The development of steroid sulphatase inhibitors

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Introduction

Steroid sulphatases regulate many important physiological processes, including the formation of neurosteroids, some aspects of reproductive function and part of the immune response. Dehydroepiandrosterone sulphatase in macrophages regulates the progression of T₈ helper cells to the Th1 phenotype (Daynes et al. 1993, Rook et al. 1994) which secrete cytokines which may be involved in the development of some autoimmune diseases. However, their pivotal role in regulating oestrogen synthesis in endocrine-dependent tumours has been the greatest stimulus in developing potent steroid sulphatase inhibitors. Carlstrom et al. (1984a) made the initial observation that danazol, an isoxazole derivative of 17α-ethinyl testosterone, possessed steroid sulphatase inhibitory properties when it was found that the ratio of dehydroepiandrosterone sulphate (DHEA-S) to unconjugated DHEA increased in women treated with this drug for endometriosis. Subsequent in vitro studies confirmed the ability of danazol to inhibit DHEA sulphatase activity in breast tissues (Carlstrom et al. 1984b). Since this finding, several research groups have set out to synthesize and develop potent steroid sulphatase inhibitors. In this article, we review the rationale which led to the development of steroid sulphatase inhibitors and detail the rapid progress that has been made in this field.

Background to the development of steroid sulphatase inhibitors

It is now generally accepted that the in situ formation of oestrogens in breast tumours, and also possibly in endometrial tumours, makes a major contribution to the high oestrogen concentrations found in these tissues (Naitoh et al. 1989, Reed et al. 1989). Concentrations of the biologically active oestrogen oestradiol are higher than those of oestrone in tumours, and levels of both oestrogens in tumours are considerably higher than the low plasma oestrogen concentrations that are found in postmenopausal women (Bonney et al. 1983, Van Landeghem et al. 1985). Although breast and endometrial cancer occur in premenopausal women, the highest incidence is in postmenopausal women, at a time when ovarian production of oestrogen has ceased. Oestrogens continue to be produced in postmenopausal women and, while adipose tissue is a major site of peripheral oestrogen synthesis, all the enzymes which are required for oestrogen formation from androgen precursors are present in breast tumours (James et al. 1987).

The enzymes that are involved in breast tumour oestrogen synthesis are shown in Fig. 1. The enzymes are aromatase, which converts androstenedione to oestrone (Fig. 2(1)), and oestradiol 17β-hydroxy-steroid dehydrogenase, which in breast tumours converts oestrone to oestradiol (McNeil et al. 1986, Luu-The et al. 1989). Much of the oestrone formed from androstenedione is metabolised to oestrone sulphate (Fig. 2(2)) by oestrone sulphotransferase (Hobkirik 1993), and can be converted back to oestrone by the action of oestrone sulphatase.

Plasma concentrations of oestrone sulphate are 10-20 times higher than those of the unconjugated oestrogens (Noel et al. 1981), and the half-life of oestrone sulphate in blood (10-12 h) is considerably longer than that of unconjugated oestrogens (20 min) (Ruder et al. 1972). Furthermore, the sulphatase pathway is thought to be the major route for breast tumour oestrogen synthesis, with at least 10 times as much oestrone in tumours originating via this route.
than is formed from androstenedione (Santner et al. 1984).

The possibility that oestrone sulphate may act as a reservoir for oestrone synthesis in postmenopausal women was first suggested by studies using aromatase inhibitors for the therapeutic treatment of women with breast cancer. These investigations revealed that while inhibitors such as aminoglutethimide or 4-hydroxy-androstenedione effectively abolished peripheral aromatase activity, plasma oestrone and oestradiol concentrations unexpectedly only decreased by 40-50% (Dowsett et al. 1989, Reed et al. 1990). Plasma oestrone sulphate concentrations remain relatively high during treatment with aromatase inhibitors (Johnston et al. 1994, Svenstrup et al. 1994), and it was reasoned that conversion of oestrone sulphate to oestrone and oestradiol was the likely source of the unconjugated oestrogens which were still detectable in the plasma of women receiving aromatase inhibitor therapy (Reed & Purohit 1993). The results from these clinical studies prompted the synthesis of the first steroid sulphatase inhibitor, oestrone-3-O-methylthiophosphonate (E1-MTP, Fig. 2(3)), which was specifically developed as a potential sulphatase inhibitor (Duncan et al. 1993).

**DHEA sulphatase regulates the synthesis of an additional potent oestrogenic hormone, androstenediol**

Another important reason for attempting to develop a potent steroid sulphatase inhibitor emerged from the knowledge that, while aromatase inhibitors were designed to reduce production of oestrone, they had no effect on the synthesis of androstenediol, another major hormone in postmenopausal women which also has oestrogenic properties. This hormone, although an androgen, can bind to the oestrogen receptor (ER) and can stimulate the growth of ER-positive breast cancer cells (Adams et al. 1981, Poulin & Labrie 1986). Androstenediol also stimulates the growth of dimethylbenzanthracene-induced mammary tumours in the rat, and is able to stimulate tumour growth in the presence of an aromatase inhibitor, showing that it does not have to undergo conversion to an oestrogen in order to be able to stimulate tumour growth (Dauvois & Labrie 1989).

The origin of androstenediol has been investigated using isotopic infusion techniques, similar to those used to measure in vivo peripheral aromatase activity (Poortman et al. 1980). More than 90% of the androstenediol produced in postmenopausal women was found to originate from DHEA-S. DHEA-S can either be converted to DHEA by DHEA sulphatase, with subsequent metabolism of DHEA to androstenediol, or converted to androstenediol sulphate, with subsequent hydrolysis to androstenediol (Fig. 1). Although the affinity of androstenediol for the ER is somewhat lower than that of oestradiol, plasma androstenediol concentrations are 100-fold higher than those of oestradiol. A significant correlation was also found between plasma DHEA-S and androstenediol concentrations in postmenopausal women, adding further evidence for DHEA-S being the major source of plasma androstenediol (Bonney et al. 1984).
Are oestrone sulphatase and DHEA sulphatase the same or different enzymes?

The ability to inhibit the hydrolysis of oestrone sulphate and DHEA-S could, therefore, make an important contribution to the therapies available to reduce the production of steroids with oestrogenic properties in women with breast cancer. However, whether the hydrolysis of these steroid conjugates is mediated by the same or different enzymes remains controversial (Reed & Purohit 1994). Mammalian aryl sulphatases are classified into three types, A, B and C, with the microsomal aryl sulphatase C (steryl sulphate sulphohydrolase, EC 3.1.6.2) being responsible for the hydrolysis of 3β-hydroxysteroids. This enzyme has been purified using several different solubilization and isolation procedures, and there is evidence from these investigations for the existence of distinct (Gniot-Szulzycka & Januszewska 1986) or identical enzymes (Burns 1983, Dibbelt & Kuss 1986) being responsible for the hydrolysis of oestrone sulphate and DHEA-S.

To further examine this controversial problem, placental steroid sulphatase cDNA was transiently transfected into COS-1 cells. The expressed enzyme was capable of hydrolysing both oestrone sulphate and DHEA-S, providing evidence for only one enzyme being responsible for the hydrolysis of both alkyl and aryl steroid sulphates (Purohit et al. 1994). Furthermore, it was possible to inhibit the hydrolysis of both oestrone sulphate and DHEA-S using a sulphatase inhibitor. These results demonstrate that it should be possible to block the synthesis of both oestrone and androstenediol using a steroid sulphatase inhibitor.

Steroid sulphatase activity in normal and malignant breast and endometrial tissues

Steroid sulphatase is found in most body tissues, and in an examination of its distribution in the rhesus monkey (Martel et al. 1994) was detected in every tissue examined except the salivary gland. The distribution of DHEA and oestrone sulphatase activities was similar, lending further support to the existence of only one enzyme which is responsible for both activities.

Several studies have now confirmed that oestrone sulphatase activity is significantly higher in malignant breast tissue than in normal breast tissue (Tseng et al. 1983, James et al. 1987, Naitoh et al. 1989, Utsumi 1989). The activity of oestrone sulphotransferase, which also has the potential to modulate tissue oestrogen availability (by converting oestrogens to their sulphated derivatives) has also been examined in breast tissues. Oestrone sulphotransferase, while detectable in malignant breast tissues, was 5-10 times less active than oestrone sulphatase (Tseng et al. 1983).

Increased oestrone sulphatase activity in endometrial cancer tissue has also been detected (Yamamoto et al. 1993). In normal endometrial tissue, oestrone sulphatase activity was detectable, but at a relatively low level (<8 nmol/mg protein per h), whereas in malignant tissue activity of up to 35 nmol/mg protein per h was found. As found in breast tumour tissue, oestrone sulphotransferase activity was considerably lower than sulphatase activity in malignant endometrial tissue.

Both oestrone sulphatase and DHEA sulphatase activities have been detected in prostatic tissue (Bartsch et al. 1990). As ERs, in addition to androgen receptors, have been detected in prostatic tissue, it is possible that steroids such as androstenediol could also have an important role in regulating the proliferation of prostatic tissue.

The activities of the aromatase, dehydrogenase and oestrone sulphatase are all increased in breast tumour tissue compared with their activities in normal breast tissues (James et al. 1987). Research to identify factors present in or produced by breast tumours which may account for the increased enzyme activities detected in breast tumours has attracted considerable interest. It has now emerged that a number of cytokines, in particular interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα), have major roles in regulating oestrogen synthesis in breast tumours (Reed et al. 1993, Macdiarmid et al. 1994, Reed et al. 1995). IL-6 and TNFα can act synergistically to increase oestrone sulphatase activity; TNFα probably increases the expression of the gp130 signal-transducing component of the IL-6 receptor complex (Snyers & Content 1992). IL-6 is produced by breast tumour-derived fibroblasts (Duncan et al. 1994), while adipocytes are a major source of the TNFα produced within the body (Hotamisligil et al. 1995). In addition to
Figure 2 Structures of steroids and steroid sulphotase inhibitors.
production of cytokines by these cells, however, it is now apparent that infiltration of breast tumours by macrophages and lymphocytes is an additional important source of cytokine production (Purohit et al. 1995a).

Endocrine drugs as potential steroid sulphatase inhibitors

As previously discussed, danazol was the first drug which, in addition to its other properties, was also found to inhibit steroid sulphatase (Carlstrom et al. 1984a). In a preliminary study the effect of danazol therapy (800 mg/day for 2 weeks) on the conversion ratio (CR) of oestrone sulphate to oestrone was investigated in three menopausal women with breast cancer (Purohit et al. 1992). For two of the three women, a 40% decrease in the CR was detected, while no change occurred in the third subject. Sulphatase activity in normal breast tissue obtained from these women decreased by 24-64% and there was a 31% decrease in plasma oestrone concentrations. From this investigation it was concluded that, while danazol could inhibit steroid sulphatase activity in vivo, it was a relatively weak inhibitor.

The ability of danazol to inhibit oestrone sulphatase activity has also been examined using breast cancer cell lines which possess oestrone sulphatase activity. Using cell homogenates, danazol (10 µM) inhibited oestrone sulphatase activity (measured using a substrate concentration of 8 µM) in MCF-7 cells by 38%, in MDA-MB-231 cells by 36% and in T47D cells by 27% (Nguyen et al. 1993). In intact MCF-7 breast cancer cells, using a physiological substrate concentration (2 nM), danazol (10 µM) inhibited sulphatase activity by 62% (Purohit & Reed 1992).

In addition to danazol, a number of other steroidal and non-steroidal drugs, which are used therapeutically, have also been examined for their potential as steroid sulphatase inhibitors. Pasqualini et al. (1989) reported that antioestrogens could decrease the concentration of oestradiol in breast cancer cells, after incubation with oestrone sulphate, suggesting that such compounds may also act as sulphatase inhibitors. In intact MCF-7 breast cancer cells, tamoxifen has also been found to exert a small stimulatory effect on oestrone sulphatase activity, while the major tamoxifen metabolite 4-hydroxy-tamoxifen increased sulphatase activity by 52% (Purohit & Reed 1992).

Using a placental microsome preparation as a source of steroid sulphatase activity, Evans et al. (1991) failed to detect any significant sulphatase inhibition with tamoxifen when tested at concentrations of up to 50 µM. In contrast to this result, but using rat mammary tumour tissue, some evidence for antioestrogens acting as sulphatase inhibitors was obtained by Santner & Santen (1993). Tamoxifen, 4-hydroxytamoxifen and the ‘pure’ antioestrogen ICI 168,384 were all found to inhibit sulphatase activity, but with relatively high $K_i$ values of 1130 µM, 290 µM and 11 µM respectively. Using a human tumour preparation, 4-hydroxytamoxifen was still found to inhibit steroid sulphatase activity ($K_i$ 1320 µM), but tamoxifen and the ‘pure’ antioestrogen did not inhibit sulphatase activity when tested at concentrations of up to 1 mM. If the steroidal and non-steroidal antioestrogens can inhibit oestrone sulphatase activity in vivo, as noted by Santner & Santen (1993), such an effect is likely to be secondary to their ability to bind to the ER and block the action of oestradiol.

In view of the results of a number of investigations suggesting that antioestrogens may possess steroid sulphatase inhibitory properties, a study was carried out to examine their effects in vivo on rat liver steroid sulphatase activity (Purohit et al. 1995c). For this investigation, rats received 4-hydroxytamoxifen or the ‘pure’ antioestrogen ICI 182,780 for 7 days (10 mg/kg s.c.). Treatment with 4-hydroxytamoxifen increased liver sulphatase activity by 43%, whilst ICI 182,780 was without effect. Results from this investigation suggest that while some in vitro studies have found evidence of sulphatase inhibition with antioestrogens when tested at high concentrations, they do not act in vivo to inhibit steroid sulphatase activity. Further support for this conclusion is provided from studies in which plasma oestrogen concentrations have been measured in postmenopausal women receiving tamoxifen therapy. Only relatively minor alterations, or no changes, in plasma oestrogen concentrations have been found in such studies in postmenopausal women (Wilking et al. 1982).

The other group of endocrine drugs to have attracted attention as potential steroid sulphatase inhibitors is the progestogens, which are widely used for oral contraceptive and hormone replacement therapies. In microsomes prepared from human
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breast cancer tissue, demegestone and chlormadinone acetate (both progesterone derivatives) inhibited oestrone sulphatase activity by 25% at 10 µM. In contrast, medroxyprogesterone acetate (MPA; which is also a progesterone derivative) simulated oestrone sulphatase activity by 23% at 10 µM (Prost-Avallet et al. 1991). MPA also stimulated oestrone sulphatase activity in intact MCF-7 breast cancer cells, by 48% at 10 µM (Purohit & Reed 1992). Promegestone (R5020; another progesterone derivative) failed to have any inhibitory effects when tested in a human breast cancer microsome preparation at up to 80 µM (Prost-Avallet et al. 1991). While results from these in vitro studies provide relatively little support for progestogens acting as steroid sulphatase inhibitors, evidence in support of such a potential role was claimed from the finding that oestrone sulphatase activity is significantly lower in normal breast tissues of premenopausal women receiving oral contraceptive therapy than in untreated women (Soderqvist et al. 1994). However, in intact MCF-7 cells, ethinylestradiol (which is widely used as the oestrogenic component for oral contraceptive therapy) markedly (81%) inhibited oestrone sulphatase activity at 10 µM (Purohit et al. 1992). It is not yet clear, therefore, whether the reduced oestrone sulphatase activity found in women receiving oral contraceptive therapy results from the oestrogenic or progestational component of such therapy.

Promegestone has recently been reported to decrease oestrone sulphatase mRNA expression significantly in ER-positive and -negative breast cancer cell lines, and also to inhibit its activity in these cells (Pasqualini et al. 1994). A proposed Phase I trial to examine the ability of promegestone to inhibit steroid sulphatase activity in vivo should soon resolve whether such progestogens will have an important role as steroid sulphatase inhibitors in the treatment of women with breast cancer. However, in rats, MPA (like 4-hydroxytamoxifen) was found to increase liver oestrone sulphatase activity significantly (Purohit et al. 1995c). Such results show that there is a need for new specific steroid sulphatase inhibitors for this form of therapy, rather than utilizing any relatively weak inhibitory properties that progestogens may have.

Development of steroid-based sulphatase inhibitors

E1-MTP (Fig. 2(3))

While evidence had accumulated to suggest that some natural steroid sulphates could inhibit steroid sulphatase activity (Reed & Purohit 1994), it was evident that if sulphatase inhibitors were to have a

<table>
<thead>
<tr>
<th>Table 1 Inhibition of oestrone sulphatase activity in MCF-7 cells by various compounds</th>
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<tr>
<td><strong>Compound</strong></td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>E1-3-O-methylsulphonate</td>
</tr>
<tr>
<td>E1-3-O-ethylsulphonate</td>
</tr>
<tr>
<td>E1-3-O-butylsulphonate</td>
</tr>
<tr>
<td>E1-3-O-sulphamate</td>
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<tr>
<td>E2-3-O-sulphamate</td>
</tr>
<tr>
<td>DHEA-3-O-sulphamate</td>
</tr>
<tr>
<td>E1-3-O-H-phosphonate</td>
</tr>
<tr>
<td>E1-3-O-(methylphosphonate)</td>
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<tr>
<td>E1-3-O-(ethylphosphonate)</td>
</tr>
<tr>
<td>E1-3-O-(phenylphosphonate)</td>
</tr>
<tr>
<td>E1-3-O-(phenylphosphoramidate)</td>
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</tbody>
</table>

E1, oestrone; E2, oestradiol; DHEA, dehydroepiandosterone.
(Data from the unpublished observations of N M Howarth, A Purohit, M J Reed & B V L Potter.)
therapeutic role, there was a need for more potent inhibitors. The first steroid-based compound to be specifically synthesized and tested as a potential steroid sulphatase inhibitor was E1-MTP (Fig. 2(3)). This compound was selected as a potential steroid sulphatase inhibitor, since it had previously been shown that it acted as a steroid sulphate structural mimic when used for the generation of antibodies which recognized oestrone sulphate (Cox et al. 1979). This compound was resistant to metabolism in vivo, suggesting that it was not an alternative substrate, and it was therefore reasoned that it might also inhibit oestrone sulphatase activity.

E1-MTP was found to be about 14 times more potent than danazol in its ability to inhibit oestrone sulphatase activity in intact MCF-7 cells, and had a \( K_i \) of 37.5 \( \mu \)M for the inhibition of sulphatase activity in microsomes prepared from breast tumour tissue (Duncan et al. 1993). Incubation of E1-MTP with liver or placental tissues confirmed its resistance to metabolism, whereas at least 50% of oestrone sulphate, incubated in a similar manner, was converted to oestrone.

E1-MTP therefore provided some indication as to the type of steroid derivatives which might be useful as steroid sulphatase inhibitors. Subsequently, a series of related steroid sulphate surrogates were synthesized, and tested for their ability to inhibit sulphatase activity. While most compounds tested had a potency similar to that of E1-MTP in their ability to inhibit oestrone sulphatase activity in MCF-7 cells, one compound, oestrone-3-O-sulphamate (EMATE, Fig. 2(4)), and its \( N \)-methylated derivatives (Fig. 2(5/6)), emerged as being extremely potent (Table 1).

**Figure 3** Irreversible inhibition of placental microsomal oestrone sulphatase activity by oestrone-3-O-sulphamate (EMATE, Fig. 2(4)). In contrast oestrone-3-O-(\( N,N \)-dimethyl)sulphamate (DEMATE, Fig. 2(6)) and oestrone-3-O-methylthiophosphonate (E1-MTP, Fig. 2(3)) did not inhibit sulphatase activity in an irreversible manner (for further details see Purohit et al. 1995b).

**EMATE (Fig. 2(4)) and related compounds**

Using intact MCF-7 cells and a physiological concentration of oestrone sulphate (2 nM) or DHEA-S (1 \( \mu \)M), EMATE (1 nM) inhibited the hydrolysis of both steroid sulphates, by 95% and 82% respectively. The IC\( _{50} \) for the inhibition of oestrone sulphatase by EMATE in MCF-7 cells was 65 pM (Howarth et al. 1994, Purohit et al. 1995b). The \( N \)-monomethylated (Fig. 2(5)) and \( N,N \)-dimethylated derivatives (Fig. 2(6)) of EMATE,
whilst still more potent than E1-MTP, were less potent than EMATE in their abilities to inhibit sulphatase activity.

**Nature of EMATE inhibition**

The ability of EMATE to effectively inhibit oestrone sulphatase activity when tested in the low nanomolar range suggested that it might be an irreversible inhibitor. This was confirmed initially by demonstrating that incubation of monolayers of MCF-7 cells with EMATE for 2 h, with subsequent removal of the medium and extensive washing, prevented restoration of oestrone sulphatase activity (Howarth et al. 1994, Purohit et al. 1995b). In contrast, after identical treatment of cells with E1-MTP, oestrone sulphatase activity was restored after removal of the medium containing the inhibitor. In a further study, placental microsomes were preincubated with EMATE, the N,N-dimethylated analogue of EMATE or E1-MTP for 30 min at 37 °C followed by overnight dialysis at 4 °C. As shown in Fig. 3, reassaying oestrone sulphatase activity indicated no recovery with EMATE, whereas activity was restored almost to control levels with the other inhibitors.

Enzyme kinetic studies subsequently confirmed that EMATE was an irreversible inhibitor when it was shown that EMATE inhibited oestrone sulphatase activity in a time- and concentration-dependent manner (Purohit et al. 1995b). The X-ray crystal structure of EMATE has been reported (Williams et al. 1996).

**Other steroid sulphate surrogates**

A series of steroid sulphatase inhibitors designed from modifications of the enzyme substrate oestrone sulphate has been synthesized and shown to inhibit sulphatase activity in placental microsomes (Li et al. 1993). Of the ten inhibitors in this series, a sulphonyl chloride (Fig. 2(7)) derivative of oestrone was a relatively strong inhibitor, inhibiting oestrone sulphatase activity in placental microsomes by 65% at 60 µM. The series of potential sulphatase inhibitors tested by these researchers has now been extended to include desoxyoestrone-3-sulphonylfluoride (Fig. 2(8)) and desoxyoestrone-3-sulphonylamide (Fig. 2(9)). In this series, the contribution of distinctive sulphate ester groups and related 3-substituents on enzyme inhibition was examined (Dibbelt et al. 1994).

The binding affinity of inhibitors was not found to change markedly if the negatively charged oxygen of the sulphate moiety was replaced by a non-charged electronegative substituent, such as fluorine. The affinity of inhibitors for the enzyme decreased markedly if the oxygen anion was replaced by an amino or methyl group. However, all the inhibitors in this series have a significantly lower binding affinity for the enzyme than the natural substrate, oestrone sulphate; this is most likely due to the deletion of the oxygen between the A-ring and surrogate group.

Of the other group of compounds which Cox et al. (1979) originally employed as oestrone sulphate mimics when attempting to generate antisera which recognised oestrone sulphate, oestrone-3-O-phosphate (Fig. 2(10a)) has also been synthesized and tested for its oestrone sulphatase inhibitory proper-

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**Table 2** IC<sub>50</sub> values for tetrahydronaphthol (THN) and diethylstilboestrol (DES) derivatives as measured in MCF-7 breast cancer cells. Values for oestrone-3-O-sulphamate (EMATE) and oestrone-3-O-methylthiophosphonate (E1-MTP) are included for comparison.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>THN-sulphamate (Fig. 2(15))</td>
<td>1 µM</td>
</tr>
<tr>
<td>THN-N-methylsulphamate (Fig. 2(16))</td>
<td>25 µM</td>
</tr>
<tr>
<td>THN-N,N-dimethylsulphamate (Fig. 2(17))</td>
<td>&gt;50 µM</td>
</tr>
<tr>
<td>DES-bis-sulphamate (Fig. 2(18))</td>
<td>10 nM</td>
</tr>
<tr>
<td>DES-bis-N,N-dimethylsulphamate (Fig. 2(19))</td>
<td>10 µM</td>
</tr>
<tr>
<td>E1-MTP (Fig. 2(3))</td>
<td>100 nM</td>
</tr>
<tr>
<td>EMATE (Fig. 2(4))</td>
<td>65 pM</td>
</tr>
</tbody>
</table>
ties (Anderson et al. 1995, Li et al. 1995, N M Howarth, A Purohit, M J Reed & B V L Potter, unpublished observations). In addition, DHEA-3-O-phosphate (Fig. 2(13), Anderson et al. 1995), oestrone-3-methylsulphonate (Fig. 2(11)) and oestrone-3-methylene sulphonate (Fig. 2(12), Li et al. 1995) have also been tested for their sulphatase inhibitory properties. Oestrone-3-phosphate is a relatively potent steroid sulphatase inhibitor with $K_i$ values of 0.89 µM (Anderson et al. 1995) and 0.3 µM (Li et al. 1995) when measured at pH 7.0. The ability of oestrone-3-O-H-phosphonate (Fig. 2(10b)) to inhibit oestrone sulphatase activity in MCF-7 cells has also been examined; it inhibited activity by 80% at 10 µM (N M Howarth, A Purohit, M J Reed & B V L Potter, unpublished observations). Replacement of the oxygen between the A-ring of the steroid and the sulphate group with a methylene group (Fig. 2(12)) increased the $K_i$ by more than a 100-fold compared with that of oestrone sulphate, indicating that the bridging oxygen atom present in oestrone sulphate is essential for high affinity binding (see also Woo et al. 1996a).

**Development of non-steroidal inhibitors**

Most of the compounds reviewed so far which have been found to possess steroid sulphate inhibitory properties have been derivatives of the oestrone or DHEA steroid nucleus. As the goal of this research is to develop a therapeutic drug for use in women with oestrogen-dependent tumours of the breast or endometrium, it may not be the best strategy to employ an oestrogen derivative as a sulphatase inhibitor. It is possible, for example, that in vivo conversion to an oestrogenic metabolite could occur. A search has therefore been started to identify and develop a non-steroidal, non-oestrogenic, steroid sulphatase inhibitor. Not only would such an inhibitor be more suitable for therapeutic use in women with breast cancer, but would also be more appropriate for use in other non-malignant conditions, such as the prevention of autoimmune disease, where drugs may need to be administered from an early age.

The first non-steroidal steroid sulphatase inhibitors were developed by Birnbock & Von Angerer (1990) and were based upon a new class of mammmary tumour inhibitory compounds including 5-acetoxy-3-(4-acetoxyphenyl)-1-ethyl-3-methylindole. The most potent inhibitor proved to be 3-methyl-1-pentafluorophenylmethyl-6-sulphooxy-2-(4-sulphooxyphenyl)-4-trifluoromethylindole (Fig. 2(14)). Using a partially purified steroid sulphatase prepared from calf uterus, this compound had an IC$_{50}$ of 80 µM.

In order to explore the structure-activity relationship of different types of steroid sulphatase inhibitors, and also to examine the possibility of developing a non-steroidal inhibitor, a series of sulphamate derivatives of tetrahydronaphthol (THN) (Fig. 2(15-17)) and diethylstilboestrol (DES) (Fig. 2(18/19)) was synthesized and these were tested for their ability to inhibit oestrone sulphatase (Table 2).

Three sulphamate derivatives of THN were synthesized. Of these the N,N-dimethylsulphamate (Fig. 2(17)) was inactive when tested at 10 µM, with THN-sulphamate (Fig. 2(15)) being the most potent, inhibiting activity by 15-79% over the 0.1-10 µM range in intact MCF-7 cells. Pretreating MCF-7 breast cancer cells with THN-sulphamate, as previously used to investigate the nature of EMATE inhibition, revealed that oestrone sulphatase activity only recovered by 22% compared with the activity in untreated cells. This result suggests that THN-sulphamate may act as a weak irreversible inhibitor. The sulphamate derivatives of DES were considerably more potent inhibitors than the THN derivatives. DES-bis-N,N-dimethylsulphamate (Fig. 2(19)) inhibited activity by more than 90% at all concentrations tested (0.1-10 µM).

The IC$_{50}$ values for sulphamate derivatives of THN and DES, as measured in MCF-7 cells, are compared with those for EMATE and E1-MTP in Table 2. Like EMATE, DES-bis-sulphamate (Fig. 2(18)) appears to act as an irreversible inhibitor. While further research is currently in progress to develop a non-steroidal non-oestrogenic inhibitor which is equipotent with EMATE, the finding that phenolic compounds other than steroids can be utilized for the development of potent inhibitors should greatly facilitate the search for a non-steroidal inhibitor. This had led to the development of a series of coumarin sulphamate derivatives which are non-oestrogenic but which have similar inhibitory properties to those of EMATE (Woo et al. 1996b).
In vivo inhibition of steroid sulphatase

The in vivo testing of most steroid sulphatase inhibitors developed so far, apart from EMATE, has not yet been reported. As previously discussed, while there is evidence from clinical observations and in vitro testing that antioestrogens and progestogens may inhibit steroid sulphatase activity, no inhibition of rat liver sulphatase activity was detected after the administration of MPA or antioestrogens (Purohit et al. 1995c).

Preliminary studies were carried out to examine the in vivo efficacy of E1-MTP, but as this compound is a competitive inhibitor (Duncan et al. 1993) it is difficult to demonstrate inhibition in tissues obtained from animals treated with this compound. This is presumably due to the fact that upon dilution and homogenization the inhibitor concentration at the active site of the enzyme is reduced, making it difficult to demonstrate inhibition. However, some evidence was obtained to indicate that E1-MTP does inhibit oestrone sulphatase activity in vivo. Plasma oestradiol concentrations were measured in samples obtained from treated (1 mg/kg per day for 7 days s.c.) and untreated animals. As shown in Fig. 4, the plasma oestradiol concentration was about 50% lower in treated rats, suggesting that E1-MTP is active in vivo. Although more potent inhibitors were subsequently developed, a full evaluation of the in vivo inhibitory potential of E1-MTP and DHEA-methyl thiophosphonate (DHEA-MTP) should be carried out. It is possible that DHEA-MTP, a non-oestrogenic inhibitor, may have a therapeutic role as a steroid sulphatase inhibitor.

As previously discussed, a major concern related to the use of oestrone derivatives as steroid sulphatase inhibitors is that such compounds may be converted in vivo to oestrogenic metabolites. To examine the possible oestrogenic effects of two possible metabolites of oestrone-based sulphatase inhibitors, 3-amino-oestrone and 3-thio-oestrone, Selcer & Li (1995) investigated their effects on the growth of the rat uterus in ovariectomized animals. These compounds were found to be well tolerated in vivo at up to 100 µg/day. Neither 3-amino- nor 3-thio-oestrone had any effect on uterine weight when tested at 25 µg/day, indicating that they were not oestrogenic. These compounds did not block the ability of oestradiol to stimulate uterine growth and, therefore, are not antioestrogenic. However, the 3-amino- and 3-thio-oestrone derivatives, when tested in vitro, only inhibited sulphatase activity by 10-15%. So far, the ability of the other compounds, developed as inhibitors by this group, to act in vivo has not yet been reported.

EMATE has been fully investigated for its ability to inhibit steroid sulphatase activity in vivo in the rat. A dose of 1 mg/kg, administered either subcutaneously or orally for 7 days, effectively abolished both oestrone and DHEA sulphatase activities in rat liver (Purohit et al. 1995c). Furthermore, EMATE has a prolonged duration of action. After a single dose (10 mg/kg) liver steroid sulphatase activity remained completely inactivated with only a small (10%) recovery in activity occurring 7 days after EMATE injection.

The main reason for developing a steroid sulphatase inhibitor is for use in the treatment of endocrine-dependent breast cancer. A preliminary study was therefore carried out to evaluate the potential of EMATE to inhibit the growth of endocrine-dependent tumours by examining its ability to reduce the growth of oestrone sulphate-stimulated nitisomethylurea-induced mammary tumours in the rat. This model has been widely used to evaluate a number of endocrine therapies (Wilkinson et al. 1986), and Santner et al. (1990) initially demonstrated that physiological concentrations of oestrone

![Figure 4](image)

**Figure 4** Effect of treatment with oestrone-3-O-methylthiophosphonate (E1-MTP, Fig. 2(3)) on plasma oestradiol concentrations in the rat. Groups of animals (n=3) received vehicle (propylene glycol) or E1-MTP in propylene glycol (1 mg/kg per day s.c. for 7 days). Values are means±S.D.
Figure 5 Proposed mechanism of (A) oestrone sulphate (E1S) hydrolysis and (B) enzyme inactivation by oestrone-3-O-sulphamate (EMATE, Fig. 2(4)). (Reproduced with permission from Purohit et al. (1995b), ©1995 American Chemical Society)
sulphate could stimulate the growth of these tumours in ovariectomized rats. After the administration of EMATE (1 mg/kg for 12 days s.c.) the rate of oestrone sulphate-stimulated growth was reduced, with tumour regression being detected by the end of the study period. Oestrone sulphatase activity in the tumours and livers of treated animals was almost completely suppressed (Purohit et al. 1995c).

**Mechanism of steroid sulphatase inhibition**

In addition to having a potential therapeutic role, the development of an irreversible inhibitor should help in identifying the active site of the steroid sulphatase enzyme and in elucidating the mechanism of aryl steroid sulphate hydrolysis.

We have recently postulated (Purohit et al. 1995b, Williams et al. 1996) that the mechanism of aryl sulphate hydrolysis may be analogous to the proposed mechanism of sulphuryl transfer by aryl steroid sulphotransferase (Chai & Lowe 1991, Chai et al. 1992). As shown in Fig. 5A, it is suggested that a possible mechanism may involve direct nucleophilic displacement from the sulphate group of oestrone sulphate, either by water in a one-step mechanism, or by a two-step mechanism involving a sulphated enzyme intermediate, which is subsequently hydrolysed by water to regenerate active enzyme. It is possible that, in the inactivation of oestrone sulphatase by EMATE, either the essential amino acid residue which is normally sulphated during catalysis, or a neighbouring residue, becomes irreversibly sulphanoylated (Fig. 5B). Support for such a mechanism, possibly involving a sulphanoylated intermediate, is provided by the finding that replacement of the bridging O atom in the sulphamate group of EMATE by other heteroatoms, i.e. as in the 3-S-sulphamate (Fig. 2(20)) and the 3-N-sulphonate (Fig. 2(21)), not only reduces the potency of EMATE as an inhibitor, but also abolishes its active site-directed mode of inhibition (Woo et al. 1996a).

**Future perspectives**

During the last 2 years there has been a considerable increase in the number of potential steroid sulphatase inhibitors that have, so far, only been tested in vitro. It is evident that further in vivo investigations, with the most potent of these compounds, need to be carried out in order to develop a full understanding of the structure-activity relationship that is required for efficient in vivo sulphatase inhibition. Another important requirement is the development of a non-oestrogenic inhibitor which has a similar potency to that of EMATE, the most active inhibitor so far developed. Having identified such an inhibitor it will be possible to proceed to a Phase I trial to evaluate the potential benefit of steroid sulphatase inhibition in the treatment of women with endocrine-dependent tumours of the breast or endometrium.

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