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Inhibition of Steroid Sulphatase Activity by Steroidal Methylthiophosphonates: Potential Therapeutic Agents in Breast Cancer

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The hydrolysis of steroid sulphates, by steroid sulphatase, is an important source of oestrogenic steroids (oestrone, oestradiol and 5-androstene-3β,17β-diol) which are found in tumours. In the present study, we have examined the effect of dehydroepiandrosterone-3-*O*-methylthiophosphonate (DHA-3-MTP), pregnenolone-3-*O*-methylthiophosphonate (pregnenolone-3-MTP) and cholesterol-3-*O*-methylthiophosphonate (cholesterol-3-MTP) on the inhibition of oestrone sulphatase as well as DHA sulphatase activities in intact MCF-7 breast cancer cells and in placental microsomes. All three methylthiophosphonates significantly ($P < 0.01$) inhibited the hydrolysis of oestrone sulphate (E_1S) in intact MCF-7 cells (31–85% inhibition at 1 μM and 53–97% inhibition at 10 μM). Significant inhibition of DHA sulphatase was also achieved. At a concentration of 50 μM, all three compounds inhibited the hydrolysis of dehydroepiandrosterone sulphate (DHAS) by >95%. Using human placental microsomes, the K_m and V_{max} of E_1S were determined to be 8.1 μM and 43 nmol/h/mg protein. The corresponding K_i values for DHA-3-MTP, pregnenolone-3-MTP and cholesterol-3-MTP were found to be 4.5, 1.4 and 6.2 μM, respectively. Such inhibitors which are resistant to metabolism may have considerable potential as therapeutic agents and may have additional advantage over aromatase inhibitors in also reducing tumour concentrations of the oestrogenic steroid, 5-androstene-3β,17β-diol, by inhibiting the hydrolysis of DHAS.

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INTRODUCTION

Until recently, steroid sulphates were thought to represent the inactive end products of steroid metabolism. It is now generally accepted that sulphate conjugates of steroids are important intermediates in the synthesis and action of steroid hormones [1–3]. Many breast tumours are hormone dependent and the potential role that steroid sulphates, in particular oestrone sulphate (E_1S) and dehydroepiandrosterone sulphate (DHAS), may have in supporting the growth of such tumours is increasingly becoming evident. Serum levels of E_1S and DHAS at 1 nM and 1 μM, respectively, are much higher than those of the unconjugated forms of these steroids [4, 5]. Significant concentrations of E_1S and DHAS have also been detected in breast tumours [5, 6]. In addition, extremely high concentrations of steroid sulphates are present in breast cyst fluid collected from women with gross cystic breast disease, a condition which may be associated with an increased risk for the development of breast cancer [7]. The half-life of steroid sulphates is much longer (7.5 h) than that of the unconjugated steroids (30 min), thus making steroid sulphates a potential circulating reservoir for the formation of active steroid hormones [8, 9]. *In vitro* steroid sulphatase activity is 1000 times or more higher than aromatase activity in most breast tissues [10, 11]. Together, these findings suggest that steroid sulphatase may have a key role in regulating the *in situ* formation of the oestrogenic steroids oestrone (E_1), oestradiol (E_2) and 5-androstene-3β,17β-diol (ADIOL) from existing sulphated precursors.

To reduce tissue exposure to oestrogen, efforts have been mainly concentrated in the development of specific inhibitors of the aromatase enzyme. While compounds such as aminoglutethimide and 4-hydroxyandrostenedione greatly reduce peripheral aromatase activity, plasma E_1 and E_1S concentrations are only

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reduced by about 50% [12, 13]. Recent interest in the development of steroid sulphatase inhibitors has also arisen because (i) aromatase inhibitors act distal to the hydrolysis of DHAS; hence, such inhibitors would not be expected to reduce levels of the oestrogenic steroid, ADIOL, and (ii) steroid sulphates of E_1 and DHA have been shown to be biologically active *in vitro* as well as *in vivo*. Using MCF-7 breast cancer cells which possess steroid sulphatase activity, Vignon *et al.* [2] demonstrated that E_1S stimulated the synthesis of well characterized oestrogen-induced proteins. Recently, Santner *et al.* [14] confirmed these findings and reported that physiological concentrations (0.1 nM) of E_1S stimulated cell proliferation. Using the NMU-induced mammary cancer model in rats, Santner *et al.* [15] showed that administration of E_1S resulted in a dose-dependent stimulation of the NMU-induced tumour, suggesting that E_1S is biologically active *in vivo*, presumably after hydrolysis of E_1 catalyzed by steroid sulphatase. Similarly, the oestrogenic effects of physiological concentrations of ADIOL, which is produced from DHAS via DHA, has been shown *in vitro* in MCF-7 human breast cancer cells [16] as well as *in vivo* in the DMBA-induced rat mammary tumours [17].

Previously we have reported the ability of oestrone-3-*O*-methylthiophosphonate (E_1-3-MTP) to efficiently inhibit the hydrolysis of E_1S in MCF-7 breast cancer cells and in placental and breast tumour preparations [18, 19]. In the present study, we report the synthesis and potent steroid sulphatase inhibitory activity of the 3-*O*-methylthiophosphonates of DHA, pregnenolone and cholesterol.

EXPERIMENTAL

Chemical and reagents

[6,7-^{3}H]E_1S (sp. act. 48 Ci/mmol), [7-^{3}H]DHAS (sp. act. 48 Ci/mmol), [4-^{14}C]E_1 (sp. act. 52 mCi/mmol) and [4-^{14}C]DHA (sp. act. 51 mCi/mmol) were purchased from NEN-Dupont (Germany). Unlabelled steroids were purchased from Sigma Chemical Co. (Poole, England). Non-polar contaminants were removed from [^{3}H]E_1S and [^{3}H]DHAS before use by extraction with toluene. All organic solvents were of A.R. grade and were supplied by BDH Ltd (U.K.). Falcon 25 cm^2 tissue culture flasks were bought from Marathon Ltd (U.K.). Reagents for cell culture were purchased from Flow Labs (Irvine, Scotland). The ER + ve MCF-7 human breast cancer cell line was a gift from Dr M. Lippman (Georgetown University, Washington, U.S.A.). This cell line was maintained in culture free of mycoplasma contamination and was routinely tested for mycoplasms (GenProbe Inc., U.S.A.).

^{1}H, ^{13}C, ^{31}P NMR spectra were run on a JEOL FX90Q and GX270 NMR spectrometers. ^{31}P resonances were referenced to external 85% H_3PO_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 of DHA-, pregnenolone- and cholesterol-3-MTP

Steroid methylthiophosphonates were essentially synthesized as described previously for E_1-3-MTP [18, 19]. Briefly, an excess of methanethiophosphonic dichloride was added dropwise to a solution of dried steroid in anhydrous pyridine at 0°C under nitrogen. The reaction was followed by TLC (ethyl acetate) and after it had gone to completion (*ca* 24 h) the intermediate steroid methylthiophosphonochloridate was quenched with an excess of dried 2-cyanoethanol. After warming to room temperature overnight the reaction mixture was poured into water and the product was extracted with ethyl acetate, dried and purified by flash column chromatography. The neutral methylthiophosphonate diester was then treated with aqueous ammonia at 65°C to remove the cyanoethyl group, the solvent evaporated and the product decolourized using activated charcoal to afford the ammonium salt of the steroid 3-*O*-methylthiophosphonate. All compounds exhibited satisfactory spectroscopic data in accord with their structure. Full details will be published elsewhere.

Cell culture

MCF-7 human breast cancer cells were maintained in Minimal Essential Medium (MEM) containing 20 mM HEPES, 5% fetal bovine serum, 2 mM glutamine, non-essential amino acids and 10 mM sodium bicarbonate. For experiments, up to 40 replicate 25 cm^2 tissue culture flasks were seeded with about 1×10^5 cells/flask. For testing the effect of DHA-3-MTP, pregnenolone-3-MTP and cholesterol-3-MTP on *in vitro* steroid sulphatase activity, the cells were allowed to grow to 80% confluency. The medium was changed on every third day.

In vitro E_1 sulphatase assay on cell monolayers

Intact monolayers of MCF-7 cells in triplicate 25 cm^2 tissue culture flasks were incubated for 20 h at 37°C with [^{3}H]E_1S (5 pmol, 7×10^5 dpm) in serum-free MEM (2.5 ml) with or without steroidal 3-*O*-methylthiophosphonates (2.5–25 nmol). Products were extracted from the medium (1 ml) with toluene (5 ml). [^{14}C]E_1 (7×10^3 dpm) was used to monitor procedural losses. The ^{3}H and ^{14}C content in the toluene was determined by scintillation spectrometry. Each batch of experiments included flasks without cells (to assess apparent nonenzymic hydrolysis of the substrate). The number of cell nuclei per flask was determined using a Coulter Counter after treating the cell monolayers with Zaponin. Unless otherwise stated, the results are expressed as the means ± SD of the total product

$(E_1 + E_2)$ formed during the incubation period (20 h) and calculated for 10^6 cells.

Unpaired Student's t test was used to test the statistical significance of our findings. One flask in each batch was used to assess cell membrane status and viability using the trypan blue exclusion method.

In vitro DHA sulphatase assay on cell monolayers

The method used was as described above for E_1 sulphatase except [^{3}H]DHAS (1 nmol, 7×10^5 dpm, 2.5 ml) was used as substrate and [^{14}C]DHA (7×10^3 dpm) was used for assessing procedural losses. Inhibitor concentrations varied from 1–50 μM.

Tissue preparation

Microsomes from a sulphatase-positive human placenta from a normal term pregnancy were prepared as described previously [20]. Protein concentration was determined by the method of Lowry *et al.* [21].

Inhibition of E_1 sulphatase activity in placental microsomes by steroidal 3-O-methylthiophosphonates

Incubations (1 ml) were carried out using a protein concentration of 200 μg/ml, four substrate concentrations varying from 2–20 μM, and an incubation time of 30 min. Each of the four substrate concentrations was tested at inhibitor concentrations of 0, 10 and 50 μM. Regression lines for the corresponding Lineweaver–Burk plot were drawn according to a least-squares fit.

RESULTS

Steroid sulphatase activity in MCF-7 human breast cancer cells has been well characterized by us [22] and others [23]. Apparent non-enzymic hydrolysis of the substrates (blanks) was found to be <0.01%. At the end of the incubation period, 95–98% of the cells were found to be viable by the trypan blue exclusion method.

Dose-dependent inhibition of E_1 sulphatase activity in intact MCF-7 cells by steroidal 3-O-methylthiophosphonates

3-*O*-Methylthiophosphonates of all three steroids (DHA, pregnenolone and cholesterol) inhibited E_1 sulphatase activity *in vitro* in MCF-7 breast cancer cells in a dose-dependent manner (Fig. 1). Significant ($P < 0.001$) inhibition of E_1 sulphatase was obtained at a concentration of 1 μM of DHA-3-MTP, pregnenolone-3-MTP and cholesterol-3-MTP (85, 51 and 31% inhibition, respectively). At 10 μM, the compounds inhibited the hydrolysis of E_1S by 97, 89 and 53%, respectively.

Dose-dependent inhibition of DHA sulphatase activity in intact MCF-7 cells by steroidal 3-O-methylthiophosphonates

Hydrolysis of DHAS to DHA was inhibited in dose-dependent manner by all three compounds (Fig. 2). Significant inhibition of DHA sulphatase activity was obtained at a concentration of 1 μM of the three methylthiophosphonate. At this concentration, cholesterol-3-MTP was the weakest inhibitor (28% inhibition) whereas DHA-3-MTP and pregnenolone-3-MTP produced 77 and 54% inhibition, respectively. At a concentration of 50 μM, all three compounds inhibited the hydrolysis of DHAS by >95%.

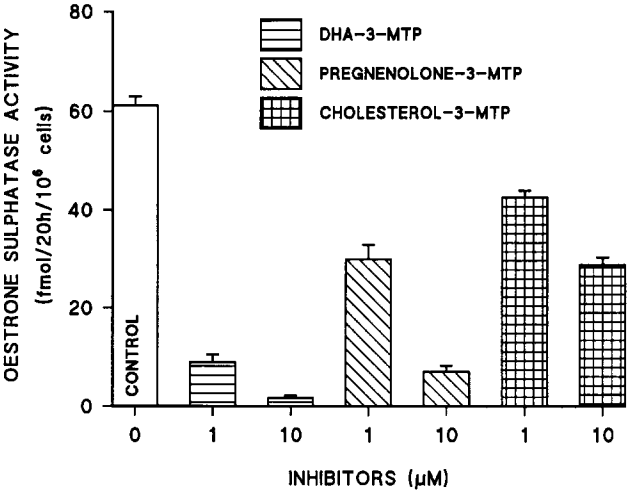

Fig. 1. Concentration-dependent inhibition of E_1 sulphatase activity in intact MCF-7 cells. Monolayers of MCF-7 cells in 25 cm^2 flasks were incubated for 20 h at 37°C with [^{3}H]E_1S (2 nM) and steroid methylthiophosphonates at 1 and 10 μM. E_1 sulphatase activity was determined by measuring the total amount of tritium-labelled E_1 and E_2 formed. *Error bars*, SD of triplicate determinations; $n = 3$.

Inhibition of placental microsomal E_1 sulphatase activity

The extent of hydrolysis of E_1S by placental microsomes was directly proportional to incubation time,

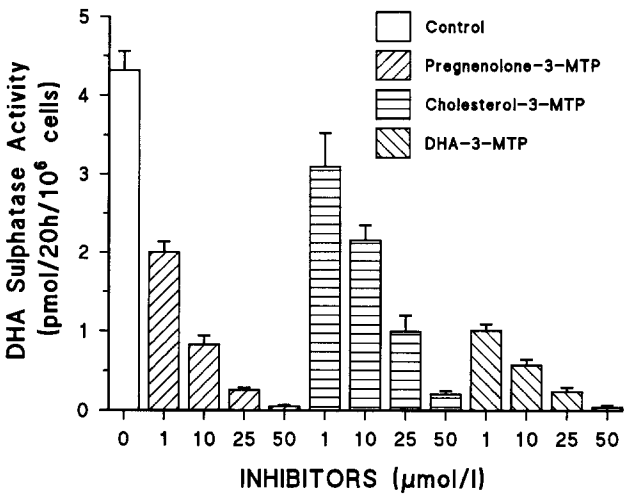

Fig. 2. Concentration-dependent inhibition of DHA sulphatase activity in intact MCF-7 cells. Monolayers of MCF-7 cells in 25 cm^2 flasks were incubated for 20 h at 37°C with [^{3}H]DHAS (1 μM) and inhibitors at varying concentrations of 1 to 50 μM. DHA sulphatase activity was determined by measuring the total amount of tritium-labelled unconjugated products formed. *Error bars*, SD of triplate determinations; $n = 3$.

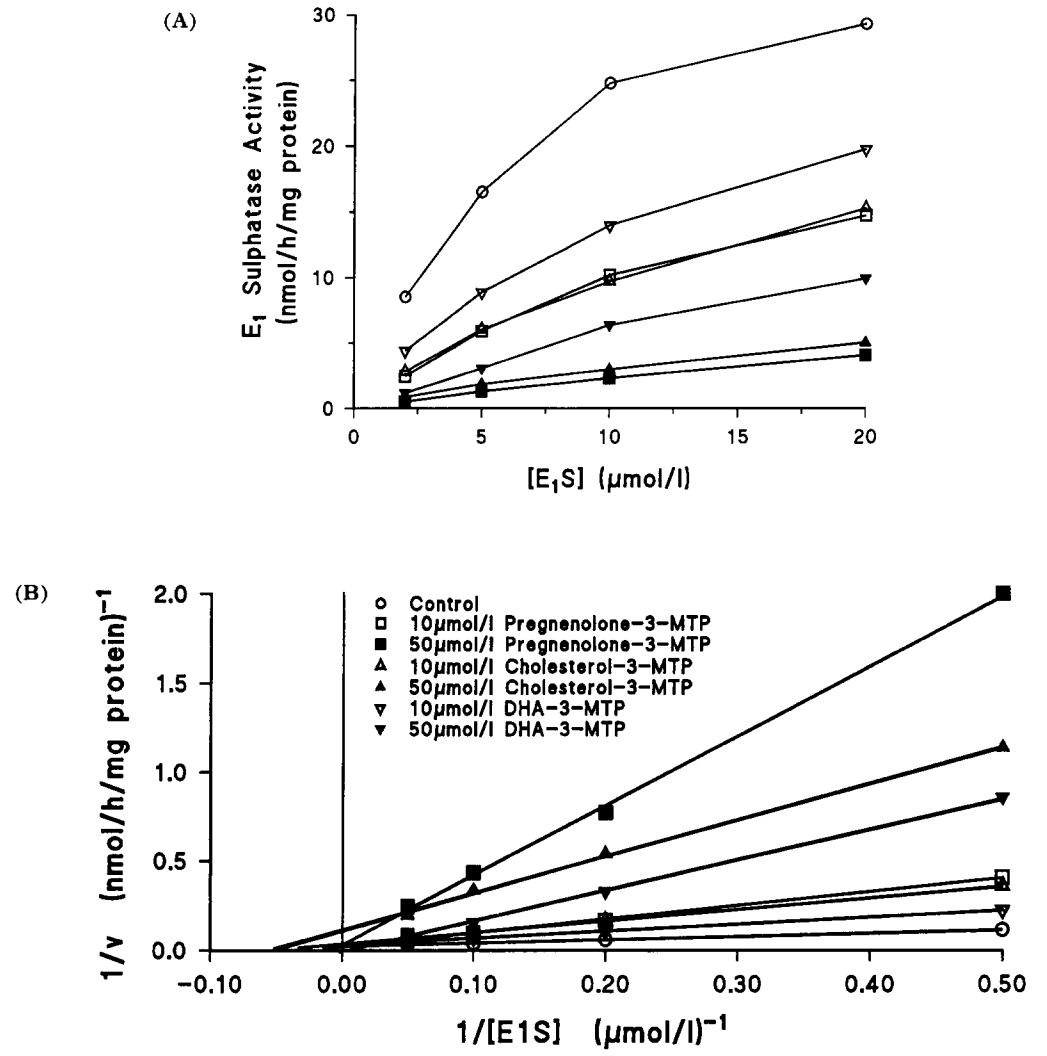

Fig. 3. (A) Inhibition of placental microsomal E_1 sulphatase activity with 3-methylthiophosphonate derivatives of DHA, pregnenolone and cholesterol. E_1S (2–20 μM) was incubated with placental microsomes (200 μg protein) in the presence and absence of DHA-3-MTP, pregnenolone-3-MTP and cholesterol-3-MTP (10 and 50 μM) for 30 min at 37°C. Symbols are described in (B). (B) Lineweaver–Burk plot of data from (A) for the inhibition of placental E_1 sulphatase activity. $K_m = 8.1$ μM, $V_{max} = 43$ nmol/h/mg protein.

protein concentration and substrate concentration within the limits assessed (data not shown). Apparent K_m and V_{max} values for E_1S determined using the Lineweaver–Burk plot were 8.1 μM and 43 nmol/h/mg protein (Fig. 3B). The inhibition plots (Figs 3A and 3B) suggested competitive inhibition by the three steroidal 3-*O*-methylthiophosphonates. The K_i values for each of the three compounds, obtained from a plot of the slope of the Lineweaver–Burk plot as a function of inhibitor concentration were 4.5 μM for DHA-3-MTP, 1.4 μM for pregnenolone-3-MTP and 6.2 μM for cholesterol-3-MTP.

DISCUSSION

The central role that E_1S and E_1 sulphatase may have in regulating the supply of E_1 to breast tumours has been suggested by several studies [6, 11]. Although E_1 and DHA sulphatase activities are considerably higher than aromatase activity in breast tumours, the development of inhibitors of steroid sulphatase activity has attracted little attention [24]. Because such an inhibitor would be of potential value as a therapeutic agent, to add to the endocrine therapies available for the treatment of hormone-dependent cancers of the breast, endometrium and prostate, we recently reported the potent inhibitory effect of E_1-3-MTP on E_1 sulphatase [18, 19]. The present study extends the inhibitory profile of steroidal 3-*O*-methylthiophosphonate. MTP derivatives of DHA, pregnenolone and cholesterol all show inhibition of E_1 sulphatase as well as DHA sulphatase. In intact MCF-7 breast cancer cells, the order of potency is DHA-3-MTP > E_1-3-MTP > pregnenolone-3-MTP > cholesterol-3-MTP. The K_i values are in the same order of magnitude and comparable to that of E_1-3-MTP [18]. Whether there is one sulphatase hydrolysing the different steroid sulphates or separate sulphatase enzymes under common genetic control is controversial [25]. The fact that both pregnenolone- and cholesterol-3-MTP are

successful in inhibiting the hydrolysis of E_1S and DHAS, suggests a single sulphatase enzyme common to all four substrates.

Although the stability of steroid-3-*O*-methylthiophosphonates *in vivo* needs to be established, these compounds are much more resistant to metabolism than naturally occurring steroid sulphates such as E_1S [18]. Furthermore, in contrast to naturally occurring steroid sulphates, sulphatase inhibitors such as steroid-3-*O*-methylthiophosphonates, which are not alternative substrates and therefore resistant to sulphatase action, should inhibit the hydrolysis of E_1S and DHAS *in vivo*.

The importance of inhibiting the hydrolysis of DHAS has not been adequately emphasized. Action of sulphatase on DHAS results in the formation of the oestrogenic steroid, ADIOL, via DHA. ADIOL can compete with E_2 for the cytoplasmic receptor and can invoke a biological response characteristic to oestrogen, i.e. increase in uterine weight and in progesterone receptors [16]. ADIOL and DHA are potent stimuli *in vivo* of DMBA-induced rat mammary tumour growth [17]. The levels of DHA and ADIOL are higher in breast tumours than in normal breast tissue [3]. Hence, inhibition of DHA sulphatase activity is vital in limiting this source of an oestrogenic stimulus. Whereas specific aromatase inhibitors available for the treatment of breast cancer fail to reduce the levels of ADIOL [17], we have demonstrated that steroidal methylthiophosphonates are potent inhibitors of DHA sulphatase activity *in vitro*. They should therefore be of further therapeutic value by reducing ADIOL levels. Evaluation of these inhibitors *in vivo* as well as the synthesis of analogues of steroid 3-*O*-methylthiophosphonates is in progress.

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