Inhibition of Estrone Sulfatase Activity by Estrone-3-methylthiophosphonate: A Potential Therapeutic Agent in Breast Cancer

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ABSTRACT

Many breast tumors are hormone dependent, and there is evidence that hydrolysis of estrone sulfate (E1S) to estrone, by estrone sulfatase, is an important source of the estrogen which is found in tumors. In this study, we have developed a novel pathway for the synthesis of estrone-3-methylthiophosphonate (E1-3-MTP) and examined its ability to inhibit estrone sulfatase activity in MCF-7 breast cancer cells and human placental and breast tumor preparations. In MCF-7 breast cancer cells, E1-3-MTP, 100 nM and 10 μM, inhibited estrone sulfatase activity by 52 and >98%, respectively. The apparent Km and Vmax for E1S were 14.6 and 32.8 μM, respectively. A comparison of the metabolism of [3H]E1S and [3H]E1-3-MTP by human placenta or rat liver revealed that, whereas 50-60% of [3H]E1S was converted to [3H]estrone, <3% of [3H]E1-3-MTP was hydrolyzed. The development of an efficient inhibitor of estrone sulfatase, which is resistant to metabolism, will allow the importance of the estrone sulfatase pathway of estrogen formation in breast tumors to be assessed and such an inhibitor may have considerable potential as a therapeutic agent.

INTRODUCTION

Breast cancer is the most prevalent type of cancer in Western countries and approximately one-third of breast tumors are hormone dependent (1). There is considerable evidence derived from epidemiological, clinical, and experimental studies suggesting that estrogens have a central role in supporting the growth of hormone-dependent tumors (2). In postmenopausal women, in whom breast cancer commonly occurs, breast tumor concentrations of estrone (Fig. 1a, compound 1) and estradiol are considerably higher than blood estrogen levels (3). While retention of estrogens in breast tumors by high-affinity-binding proteins will contribute to the level of estrogens in tumors, estrogen concentrations are higher than plasma levels in both receptor-positive and receptor-negative tumors (4). In situ formation of estrogen from estrogen precursors within tumors is therefore likely to make a major contribution to the estrogen content of breast tumors.

The enzymes required for estrogen synthesis (i.e., aromatase, dehydrogenase, and sulfatase) are present in normal and malignant breast tissues (5). Blood concentrations of estrone sulfatase (Fig. 1a, compound 1) and estradiol are considerably higher than blood estrogen levels (5). While retention of estrogens in breast tumors by high-affinity-binding proteins will contribute to the level of estrogens in tumors, estrogen concentrations are higher than plasma levels in both receptor-positive and receptor-negative tumors (4). In situ formation of estrogen from estrogen precursors within tumors is therefore likely to make a major contribution to the estrogen content of breast tumors.

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INHIBITION OF ESTRONE SULFATASE ACTIVITY

\[^{1}H, \^{13}C, 3^{1}P\] NMR spectra were run on Jeol FX90Q and GX270 NMR spectrometers. \[^{31}P\] resonances were referenced to external 85% H\(_3\)PO\(_4\); chemical shifts are positive when downfield from this reference. Melting points were determined on a Reichert-Jung Thermo Galen Kofler block and are uncorrected. Mass spectra were recorded by the Mass Spectrometry Service, University of Bath. TLC was performed on silica gel 60F (Merck) plates with detection by UV light or with methanolic phosphomolybdic acid.

**Synthesis E\(_3\)-3-MTP**

*Initial Synthesis*

E\(_3\)-3-MTP (Fig. 1a, compound 3) was initially synthesized according to the method of Cox et al. (12). However, a number of problems were encountered with this method, the main one being the purification of the final product. Since E\(_3\)-3-MTP is charged, it could not be easily purified by standard chromatographic techniques. Other difficulties were also experienced in separating the crude compound from the pyridine used in the reaction mixture; even at pH 2, some of the desired material still existed as the pyridinium salt. \[^{3}H\]E\(_3\)-3-MTP was prepared by this method and purified by TLC using the system ethyl acetate/methanol/ammonium hydroxide (75:25:2).

Therefore, we decided to modify the original synthesis. The ammonium salt of E\(_3\)-3-MTP was prepared instead from estrone by a two-step pathway involving the formation of the 2-cyanoethyl thiophosphonate diester, a protected neutral intermediate similar to that used in DNA synthesis (13) and which allowed for effective purification, followed by \(\beta\)-elimination of the cyanoethyl function with aqueous ammonia.

*Modified Synthesis*

Preparation of Estrone-3-O-(2-cyanoethyl)methylthiophosphonate (Fig. 1b, Compound 6). Methylthiophosphonic dichloride (2.90 ml; 27.74 mmol; 3 Eq) was added dropwise to a stirred solution of estrone (2.50 g; 9.25 mmol; 1 Eq) in anhydrous pyridine (40 ml) at 0\(^\circ\) under nitrogen. The reaction mixture was allowed to warm to room temperature, and stirring was continued for an additional 24 h. The reaction mixture was then cooled to 0\(^\circ\), and anhydrous 2-cyanoethanol (3.79 ml; 55.47 mmol; 6 Eq) was added dropwise. The reaction was again allowed to warm to room temperature, and stirring was continued for another 24 h.

Subsequently, the reaction mixture was poured into water (100 ml), and the resulting aqueous solution was extracted with ethyl acetate (4 \(\times\) 75 ml). The combined organic extracts were dried over anhydrous MgSO\(_4\), and the solvent was removed \(\textit{in vacuo}\). Final traces of pyridine were removed by repeated coevaporation with toluene (3 \(\times\) 30 ml).

Purification of the crude material was effected by flash chromatography on Sorbsil C60 Silica gel (98:2, chloroform/methanol) to afford compound 6 (Fig. 1b) as a pale yellow oil, which solidified on standing \(\text{[2.14 g; 55%; m.p., 88-90}\(^\circ\); \text{Rf. 0.59 (98:2, chloroform:methanol)\].}

\[^{1}H\] NMR (270 MHz; CDC\(_3\)): 0.91 (s, 3H, C\(_8\)-Me), 1.40-1.75 (m, 6H), 1.94-2.60 (series of m, 6H), 2.04 (d, 3H, \(J = 15.39\) Hz, P-Me), 2.67-2.78 (m, 2H), 2.92 (m, 2H), 4.30 (m, 2H, -OCH\(_2\)CH\(_2\)CN), 6.92 (br d, 2H, \(J = 13.73\) Hz, phenyl), 7.26 (d, 1H, \(J = 9.16\) Hz, phenyl).

\[^{13}C\] NMR (67.8 MHz; CDC\(_3\)): 13.78 (q, C\(_{18}\)-Me), 19.69 (t), 21.50 (t), 22.64 (q, P-Me), 25.66 (t), 26.24 (t), 29.32 (t), 31.46 (t), 35.78 (t), 37.88 (d), 44.01 (d), 47.87 (s), 50.34 (d), 60.91 (d), 61.00 (s), 118.61 (d), 121.44 (d), 126.53 (d), 137.14 (s), 138.34 (s), 147.94 (s), 220.69 (s, C = O).

\[^{31}P\] NMR (109.37 MHz; CDCl\(_3\))\(_{[\text{H-decoupled}]\}): 95.16 and 95.19 (ratio of diasteroisomers, 1.4:1).

m/z(%): 417(4)(M\(^+\)), 312(59), 270(10), 259(17), 242(17), 206(48), 165(31), 148(72), 132(25), 112(90), 95(60), 83(38), 71(55), 57(100), 43(62), 29(19).

Preparation of the Ammonium Salt of Estrone-3-Methylthiophosphonate (Fig. 1a, Compound 3). A solution of aqueous ammonia (specific gravity, 0.88; 25 ml) was added dropwise to a stirred solution of estrone-3-MTP (2.50 g; 9.25 mmol) in ethyl acetate/methanol/ammonium hydroxide (75:25:2) at 0\(^\circ\). Subsequently, the solvent was removed \(\text{in vacuo}\), the white residue was subjected to coevaporation with toluene (3 \(\times\) 30 ml) in order to remove the final traces of water. Minor colored impurities were removed from the orange/cream wax solid by treatment of a solution of this compound in methanol with activated charcoal. When the solvent had evaporated \(\text{in vacuo}\), compound 3 was obtained as a cream foamy solid (0.46 g; 91%).

**Reagents and conditions:**

(i) \(a, \text{MePSCl/Pyridine/24 h; b, HOCH\(_2\)CH\(_2\)CN/24 h}\)

(ii) \(NH\(_2\)OH/65\(^\circ\)/5 h\)

Fig. 1. Structures. \(a\): compound 1, estrone; compound 2, estrone-3-sulfate; compound 3, estrone-3-methylthiophosphonate; compound 4, aminoglutethimide; compound 5, 4-hydroxyandrostenedione; \(b\): compound 1, estrone; compound 3, estrone-3-methylthiophosphonate; compound 6, estrone-3-O-(2-cyanoethyl)methylthiophosphonate.
In Vitro flasks were seeded with about 1 \times 10^6 cells/flask using the medium described containing fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 0.075% containing 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5% changed on every third day.

Monolayers of MCF-7 cells in triplicate 25-cm² tissue culture flasks were seeded with about 1 \times 10^6 cells/flask using the medium described above. For testing the effect of EI-3-MTP on in vitro estrone sulfatase activity, the cells were allowed to grow to 80% confluency and the medium was changed every third day.

**In Vitro Estrone Sulfatase Assay on Cell Monolayers**

Intact monolayers of MCF-7 cells in triplicate 25-cm² tissue culture flasks were washed with Earle’s balanced salt solution and incubated for 20 h at 37°C with [³H]EjS (5 pmol, 7 x 10^5 dpm) in serum-free minimal essential medium containing 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 0.075% sodium bicarbonate. For experiments, up to 30 replicate 25-cm² tissue culture flasks were seeded with about 1 \times 10^6 cells/flask using the medium described above. For testing the effect of EI-3-MTP on in vitro estrone sulfatase activity, the cells were allowed to grow to 80% confluency and the medium was changed on every third day.

**Cell Culture**

MCF-7 breast cancer cells were maintained in minimal essential medium containing 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5% fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 0.075% sodium bicarbonate. For experiments, up to 30 replicate 25-cm² tissue culture flasks were seeded with about 1 \times 10^6 cells/flask using the medium described above. For testing the effect of EI-3-MTP on in vitro estrone sulfatase activity, the cells were allowed to grow to 80% confluency and the medium was changed on every third day.

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**RESULTS**

Dose-dependent Inhibition of Estrone Sulfatase Activity in Intact MCF-7 Cells by EI-3-MTP. Estrone sulfatase activity in MCF-7 breast cancer cells has been well characterized by us (15) and others (16). EI-3-MTP inhibited estrone sulfatase activity in vitro in MCF-7 breast cancer cells in a dose-dependent manner (Fig. 2). Significant (P < 0.001) inhibition of estrone sulfatase was obtained at a concentration of 100 nM of EI-3-MTP. At 10 µM, EI-3-MTP inhibited the hydrolysis of EI₃ by >98%.

**Kₘ and V_max Values for EI₃ in Human Placenta and Human Breast Tumor.** The extent of hydrolysis of EI₃ by both tissues was directly proportional to incubation time, protein concentration, and substrate concentration within the limits assessed (data not shown). Apparent Kₘ and V_max values were determined under initial rate conditions using the Lineweaver-Burk plot. The apparent Kₘ and V_max for EI₃ were 4.8 µM and 148 pmol/min/mg protein in the placenta (Fig. 3a) and 16.9 µM and 38 pmol/min/mg protein in breast tumor (Fig. 4a).

**In Vitro Metabolism of [³H]EjS by Placental and Liver Tissue**

In order to assess whether the methylthiophosphonate group was stable and resistant to hydrolysis by sulfatase, [³H]EjS-EI-3-MTP (150,000 dpm) was incubated separately with placental or rat liver 2000 × g supernatants 20 and 100 µg protein, respectively, for 4 h. Nonpolar products were extracted with toluene, the organic phase was evaporated, and the products were separated by TLC in the system dichloromethane:ethyl acetate (4:1 v/v). [³H]EjS was used as a positive control in these experiments.

**Error bars,** SD of triplicate determinations; n = 3.

**PRODUCT FORMED**

**INHIBITOR (µM)**

![Fig. 2. Concentration-dependent inhibition of estrone sulfatase activity in intact MCF-7 cells by EI-3-MTP and DANAZOL.](image_url)

**CONCLUSIONS**

Estrone sulfatase activity in MCF-7 breast cancer cells has been well characterized by us (15) and others (16). EI-3-MTP inhibited estrone sulfatase activity in vitro in MCF-7 breast cancer cells in a dose-dependent manner (Fig. 2). Significant (P < 0.001) inhibition of estrone sulfatase was obtained at a concentration of 100 nM of EI-3-MTP. At 10 µM, EI-3-MTP inhibited the hydrolysis of EI₃ by >98%.

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estrone to breast tumors (9, 16, 17). An efficient inhibitor of estrone sulfatase activity is therefore required to assess the importance of the estrone sulfatase pathway of estrone synthesis in tumors. Such an inhibitor would also be of potential value as a therapeutic agent, to add to the endocrine therapies available for the treatment of breast cancer.

Although estrone sulfatase activity is considerably higher than aromatase activity in breast tumors, the development of inhibitors of estrone sulfatase activity has attracted little attention (18). Earlier investigations into the control of estrone sulfatase activity indicated that some unconjugated steroids, such as progesterone or dehydroepiandrosterone, could partially inhibit steroid sulfatase activity (19). The addition of a sulfate moiety to the steroid nucleus (e.g., pregnenolone sulfate) was also found to increase the extent of inhibition of estrone sulfatase activity (20). From the in vitro studies of MacIndoe et al. (21), physiological concentrations of naturally occurring steroid sulfates, such as dehydroepiandrosterone sulfate, might be expected to inhibit in vivo estrone sulfatase activity. However, because of the ubiquitous distribution of steroid sulfatases throughout the body, dehydroepiandrosterone sulfate may be hydrolyzed in the liver and at the plasma membrane of tumor cells and may therefore not be available.

**Kinetics of Inhibition of Placental and Breast Tumor Estrone Sulfatase Activity with E1-3-MTP.** To assess and classify the inhibitory effect of E1-3-MTP on the hydrolysis of EtS, increasing quantities of this substrate (2–50 μM) were each incubated with E1-3-MTP (0, 2, 5, 10, 20, 40 μM). The Lineweaver-Burk plots (Figs. 3a and 4a) indicated competitive inhibition by E1-3-MTP of the hydrolysis of EtS in both placenta and breast tumor tissue. The Ki for E1-3-MTP obtained from a plot of the slope of the Lineweaver-Burk plot as a function of E1-3-MTP concentration was 14.6 μM in the placenta (Fig. 3b) and 32.8 μM in breast tumor tissue (Fig. 4b).

**Metabolism of [3H]E1-3-MTP.** The metabolism of [3H]E1-3-MTP to [3H]estrone by placental and rat liver 2000 × g supernatants is shown in Fig. 5 along with that of [3H]EtS for comparison. While [3H]EtS is hydrolyzed to [3H]estrone to a large extent by both tissues (50–60%), [3H]E1-3-MTP is resistant to hydrolysis to form [3H]estrone (<2.5% [3H]EtS formed). In addition to [3H]estrone, no other products were detected.

**DISCUSSION**

Recent investigations have indicated the central role that estrone sulfate and estrone sulfatase may have in regulating the supply of estrone to breast tumors (9, 16, 17). An efficient inhibitor of estrone sulfatase activity is therefore required to assess the importance of the estrone sulfatase pathway of estrone synthesis in tumors. Such an inhibitor would also be of potential value as a therapeutic agent, to add to the endocrine therapies available for the treatment of breast cancer.

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jugates were extracted with toluene and separated by TLC (dichloromethane:ethyl acetate, 4:1, to inhibit estrone sulfatase inhibitors, such as Ew3-MTP, which are not alternative substrates and estrone, one of which was E~-3- MTP, to bovine serum albumin, to act liver preparations (20 and 100 lag protein, respectively) for 4 h. Nonpolar products therefore resistant to sulfatase action, should inhibit the development of antisera to estrone sulfate. Such antisera were required in cancer cells and in placental and breast tumor cytosol preparations. Values for the Km and 40 laM, respectively (20). The Ki value for EI-3-MTP using breast tumor cytosol is 33 µM. However, as previously discussed, the ability of compounds such as 5-androstenediol-3-sulfate to inhibit estrone sulfatase activity in vivo is doubtful due to the instability of the sulfate group. Although the stability of E1-3-MTP remains to be established, results from the study with liver microsomes suggest that this compound is much more resistant to metabolism than naturally occurring steroid sulfates, such as estrone sulfate.

In addition to the preliminary inhibition studies with danazol (22), a number of derivatives of 2-(hydroxyphenyl)-indole 1% have recently been shown to inhibit estrone sulfatase activity (23). This series of inhibitors was based on a new class of mammmary tumor inhibitor compounds, one of which was shown to be metabolized to a sulfate conjugate in vivo. The concentrations producing 50% inhibition for this series of hydroxyphenyl indole derivatives ranged from 80–4000 µM, using a calf uterus cytosol preparation. For comparison, the concentrations producing 50% inhibition for E1-3-MTP, using placental and breast tumor cytosols, were 43 and 36 µM, respectively.

In conclusion, we have shown that E1-3-MTP, which was originally synthesized for the production of antisera reactive with estrone sulfate, also efficiently inhibits estrone sulfatase activity in MCF-7 breast cancer cells as well as in placental and breast tumor cytosol preparations. Having identified an efficient inhibitor of estrone sulfatase activity, it should now be possible to assess the importance of the estrone sulfatase pathway in breast tumor estrogen synthesis. In addition, the synthesis of analogues of E1-3-MTP may lead to the development of more efficient inhibitors of estrone sulfatase activity. This is in progress.

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