmol) in THF (20 mL) was added, and the mixture was allowed to warm to ambient temperature. After 20 h, the mixture was partitioned between Et_2O (150 mL) and water (150 mL). The aqueous phase was extracted with Et_2O (2 × 100 mL). The combined organic extracts were washed with water (2 × 50 mL) and concentrated in vacuo. The oil obtained was dissolved in EtOH (40 mL), and concentrated aq. HCl (10 mL) was added. The mixture was heated with reflux for 3 h, and then evaporated in vacuo. The residue was dissolved in 10% Na_2CO_3 (100 mL) and extracted with Et_2O (3 × 100 mL). The combined extracts were washed with water (100 mL), dried (K_2CO_3), and concentrated in vacuo. Chromatography ($CHCl_3$/MeOH, 5/1) gave the crude product as a mixture of E and Z isomers. Recrystallization (ex hexane) gave the pure E isomer **4** as white crystals (1.84 g, 37%): UV (EtOH) λ_{max} = 244 (ε = 14200), 280 (sh) (ε = 9200); ^{1}H NMR ($CDCl_3$) δ_H 0.90 (t, J = 7.4 Hz, 3, CH_3CH_2), 2.3 (s, 6, NMe_2), 2.45 (q, J = 7.4 Hz, 2, CH_3CH_2), 2.65 (t, J = 5.7 Hz, 2, OCH_2CH_2N), 3.95 (t, J = 5.7 Hz, 2, OCH_2CH_2N), 6.55 (d, J = 9 Hz, 2, ArH ortho to OCH_2), 6.75 (d, J = 9 Hz, ArH meta to OCH_2), 7.0 (d, J = 8 Hz, 2, ArH meta to I), 7.05–7.2 (m, 5, Ph), 7.65 (d, J = 8 Hz, ArH ortho to I).

(Z)-1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1-[4-(3-amino-1-propenyl)phenyl]-2-phenyl-1-butene (5)

A mixture of 4-iodotamoxifen **4** (1.84 g, 3.82 mmol), 3-trifluoroacetamido-1-propene (0.974 mL, 7.64 mmol), tetramethylethylenediamine (1.153 mL, 7.64 mmol), palladium acetate (43 mg, 0.382 mmol) and triphenylphosphine (100 mg, 0.764 mmol) was heated in a flask equipped with a reflux condenser, at 100°C for 20 h under a nitrogen atmosphere. After cooling, the reaction mixture was diluted with $CHCl_3$ (100 mL). The insoluble material was separated by filtering and washed with $CHCl_3$ (50 mL). The chloroform solution was extracted with water (3 × 100 mL) and evaporated. The residue was dissolved in THF (50 mL), and concentrated aqueous NH_3 (5 mL) was added. The mixture was incubated at room temperature for 50 h. The solvent was removed in vacuo, and the residue was evaporated with anhydrous EtOH (2 × 20 mL). The product **5** was purified by chromatography ($CHCl_3$/MeOH/Et_3N, 50/15/1). Evaporation gave **5** as yellow oil (0.86 g, 53%): UV (EtOH) λ_{max} = 259 (ε = 16200), 290 (sh) (ε = 11700); ^{1}H NMR ($CDCl_3$) δ_H 0.88 (t, J = 7 Hz, 3, CH_3CH_2), 2.27 (s, 6, NMe_2), 2.42 (q, J = 7 Hz, 2, CH_3CH_2), 2.67 (t, J = 5.2 Hz, 2, OCH_2CH_2N), 3.51 (d, J = 7.5 Hz, 2, CH_2NH_2), 3.89 (t, J = 5.2 Hz, 2, OCH_2CH_2N), 6.15–6.35 (m, 2, CH=CH), 6.45 (s, 2, NH_2), 6.50 (d, J = 9 Hz, 2, ArH ortho to OCH_2), 6.72 (=8 Hz, 2, ArH meta to OCH_2), 7.0–7.1 (m, 5, Ph), 7.12 (d, J = 8 Hz, 2, ArH meta to CH=CH), 7.29 (d, J = 8 Hz, 2, ArH ortho to CH=CH).

(Z)-1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1-[4-(3-ferrocenoylamino-1-propenyl)phenyl]-2-phenyl-1-butene (3)

To a stirred solution of ferrocenecarboxylic acid (100 mg, 0.435 mmol) in anhydrous THF (2 mL) were added N-hydroxysuccinimide (52 mg, 0.44 mmol) and dicyclohexylcarbodiimide (93 mg, 0.44 mmol); stirring was continued for 4 h. The precipitate of dicyclohexylurea was filtered off, and the solution of O-(N-succinimidyl) ester of ferrocenecarboxylic acid was added to the solution of **5** (164 mg, 0.384 mmol) in anhydrous THF (1 mL). Et_3N (60 μL, 0.4 mmol) was added, and the reaction mixture was incubated overnight at room temperature. Then the solvent was evaporated, and the product **3** was purified by chromatography ($CHCl_3$/MeOH, 5/1). Recrystallization (ex $CHCl_3$/Et_2O) gave **3** as yellow crystals (37 mg, 15%): UV (EtOH) λ_{max} = 265 (ε = 29500), 290 (sh) (ε = 23100); ^{1}H NMR ($CDCl_3$) δ_H 0.90 (t, J = 7.3 Hz, 3, CH_3CH_2), 2.34 (s, 6, NMe_2), 2.44 (q, J = 7.3 Hz, 2, CH_3CH_2), 2.53 (t, J = 5.4 Hz, 2, OCH_2CH_2N), 3.97 (t, J = 5.4 Hz, 2, OCH_2CH_2N), 4.14 (d, J = 7.5 Hz, CH_2NH), 4.20 (s, 5, C_5H_5), 4.34 (t, J = 1.9 Hz, 2, CH_2 meta to C=O), 4.67 (t, J = 1.9 Hz, 2, CH_2 ortho to C=O), 6.15–6.4 (m, 2, CH=CH), 6.52 (d, J = 8 Hz, 2, ArH ortho to OCH_2), 6.73 (d, J = 8 Hz, 2, ArH meta to OCH_2), 7.05–7.2 (m, 7, Ph and ArH meta to CH=CH), 7.34 (d, J = 9 Hz, 2, ArH ortho to CH=CH).

Cell culture experiments

MCF-7 human breast cancer cells (American Type Culture Collection, Rockville, MD, USA) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, glutamine (2 mM), and penicillin/streptomycin (100 units mL^{-1}) and incubated at 37°C in 5% CO_2/95% air. When the cells reached 50% confluence they were removed by trypsinization (0.05% trypsin, 0.02% EDTA in phosphate-buffered saline (PBS)) for 3–5 min, resuspended in growth medium, centrifuged for 5 min at 1000 rpm, and then plated in 6-well plates at 4×10^4 cells per well. After 24 h, cells were treated with tamoxifen (Tam, 15 μM), ferrocene (Fer, 15 μM), or the ferrocene–tamoxifen

complex (Tam-Fer, 15 μM) and incubated for an additional 24 h. Some cells were treated with oestradiol 17-β (0.1 μM) in the absence or presence of Tam-Fer.

Assessment of apoptosis

Cells were washed twice with Eagle's MEM and prepared for measurment of apoptosis. Apoptosis-associated internucleosomal DNA fragmentation was determined using a TUNEL assay as described previously (Wijsman et al 1993; Albright et al 1995).

Measurement of reactive oxygen species (ROS)

In order to assess the production of reactive oxygen species (ROS), cells treated as described above were incubated for 1 h with avidin-FITC (1:200 in PBS; Sigma, St Louis, MO, USA). Avidin has a high affinity for biotin and structural similarities to 8-oxo-deoxyguanine and biotin, and binds with a high specificity to oxidatively modified guanine residues (Struthers et al 1998). Avidin-FITC reacts specifically with 8-oxo-deoxyguanine induced by the oxidation of guanine residues following accumulation of intracellular ROS. Cells were washed with PBS (×3) then mounted in GelMount (Biomeda, Foster City, CA, USA). Image analysis of avidin-FITC labelled cells was performed using a Nikon FXA microscope equipped with an Optronics DEI 750 low-light integrating CCD camera (Optronics Engineering, Goleta, CA, USA) connected to an Apple Macintosh G3 computer utilizing a Scion CG7 image capture card for digital image capture of epifluorescence images and the public domain NIH Image program version, 1.61 as described previously (Struthers et al 1998). Avidin-FITC labelling (green fluorescence) indicative of increased generation of ROS was only detected in cells with characteristic apoptotic morphology confirmed by phase contrast morphology and TUNEL labelling.

Statistical analysis

Differences between the various treatments were examined using analysis of variance. Point and interval estimates of mean ROS generation (determined via optical density) and apoptosis in MCF cells (determined by TUNEL method) were calculated for ROS generation outcomes in four treatment groups and apoptosis outcomes in eight treatment groups (12 all together). Bonferroni's correction was used to obtain 95% overall confidence intervals: each interval was computed with confidence 99.6% = 100% – 5%/12.

Results and Discussion

Experiments on transgenic mice developing malignant tumours have shown that an increase in the production of ROS induces oxidative damage of DNA bases, which promotes apoptotic death of cancer cells, resulting in a large decrease in tumour volume and significant reductions in the rate of metastases (Salganik et al 2000; Albright et al 2004). Selective accumulation of hydrogen peroxide in cancer cells leads to targeted apoptotic death without damage to normal cells. The generation of hydroxyl radicals in cancer cells by ferrocene reacting with hydrogen peroxide and other peroxides is considered to be a cause of the anticancer activity of ferrocenes (Köpf-Maier et al 1984). It seems probable that in cells ferrocene and its derivatives can be repeatedly oxidized by hydrogen peroxide and then reduced back, thus maintaining high levels of hydroxyl radicals. Thus, innate overaccumulation of hydrogen peroxide in cancer cells might serve as a promising prodrug interacting with ferrocene-delivered iron ions.

Tamoxifen (Figure 1) is an oestradiol mimetic that binds specifically to oestrogen ERα receptors, which are abundant in breast cancer cells. The binding of tamoxifen to oestradiol receptors in breast cancer cells leads to the temporary blockage of cell division. Thus tamoxifen acts primarily as a cytostatic but not cytotoxic agent. Top et al (1996) synthesized a tamoxifen analogue, ferrocifen (Figure 1) that contains a ferrocene residue, which substitutes tamoxifen's aromatic β ring. These authors reported that ferrocifen has cytotoxic activity, and suggested that this might be due to the damage caused by hydroxyl radicals produced by the reaction of ferrocene residue with hydrogen peroxide (Top et al 2001). However, this reasonable suggestion was not proven by the authors. Although the generation of hydroxyl radicals is highly probable, the structure of the residue of tamoxifen within ferrocifen is significantly different from that of native tamoxifen; thus, the binding of ferrocifen to other molecular targets cannot be ruled out as a cause of its cytotoxicity.

Generation of hydroxyl radicals by the reaction between ferrocene derivatives and hydrogen peroxide is a simple redox

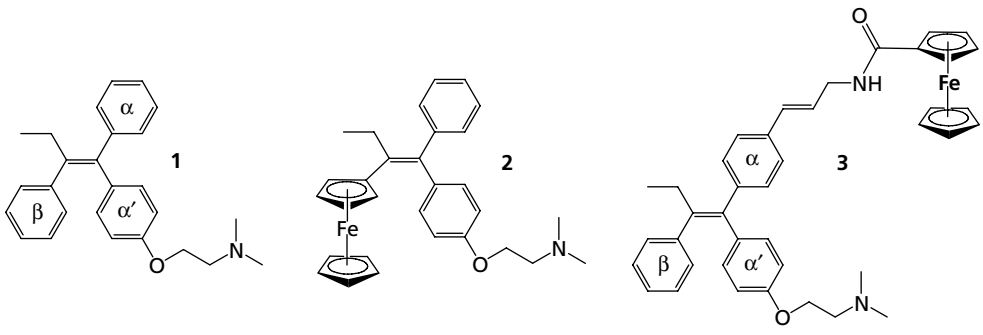

Figure 1 Schematic representation of tamoxifen (1) ferrocifen (2) and ferrocene–tamoxifen complex (3).

process that does not require interaction with cell proteins. On the other hand, any potential enzyme-dependent mechanism of ferrocifen cytotoxicity would inevitably include a step of molecular recognition of ferrocifen by one of the cell proteins.

We synthesized a molecule which, in contrast to ferrocifen, contains essentially the same building blocks present in the original configuration of tamoxifen. In our construct (Tam-Fer, Figure 1), ferrocene is not incorporated into the β ring of tamoxifen, as in ferrocifene, but instead is attached to the α ring via a linker. This construct is unlikely to bind to the same range of targets as ferrocifen but should, like ferrocifen, retain the ability to bind to oestrogen receptors.

Synthesis of the suggested structure of Tam-Fer is outlined in Figure 2. 4-Iodotamoxifen **4** was converted into compound **5** according to Heck's procedure (Dieck & Heck 1974) followed by the removal of the trifluoroacetyl protecting group. Compound **5** was conjugated with ferrocene carboxylic acid in the presence of O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate as a condensing agent or by reacting with O-(N-hydroxysuccinimidyl) ester of ferrocenecarboxylic acid.

We have determined the ability of Tam-Fer to generate free radicals in cancer cells and to induce apoptosis in experiments on MCF-7 breast cancer cells expressing oestradiol receptors. Using staining with avidin-FITC, we observed that incubation of cells with Tam-Fer conjugate **3** increases the production of free radicals in these cells by a factor of three, compared with an untreated control (Figure 3A). Avidin-FITC reacts selectively with 8-oxo-deoxyguanine that appears in cells because of the oxidation of DNA guanine residues by hydroxyl radicals.

Oxidatively modified DNA is a potent inducer of apoptosis. The number of apoptotic events in Tam-Fer treated cells

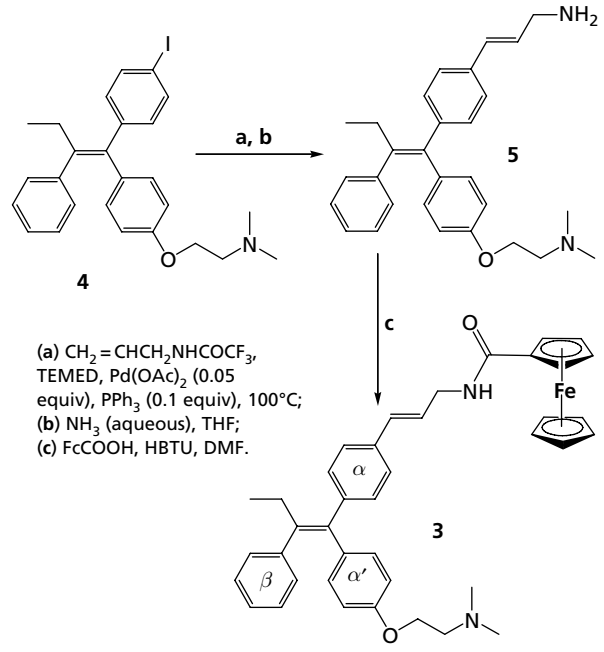

Figure 2 Schematic representation of the synthesis of the ferrocene–tamoxifen complex.

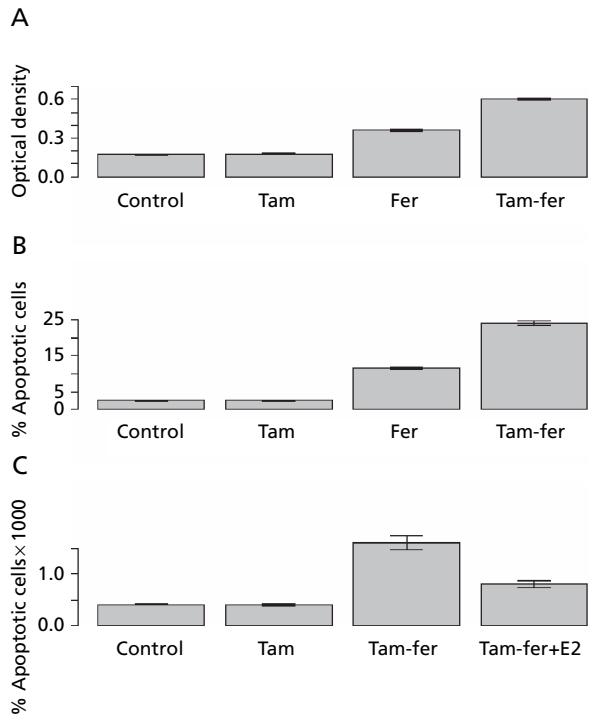

Figure 3 The effect of the tamoxifen–ferrocene (Tam-Fer)complex on the generation of reactive oxygen species (ROS) and apoptosis in MCF-7 breast cancer cells. Cells were incubated with ferrocene (Fer), tamoxifen (Tam) or Tam-Fer (15 μM) for 24 h at 37°C. **A** Generation of ROS (determined by oxidation rate of deoxyguanine that results in formation of 8-oxo-deoxyguanine in DNA, which selectively binds avidin-FITC and are quantified using a fluorescent plate reader (excitation 485 nm, emission 535 nm). Optical density reflects the level of ROS production. **B** Number of apoptotic events observed in cells (determined by the TUNEL assay). **C** Apoptosis rate in cells treated with oestradiol (0.1 μM) in the absence or presence of Tam-Fer (Tam-Fer + E2).

was about 7 times higher than in controls (Figure 3B). These results clearly show that cytotoxicity of Tam-Fer is caused by the production of hydroxyl radicals that induce apoptosis. The targeting of ferrocene to breast cancer cells expressing oestradiol receptors by the attachment of ferrocene to tamoxifen greatly increases free-radical generation and, accordingly, apoptosis induced by ferrocene. The role of oestradiol receptors in cancer cells in the interaction with Tam-Fer and its high pro-apoptotic activity is supported by the data from a separate set of experiments showing that oestradiol competitively inhibits the pro-apoptotic activity of Tam-Fer (Figure 3C).

Conclusion

Despite being known for a number of years, the selective accumulation of hydrogen peroxide in cancer cells has not attracted attention as a potential target for anticancer prodrug therapy. Here we show that this feature can be exploited in-situ to generate free radicals, which trigger apoptosis. Ferrocene and targeted ferrocene derivatives are the first compounds to exploit the overproduction of hydrogen peroxide in cancer cells as a natural prodrug to enable selective killing of cancer cells by induction of apoptosis.

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