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

L. Duncan, A. Purohit, N. M. Howarth, B. V. L. Potter, and M. J. Reed

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 K_M and V_max 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 K_I values for placental and tumor preparations being 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 metalloporphyrins, 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 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 estrone 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-nitrosomethylurea-induced rat mammary tumor model, to result in a dose-dependent stimulation of tumor growth (8). Estrone sulfatase in N-nitrosomethylurea-induced tumors is significantly reduced after ovariectomy, in contrast to hepatic estrone sulfatase activity (9), suggesting that intra-tumoral, 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. (Pooles, Dorset, United Kingdom). E1S (specific activity, 60 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [4,6-14C]Estrone (specific activity, 52 Ci/mmol) and [6,7-3H]Estrone (specific activity, 97 Ci/mmol) were obtained from the Amersham International Radiochemical Centre (Amerham, 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 (Irvin, 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.
INHIBITION OF ESTRONE SULFATASE ACTIVITY

$^1$H, $^3$C, $^{31}$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 (Merk) plates with detection by UV light or with methanolic phosphomolybdic acid.

Synthesis $E$,-$3$-MTP

**Initial Synthesis**

$E$,-$3$-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 $E$,-$3$-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$]E$,-$3$-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 $E$,-$3$-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°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 x 75 ml). The combined organic extracts were dried over anhydrous MgSO$_4$, and the solvent was removed in vacuo. Final traces of pyridine were removed by repeated coevaporation with toluene (3 x 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 [2.14 g; 55%; m.p., 88-90°C; Rf, 0.59 (98:2, chloroform:methanol)].

$^1$H NMR (270 MHz; CDCl$_3$): 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; 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 (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$_3$)[$[^{1}$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 x 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/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 flasks were seeded with about 1 x 10⁶ cells/flask using the medium described containing fetal bovine serum, 2 mM glutamine, nonessential amino acids, and 0.075% sodium bicarbonate. The cells were allowed to grow to 80% confluency and the medium was replaced every 2 days.

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).

The specific activity of the substrate. Each batch of experiments included a positive control (EtS) and flasks without cells (to assess apparent nonenzymic hydrolysis of EtS by >98%).

RESULTS

Dose-dependent Inhibition of Estrone Sulfatase Activity in Intact MCF-7 Cells by E₁₃-MTP. Estrone sulfatase activity in MCF-7 breast cancer cells has been well characterized by us (15) and others (16). E₁₃-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 E₁₃-MTP. At 10 μM, E₁₃-MTP inhibited the hydrolysis of EtS by >98%.

Kₘ and Vₘₐₓ Values for EtS in Human Placenta and Human Breast Tumor. The extent of hydrolysis of EtS by both tissues was directly proportional to incubation time, protein concentration, and substrate concentration within the limits assessed (data not shown). Apparent Kₘ and Vₘₐₓ values were determined under initial rate conditions using the Lineweaver-Burk plot. The apparent Kₘ and Vₘₐₓ for EtS 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. 3b).

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 [²H]EtS (5 pmol, 7 x 10⁵ dpm) in serum-free minimal essential medium (2.5 ml) with or without E₁₃-MTP (0.25–25 nmol). Substrate and inhibitor were added to medium in ethanol, with the final concentration being <1%. After incubation, each flask was cooled, and the medium (1 ml) was pipetted into separate tubes containing [¹⁴C]estrone (7 x 10⁶ dpm). The mixture was shaken vigorously for 30 s with toluene (5 ml). Preliminary experiments showed that >90% [¹⁴C]estrone and <0.1% [²H]EtS was removed from the aqueous phase by this treatment. A portion (2 ml) of the organic phase was evaporated, and the [³H] and [¹⁴C] content of the residue was determined by scintillation spectrometry. The mass of EtS hydrolyzed was calculated from the [³H] counts obtained (corrected for the volumes of the medium and organic phase used and for recovery of [¹⁴C]estrone added) and the specific activity of the substrate. Each batch of experiments included incubations of microsomes prepared from a sulfatase-positive human placenta (positive control) and flasks without cells (to assess apparent nonenzymic hydrolysis of EtS by >98%).

Tissue Preparation

A sulfatase-positive human placenta from a normal term pregnancy, a breast tumor from a postmenopausal woman with advanced breast cancer, and normal livers from 200-g female rats were treated separately in an identical manner as follows. Tissue was thoroughly minced with scissors and washed once with cold phosphate buffer (pH 7.4, 50 mM) and resuspended in cold phosphate buffer (5 ml/g tissue). Homogenization was accomplished with an Ultra-Turrax homogenizer, using three 10-second bursts separated by 2-min cooling periods in ice. Nuclei and cell debris were removed by centrifuging (4°C) at 2000 × g for 30 min, and portions (2 ml) of the supernatant were stored at -20°C. The protein concentration of the supernatants was determined by the method of Bradford (14). For enzyme kinetic measurements, substrate and inhibitor were added to tumor or placental preparations in ethanol, with the final concentration being <1%.

Determination of Apparent Kₘ Values

Incubations (1 ml) were carried out using a protein concentration of 125 μg/ml (placenta) or 200 μg/ml (breast tumor), nine substrate concentrations varying from 2–50 μM, and an incubation time of 20 min (placenta) or 90 min (breast tumor). In both tissues, the apparent Kₘ value and maximum velocity for EtS hydrolysis were obtained from corresponding Lineweaver-Burk plots. Regression lines for all plots were drawn according to a least-squares fit.
INHIBITION OF ESTRONE SULFATASE ACTIVITY

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

Metabolism of [³H]EI-3-MTP. The metabolism of [³H]EI-3-MTP to [³H]estrone by placental and rat liver 2000 x g supernatants is shown in Fig. 5 along with that of [³H]EI-S for comparison. While [³H]EI-S is hydrolyzed to [³H]estrone to a large extent by both tissues (50-60%), [³H]EI-3-MTP is resistant to hydrolysis to form [³H]estrone (<2.5% [³H]EI 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.

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Fig. 3. A, Lineweaver-Burk plot for the inhibition of placental estrone sulfatase activity with EI-3-MTP. EI-S (2-50 μM) was incubated with a placental preparation (125 μg protein) in the presence of EI-3-MTP (0 (C), 2 (A), 5 (O), 10 (O), 20 (.), or 40 (I) μM) for 20 min at 37°C in a final incubation volume of 1 ml. V_max 148 pmol/min/mg protein. In B, to obtain apparent Kᵢ (for EI-3-MTP), the slopes of the lines from A were plotted as a function of the concentration of EI-3-MTP. Kᵢ 14.6 μM.

Fig. 4. A, Inhibition of breast tumor estrone sulfatase activity. The assay conditions were essentially the same as those described for Fig. 3A for the inhibition of placental estrone sulfatase activity except that breast tumor preparation was used at a concentration of 200 μg protein and the incubation time was 90 min. EI-3-MTP was again used at 0 (C), 2 (A), 5 (.), 10 (O), 20 (.), or 40 (I) μM. V_max 38 pmol/min/mg protein. In B, Apparent Kᵢ for EI-3-MTP was obtained from a plot of the slopes of lines from A as a function of the concentration of EI-3-MTP. Kᵢ 32.8 μM.

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to inhibit estrone sulfatase in vivo. In contrast, steroid sulfatase inhibitors, such as E1-3-MTP, which are not alternative substrates and therefore resistant to sulfatase action, should inhibit the in vivo hydrolysis of E1S.

The problem associated with the instability of steroid sulfate conjugates in vivo had previously been examined in relation to the development of antisera to estrone sulfate. Such antisera were required to allow the direct measurement of estrone sulfate in plasma, in an attempt to eliminate the need for enzyme hydrolysis and solvent partition procedures. Early attempts to raise antisera to estrone sulfate were unsuccessful due to the removal of the sulfate moiety from estrone in vivo. To overcome this, Cox et al. (12) developed an alternative approach to the problem of the instability of sulfate derivatives. Cox et al. (12) linked two phosphorus-containing derivatives of estrone, one of which was E1-3-MTP, to bovine serum albumin, to act as structural mimics of sulfate esters. The reasoning underlying such an approach is that phosphorus-containing compounds would be more stable in vivo than sulfate derivatives and yet closely resemble sulfates in group size and charge. The use of an E1-3-MTP-bovine serum albumin hapten led to the successful production of antisera directly reactive with estrone sulfate.

We reasoned that E1-3-MTP might also inhibit estrone sulfatase activity. In the present study, we have shown that E1-3-MTP indeed acts as a potent inhibitor of estrone sulfatase activity in MCF-7 breast cancer cells and in placental and breast tumor cytosol preparations. Although it proved possible to synthesize E1-3-MTP according to the method as originally described by Cox et al. (12), we have developed a novel pathway for its synthesis which has overcome a number of problems associated with their method. In particular, the intermediate neutral thiophosphonate diester (compound 6) allows ready purification and, on deprotection of the 2-cyanoethyl group by base a volatile by-product, is produced to give the corresponding salt 10.

The ability of E1-3-MTP to inhibit estrone sulfatase activity was examined using MCF-7 breast cancer cells and placental and breast tumor cytosol preparations. Values for the $K_a$ and $V_{max}$ for E1S in these systems are in good agreement with values previously reported (21). Our results show that, using MCF-7 breast cancer cells, E1-3-MTP is a more efficient inhibitor of estrone sulfatase than danazol, which has previously been found to inhibit the activity of this enzyme complex (22). With a breast tumor cytosol, naturally occurring steroid sulfates (e.g., 5-androstenediol-3-sulfate) and unconjugated steroids (e.g., 5a-androstanediol) have been reported to have $K_i$ values of 2.0 and 40 μM, respectively (20). The $K_i$ value for E1-3-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 in vivo 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 mammary tumor inhibitory 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 estrone 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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