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. Estrone sulfatase activity 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 4.8 μM and 148 pmol/min/mg for placental and 16.9 μM and 38 pmol/min/mg for breast tumor preparations. Kinetic studies revealed that E1-3-MTP inhibited estrone sulfatase in a competitive manner with the Km values for placental and tumor preparations being 14.6 and 32.8 μM, respectively. A comparison of the metabolism of [3H]E1-3-MTP 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 estrone 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 plasma concentrations (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 sulfate (Fig. 1a, compound 2) are 8- to 10-fold greater than those of unconjugated estrone, and estrone sulfatase activity, responsible for the conversion of estrone sulfate to estrone, is a thousand-fold higher than aromatase activity in breast tissues (5, 6). Together, these findings suggest that estrone sulfatase may have a key role in regulating the formation of estrogens within breast tumors. Vignon et al. (7), using MCF-7 breast cancer cells which possess estrone sulfatase activity, originally demonstrated that estrone sulfate stimulates the synthesis of well-characterized estrogen-induced proteins. Administration of estrone sulfate has been shown, using the N-nitroso-methylurea-induced rat mammary tumor model, to result in a dose-dependent stimulation of tumor growth (8). Estrone sulfatase in N-nitroso-methylurea-induced tumors is significantly reduced after ovarectomy, in contrast to hepatic estrone sulfatase activity (9), suggesting that intratumoral, rather than peripheral, estrone sulfatase activity may have a major role in regulating the supply of estrogen to promote tumor growth.

The development of specific inhibitors of estrogen synthesis, to reduce tissue exposure to estrogen, is an important advance in the therapies available to treat women with breast cancer. So far, considerable effort has gone into the development of efficient inhibitors of aromatase activity, the complex responsible for the conversion of androstenedione to estrone. While compounds such as aminoglutethimide (Fig. 1a, compound 4) and 4-hydroxyandrostenedione (Fig. 1a, compound 5) greatly reduce peripheral aromatase activity, plasma estrone and estrone sulfate concentrations are only reduced by about 50% (10, 11). It is therefore possible that inhibitors of estrone sulfatase activity, used alone or in combination with an aromatase inhibitor, may enhance the response to this form of endocrine therapy. In contrast to the many aromatase inhibitors currently undergoing evaluation, the development of inhibitors of estrone sulfatase activity is at an early stage. We have therefore attempted to develop an efficient inhibitor of estrone sulfatase activity, and report the ability of E1-3-MTP (Fig. 1a, compound 3) to inhibit estrone sulfatase activity in MCF-7 breast cancer cells and a placental preparation containing estrone sulfatase activity.

MATERIALS AND METHODS

Reagents

Unlabeled estrone sulfate and danazol were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). E1S (specific activity, 60 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [4,14C]Estrone (specific activity, 52 mCi/mmol) and [6,7-3H]Estrone (specific activity, 97 Ci/mmol) were obtained from the Amersham International Radiochemical Centre (Amersham, United Kingdom). Nonpolar contaminants were removed from labeled and unlabeled E1S before use by extraction with toluene or diethyl ether. Methylthiophosphonic dichloride was purchased from CN Biochemicals Ltd. (High Wycombe, Bucks, United Kingdom). All organic solvents were of A.R. grade and were supplied by Fisons Plc (Loughborough, England) as were the Corning 25-cm2 tissue culture flasks. Fetal bovine serum, minimal essential medium, and supplements were purchased from Flow Laboratories (Irvine, Scotland). Estrone was purchased from Sigma and was dried under vacuum at 65°C prior to use. Pyridine was dried by distillation from potassium hydroxide.

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3 The abbreviations used are: E1-3-MTP, estrone-3-methylthiophosphonate; E1S, [6,7-3H]estrone sulfate; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; q, quadruplet; bd, broad doublet.
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$^1$H, $^{13}$C, $^3$P NMR spectra were run on Jeol FX90Q and GX270 NMR spectrometers. $^3$P resonances were referenced to external 85% H$_3$PO$_4$; chemical shifts are positive when downfield from this reference. Mass spectra were recorded by the Mass Spectrometry Service, University of Bath. TLC was performed on silica gel 60F (Merk) plates with detection by UV light or with methanolic phosphomolybdic acid.

Synthesis E$_r$-3-MTP

Initial Synthesis

E$_r$-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$_r$-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]$E_r$-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$_r$-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 β-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°C 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°C, 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 × 75 ml). The combined organic extracts were dried over anhydrous MgSO$_4$, and the solvent was removed in vacuo.

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 (2.14 g; 55%; m.p., 88-90°C, Re, 0.59 (98:2, chloroform:methanol)).

$^1$H NMR (270 MHz; CDCl$_3$): 0.91 (s, 3H, C$_{18}$-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; CDCl$_3$): 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 (s), 61.00 (s), 118.61 (d), 121.44 (d), 126.53 (d), 137.14 (s), 147.94 (s), 220.69 (s, C = O).

$^{31}$P NMR (109.37 MHz; CDCl$_3$) [H-decoupled]: 95.16 and 95.19 (ratio of diastereoisomers, 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(62), 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 to compound 6 (0.55 g; 1.32 mmol), and the resulting suspension was heated at 65°C for 5 h. Subsequently, the solvent was removed in vacuo, and the white residue was subjected to coevaporation with toluene (3 × 30 ml) in order to remove the final traces of water. Minor colored impurities were removed from the orange/cream waxy solid by treatment of a solution of this compound in methanol with activated charcoal. When the solvent had evaporated in vacuo, compound 3 was obtained as a cream foamy solid (0.46 g; 91%).

Reagents and conditions:

(i) a, MePSCl$_3$/pyridine/24 h; b, HOCH$_2$CH$_2$CN/24 h

(ii) NH$_4$OH/65°C/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 fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 0.075% containing 20 \( \mu \)M N-2-hydroxyethylpiperazine-N\( \beta \)-2-ethanesulfonic acid, 5%
the cells were allowed to grow to 80% confluency and the medium was
223.51 (s, C = O).

P-Me), 26.95 (t), 27.57 (t), 30.42 (t), 32.76 (t), 36.68 (t), 39.57 (d), 45.34 (d),
7.20 (br d, 1H, J = 8.24 Hz, phenyl).

Cell Culture

MC-7 breast cancer cells were maintained in minimal essential medium
(Raw Text End)
INHIBITION OF ESTRONE SULFATASE ACTIVITY

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 E1S, 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 E1S 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 2000x g supernatants is shown in Fig. 5 along with that of [3H]E1S for comparison. While [3H]E1S 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]E1 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.

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...
jugates were extracted with toluene and separated by TLC (dichloromethane:ethyl acetate, 4:1, to inhibit estrone sulfatase partition procedures (12). Early attempts to raise antisera to estrone estrone, one of which was E~3- MTP, to bovine serum albumin, to act whether the methylthiophosphonate group was stable and resistant to hydrolysis by drolysis of E~S.

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 sulfate activity in MCF-7 breast cancer cells as well as in placental and breast tumor cytosol preparations. Having identified an efficient inhibitor of estrone sulfate activity, it should now be possible to assess the importance of the estrone sulfate 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 sulfate activity. This is in progress.

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