Steroid Sulfatase: Molecular Biology, Regulation and Inhibition

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Abstract

Steroid sulfatase (STS) is responsible for the hydrolysis of aryl and alkyl steroid sulfates and therefore has a pivotal role in regulating the formation of biologically active steroids. The enzyme is widely distributed throughout the body and its action is implicated in physiological processes and pathological conditions. The crystal structure of the enzyme has been resolved but relatively little is known about what regulates its expression or activity. Research into the control and inhibition of this enzyme has been stimulated by its important role in supporting the growth of hormone-dependent tumors of the breast and prostate. STS is responsible for the hydrolysis of estrone sulfate and dehydroepiandrosterone sulfate to estrone and dehydroepiandrosterone respectively, both of which can be converted to steroids with estrogenic properties (i.e. estradiol and androstenediol) that can stimulate tumor growth. STS expression is increased in breast tumors and has prognostic significance. The role of STS in supporting tumor growth prompted the development of potent STS inhibitors. Several steroidal and non-steroidal STS inhibitors are now available with the irreversible type of inhibitor having a phenol sulfamate ester as their active pharmacophore. One such inhibitor, 667 COUMATE, has now entered a Phase I trial in postmenopausal women with breast cancer. The skin is also an important site of STS activity and deficiency of this enzyme is associated with X-linked ichthyosis. STS may also be involved in regulating part of the immune response and some aspects of cognitive function. The development of potent STS inhibitors will allow the role that this enzyme has in physiological and pathological processes to be investigated.

Keywords:

Steroid sulfatase, estrone sulfate, dehydroepiandrosterone sulfate, breast cancer, steroid sulfatase inhibitor, estrone sulfamate, 667 COUMATE
**Abbreviations:**

STS, steroid sulfatase; DHEAS, dehydroepiandrosterone sulfate; E1S, estrone sulfate; URE, upstream regulatory element; IL-6, interleukin 6; TNFα, tumor necrosis factor α; BCF, breast cyst fluid; E1, estrone; E2, estradiol; Adiol, 5-androstenediol; E2S, estradiol sulfate; X-LI, X-linked ichthyosis; EMATE, estrone-3-O-sulfamate; 17βHSD, 17β-hydroxysteroid dehydrogenase; OATP, organic anion transporter polypeptide; DASI, dual aromatase - sulfatase inhibitor; 2-MeOE1, 2-methoxyestrone; 2-MeOE2, 2-methoxyestradiol; 2-MeOEMATE, 2-methoxyestrone-3-O-sulfamate; 2-MeOE2-bisMATE, 2-methoxyestradiol-bis-sulfamate; rbcs, red blood cells, CA, carbonic anhydrase.
1. Introduction
After more than a decade’s research to develop potent steroid sulfatase (STS) inhibitors at least one inhibitor has now entered clinical trials to test its efficacy in postmenopausal women with breast cancer. It is therefore timely to review the role that this enzyme has in physiological and pathological conditions and to examine the rapid progress that has recently been made in developing potent STS inhibitors. STS (EC 3.1.6.2, aryl sulfatase C) is the enzyme responsible for the hydrolysis of alkyl (e.g. dehydroepiandrosterone sulfate, DHEAS) and aryl steroid sulfates (e.g. estrone sulfate, E1S) to their unconjugated forms. E1S was one of the first steroid conjugates to be isolated from the urine of pregnant mares in 1938 (1). In the past steroid sulfates were generally considered to be end products of metabolism with their water solubility aiding excretion. However, during the last decade there has been a resurgence of interest in the roles that steroid sulfates, such as DHEAS and E1S, may have as precursors for the formation of biologically active hormones.

A major impetus to the development of STS inhibitors was to identify new drugs for use in the treatment of hormone-dependent breast cancer. These tumors in postmenopausal women are initially treated with endocrine therapy, such as anti-estrogens, or more recently aromatase inhibitors. Many breast tumors will either fail to respond to such therapies, or progress after a relatively short period of time, making it necessary to continue the search for new effective therapeutic agents. While the search for STS inhibitors was in progress it became apparent that they may also have therapeutic applications in a number of other, non-oncological conditions, including regulation of part of the immune response, dermatology and cognitive function. In this paper we review the recent advances that have been made in understanding the molecular biology and structure of the STS enzyme. The roles that STS may have in regulating the formation of biologically active hormones are also considered. The research leading to the development of potent STS inhibitors is discussed together the potential therapeutic importance of this new class of drug.

2. Molecular biology of steroid sulfatase
STS is a member of a superfamily of 12 different mammalian sulfatases (2,3). The gene for the human STS is located on the distal short arm of the X chromosome and
maps to Xp22.3-Xpter; the gene is pseudoautosomal and escapes X-inactivation. On
the Y chromosome there is a pseudogene for STS, which is transcriptionally inactive
as the promoter and several exons have been deleted. Sequence divergence has
produced numerous stop codons in this pseudogene and there are several large
insertions. The extent of sequence similarity between the two genes suggests that
they have been diverging for approximately 40 million years (4).

The locus for the human STS gene on the X chromosome has been cloned,
characterised and sequenced (4) (GenBank M23945, Ensembl ENSG00000101846).
The structure of the gene is shown in Fig 1. The gene consists of 10 exons and spans
over 146Kb, with the intron sizes ranging from 102bp up to 35Kb. Variable mRNA
transcripts are detected by Northern blotting which are due to the use of alternative
polyadenylation sites within exon 10 and are not thought to be caused by splice
variants (3). The cDNA for STS has been cloned and sequenced (5,6) (GenBank
M16505, GenBank J04964). It encodes a protein of 583 amino acids, with a signal
peptide of 21-23 peptides and four potential glycosylation sites of which at least two
are utilised, at asparagine residues 47 and 259.

Inactivation of the STS gene results in X-linked ichthyosis, one of the most prevalent
Human inborn errors of metabolism (7). In 80-90% of cases the X-linked ichthyosis
is due to complete deletions of the 146Kb STS gene and substantial flanking regions
from the distal short arm of the X chromosome. However, some patients have been
identified who have normal hybridisation patterns for genomic DNA and mRNA
when probed with STS cDNA (7). Further investigation of these patients led to the
identification of six point mutations within the coding sequence of the STS gene (8,9).
These mutations lead to the production of catalytically inactive STS. The loss of
activity does not appear to be caused by incorrect localization or post-translational
modifications but maybe due to a shortened half-life and/or loss of the substrate
binding site (9). A further six mutations have now been identified all of which lead to
catalytically inactive STS (10, 11). To date all the point mutations reported are
located in the carboxyl region of the STS enzyme, which is thought to be important
for substrate binding (12).
So far there has only been a very limited study of the molecular regulation of STS. The cytokines tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) both up-regulate STS enzyme activity in MCF-7 breast cancer cells. However, upon further investigation this up-regulation appeared to be post-translationally mediated rather than occurring via any changes in gene transcription or mRNA stability (13). The promoter region of the STS gene has been characterized and some potential tissue-specific regulatory elements identified (14). The promoter is unusual, as it neither resembles a housekeeping gene nor a tightly regulated gene. It lacks a TATA box, is not GC rich and lacks binding sites for Sp1 and other known transcription factors. The transcription start sites were mapped by primer extension and S1 Nuclease protection assays. The major start site is at -221 with respect to the A nucleotide of the initiating methionine with other minor transcription start sites mapped to -197, -206 and -241. The basal promoter region was identified as a 110bp region from -192 to -302 using transient transfection reporter gene assays. Four other upstream regulatory elements (UREs) were identified, URE1 -305 to -572, URE2 -870 to -1086 and URE3-1087 to -1253 which all act as enhancers. The three-enhancer regions are counterbalanced by the presence of a negative regulatory region at -1253 to -1458. The basic promoter and URE activities could only be detected in the human choriocarcinoma JEG-3 cells which are of placental origin and have high STS activity. Transfections of the basic promoter and UREs into COS-1, HeLa and B82 cells gave no activity, suggesting that tissue-specific factors are required for activity of the STS promoter.

3. Localization of steroid sulfatase

STS activity was first demonstrated in rat liver microsomes by Dodgson and colleagues (15). Since then it has been found in testis, ovary, adrenal glands, placenta, prostate, skin, brain, fetal lung, viscera, endometrium, peripheral blood lymphocytes, aorta, kidney and bone. It is believed to be virtually ubiquitous in small quantities. The organ and tissue distribution varies considerably between different mammals. It is reported to be absent in the guinea pig and some marsupial livers and is undetectable in erythrocytes. The richest source of STS is the placenta. STS has been detected in various tissues by (i) immunohistochemistry, (ii) biochemical analysis of hydrolytic products of various sulfated substrates (by colorimetric,
fluorimetric or radiometric methods), and more recently in combination with mRNA expression levels using reverse transcriptase-polymerase chain reaction (RT-PCR).

**Immunocytochemical localization of steroid sulfatase**

Using an azo-coupling histochemical method, Partanen was unable to demonstrate the presence of STS in the epithelium of ducts and lobules of the normal breast although activity was detectable in some samples of benign and malignant breast tissues (16). With the availability of purified preparations of STS (particularly human placental STS), anti-STS (polyclonal and monoclonal) antibodies were obtained and specific immunohistochemical methods were developed to examine the subcellular localization of STS. In cultured human skin fibroblasts, STS was localised on the rough endoplasmic reticulum, Golgi cisternal, trans-Golgi reticulum and to a lesser extent in plasma membranes and components of the endocytic pathway (i.e. coated pits, endosomes and multivesicular endosomes). No STS immunostaining was detected in lysosomes (17). Immunohistochemistry with a monoclonal antibody to placental STS, combined with electron microscopy, also localised STS to the membranes of the endoplasmic reticulum, the nuclear envelope in rat hepatocytes, the proximal tubules in the kidney, and in the pineal gland, choroid plexus and adenohypophysis of the rat brain (18). More recently, immunohistochemical evidence for the presence of STS has been obtained in the cytoplasm of ovarian clear cell adenocarcinomas (19), in glandular epithelial cells of the basilar layer of the endometrium but not the myometrium (20), and in vascular smooth muscle cells from the aorta (21). Immunohistochemistry has also been combined with RT-PCR to examine the localization and expression of STS in human fallopian tubes (22). STS was found to be localised in the secretory cells of fallopian tubes and a higher number of positive cells were found in tissues obtained during the early luteal phase than in tissues collected during the follicular phase of the menstrual cycle. In agreement with these findings, abundant expression of STS mRNA was found in tissues from the early luteal phase (22). In another study STS mRNA, enzyme activity and immunoreactivity were assessed in normal human adult and fetal tissues (23). Amplified STS mRNA transcripts were weakly expressed in adult lung, aorta, liver, thyroid, testis, uterus and all fetal tissues examined. Relatively high levels of STS activity were found in adult liver and the adrenal gland. The highest activity was detected in the placenta but in keeping with the lower sensitivity of this technique,
STS immunoreactivity was only detected in placental syncytiotrophoblasts. The same researchers detected STS immunoreactivity in breast carcinoma cells in 74% of cases and this was significantly associated with its mRNA level and enzyme activity (24). An affinity purified monoclonal antibody (KW 1049), raised against STS purified from human placenta which did not cross react with arylsulfatases A or B, was used for the investigations in normal and malignant human tissues (23, 24).

ii Biochemical localization of steroid sulfatase
Historically, STS has been detected in microsomes or whole tissue homogenates using biochemical or radiometric assays of substrate hydrolysis. For specific measurements of STS activity, [6,7-3H]-estrone sulfate or [7-3H]-DHEAS are used in buffer at pH 7.4 (based on Burstein and Dorfman, (25). Phosphate buffer is preferred as it completely inhibits arylsulfatases A and B. The activities of both of these enzymes are relatively low at pH 7.4 which further improves the specificity of the assay. These assays have been used to identify and characterise STS activities in human leukocytes (26), brain (27, 28), osteoblast cell lines (29, 30), ovarian granulosa cells, (31, 32) and rat testis (33).

The central role of placental STS for the formation of estriol in the feto-placental unit, its abundance in the placenta and the virtual absence of detectable activity in cases of the inherited disorder of placental steroid sulfatase deficiency and recessive X-linked ichthyosis (X-LI) has led to the enzyme from human placenta being extensively investigated. Human placental STS has been purified to homogeneity and has been well characterised. Depending on the extent of glycosylation, the purified STS has a molecular weight of around 65 KDa (6). While evidence from early investigations suggested that aryl sulfatase c and STS may have been different enzymes biochemical and genetic analyses have confirmed that there is only one enzyme. Chromatography of placental microsomal extracts has revealed that both activities co-localize in the same fractions (34-36). Purified STS hydrolyses aryl sulfates (eg p-nitrophenyl-sulfate, E1S) as well as alkyl sulfates (DHEAS, pregnenolone sulfate, deoxycorticosterone sulfate, cholesterol sulfate) and to a lesser extent iodothyronine sulfates (37-39). In addition, since the first observation by Jobis and colleagues (40), that boys born from women with sulfatase deficient placentas develop X-LI, the link
between the deficiency of microsomal STS and X-LI has been confirmed by several groups. In these subjects, enzyme activity towards both aryl – and alkyl – steroid sulfates was lacking in all tissues examined. In keeping with the lack of STS activity, plasma concentrations of all steroid sulfates are elevated. In subjects with STS deficiency, the activities of aryl sulfatases A and B are normal (41-43). Furthermore, when the cDNA for human placental STS was transfected into COS-1 cells, the expressed protein hydrolysed aryl (E1S) and alkyl (DHEAS) steroid sulfates with the hydrolysis of both substrates being blocked by a single inhibitor (44). Although there is only one gene for STS, some evidence has emerged that different isoforms of the enzyme may exist in rodents and humans. Following the observation of Nelson and his colleagues that two isoforms may exist in mice (45), two isoforms (microsomal and nuclear) were shown to exist in rat livers and human placentas (46,47). In humans two isoforms (slow and fast) were identified in fibroblasts (48-50). It is possible that these isoforms are the result of post-translational modifications. Hence, it is apparent from biochemical and immunohistochemical localization studies that STS is mainly found in target tissues of the reproductive tract (i.e. endometrium, ovarian, prostate, testis, placenta), the breast, skin, the brain, the bone and the blood. The biological role of STS in these tissues/organs is discussed in Section 5.

4 Regulation of steroid sulfatase activity
The action of steroid sulfatase makes a major contribution to in situ estrogen production in hormone-dependent malignant tissues. Although expression of STS mRNA and STS activity are increased in malignant breast (and endometrial) tissues compared with non-malignant tissues, little is known about the regulation of its expression or activity. As the expression of other enzymes of steroidogenesis (such as aromatase) is known to be regulated by cytokines, growth factors, steroids and prostaglandin E2, some of these factors have also been tested to assess whether they would induce STS.

i Cytokines and growth factors
The cytokines IL-6 and TNFα act synergistically to increase STS activity in breast cancer cells (51, 52). Furthermore, these cytokines increase STS activity without the use of promoter/enhancer elements suggesting that the control of STS activity is via
post-translational modification of cysteine to formyl glycine in the active site or indirectly via changes in membrane fluidity or organic anion transporters, allowing increased uptake of the hydrophilic substrate (13). In contrast, the inflammatory cytokine IL-1β decreases the activity and expression of STS mRNA in human endometrial stromal cells, in a dose-dependent manner, and this effect is antagonised by the IL-1 receptor antagonist (53). IL-1β also suppressed STS activity and mRNA expression in vascular smooth muscle cells derived from human aortae (21). The presence of these cytokines in breast cyst fluid (BCF) may explain the differential regulation of STS in breast cancer cell lines by BCF (54). In a separate study, both basic fibroblast growth factor and insulin-like growth factor type 1 were found to increase STS activity in a dose – and time-dependent manner in MCF-7 and MDA-MB-231 breast cancer cells. This induction was inhibited by cycloheximide, indicating the requirement for new protein synthesis (55). These growth factors, which are thought to be secreted by breast tumors, may therefore increase local production of estrogens.

ii Steroids

Schneider and colleagues first reported that in utero androgen exposure is required for induction of androgen – responsive hepatic STS in male rats (56). Lam and Polani used exogenous testosterone treatment and concluded that STS induction is, in part, controlled by the male hormones in the mouse (57). Moutaouakkil and colleagues observed that STS was highest in the uteri of pregnant guinea pigs compared with that in the uteri of fetal, castrated or mature females suggesting estrogenic regulation (58). The possibility of substrate induction of in vivo STS activity in liver and white blood cells in ovariectomized rats was confirmed by administration of exogenous E1S to ovariectomized rats (59). In contrast, a decrease in STS mRNA levels was found when MCF-7 breast cancer cells were treated with the progestagen Promegestone (R-5020) (60). However, exposure of MCF-7 and MDA-MB-231 breast cancer cells to the progestagen, medroxyprogesterone acetate (MPA), stimulated STS activity in these cells (61). As MPA is known to affect membrane fluidity, the enhanced STS activity might be explained by increased substrate availability from the medium. In addition, the availability of sulfated substrates may be increased by the induction of specific high-affinity transporters. It has also been reported that progesterone increased the uptake of inorganic sulfate in endometrial epithelial cells through
induction of a high-affinity transport system (62). Whether progesterone or other steroids induce specific transporters for sulfated steroids in endometrial and/or other tissues remains to be explored. Recently, retinoids and 1, 25-dihydroxy vitamin D₃ have been reported to induce STS activity and expression in HL-60 promyelocytic cells (63). However, the molecular mechanisms underlying cytokine or steroid induction of STS activity and/or expression still remains to be explored. Furthermore, factors governing the extent of post-translational modification of cysteine-formyl glycine, glycosylation and translocation to the endoplasmic reticulum are all likely to influence the activity of STS.

5. Biological roles of steroid sulfatase  
A. In hormone-dependent breast cancer  
i. Hydrolysis of estrone sulfate  
Estrogens have a major role in supporting the development and growth of tumors in hormone-dependent tissues such as the breast and endometrium (64, 65). The highest incidence of breast cancer occurs in postmenopausal women after cessation of ovarian production of estrogens. However, estrogens continue to be produced in postmenopausal women by the peripheral conversion of androstenedione (Adione) to estrone (E1), a reaction mediated by the aromatase enzyme complex (66, 67). In postmenopausal women the production rates for E1 and estradiol (E2) are approximately 40µg/24h and 6µg/24h respectively (68). Much of the estrogens that are formed can be converted to estrogen sulfates by the actions of estrone sulfotransferase and phenol sulfotransferase (69-72). Sulfation of estrogens changes them from being hydrophobic to hydrophilic molecules. In addition, as estrogen sulfates are unable to bind to the estrogen receptor (ER) they are biologically inactive. Circulating concentrations of E1S are much higher than that of the unconjugated estrogens (73, 74). Estrogen sulfates bind to albumin and have a prolonged half-life in blood (up to 9h) compared with the much shorter half-lives of E1 and E2 (75). The high circulating concentrations of E1S together with its prolonged half-life has given rise to the view that E1S may act as a reservoir for the formation of biologically active estrogens via the action of STS (76-79).
In contrast to the low circulating levels of E1 and E2 in postmenopausal women there is now general agreement that their concentrations are much higher in normal and malignant breast tissues (80, 81). Concentrations of E1 and E2 in malignant breast tissues can be up to 10-times higher than those found in plasma. There is also evidence for high levels of E1S and estradiol sulfate (E2S) in breast tumors (74). Surprisingly, although plasma estrogen concentrations in postmenopausal women are much lower than in premenopausal women, breast tumor estrogen levels are similar in both groups of women (82, 83). The origin of estrogens in breast tumors has been the subject of intensive research during the last decade. There are two possible mechanisms which could account for this, uptake from the circulation and binding with high affinity to ERs, or in situ synthesis from estrogen precursors. Although uptake and binding to ERs may make an important contribution to tissue estrogen concentrations the finding that levels are similar in ER positive (ER+) and ER negative (ER-) tumors suggests that local synthesis makes a major contribution to breast tumor estrogen concentrations (84, 85).

Three enzyme systems are required for the formation of E2 from androgen precursors in breast tissues and include the aromatase, which converts Adione to E1 and 17βHSD type 1 which reduces E1 to E2, the biologically active estrogen that interacts with the ER. In addition, STS can act on E1S, formed as a result of sulfotransferase activity, to form E1 which can subsequently be converted to E2 (Fig 2). All of these enzymes have been identified in malignant breast and endometrial tissues (86, 87). However, while aromatase activity is detected in only 40-60% of breast tumors, STS activity is present in most breast tumors (86, 88). Furthermore, the activity of STS is considerably higher than that of the aromatase enzyme in breast tumors (86). Using the appropriate substrate concentrations it was found that as much as 10-times more E1 could originate from E1S, via the sulfatase pathway, than from Adione by the aromatase route (89).

Recently real time RT-PCR techniques have been used to examine STS mRNA expression in breast tissues and to relate expression to a number of clinico-pathological variables. Using this technique it was shown that the level of STS mRNA expression in malignant breast tissue (1458 amols/mg RNA) was significantly higher than in normal tissue (536 amols/mg RNA) (90). This finding is consistent with the
higher STS enzymatic activity that has been detected in malignant breast tissue (86,91).

STS mRNA expression was found to be an independent prognostic indicator in predicting relapse free survival with high levels of expression being associated with a poor prognosis (92). One possible explanation for this finding was suggested i.e. in breast tissues expressing high levels of STS mRNA, tumor cells that escape surgical removal may grow very fast and therefore patients may relapse earlier. While previous investigations found no link between time to relapse and STS activity in breast tumors (93, 94) the original findings of Utsumi and colleagues (92) have now been confirmed in two further investigations (24, 95). In one study it was found that the association between STS mRNA expression and prognosis only applied to ER+ tumors. Interestingly, high STS mRNA expression was associated with a poor prognosis in both pre- and postmenopausal women. This finding led to the suggestion that even in premenopausal women, intra-tumoral estrogen synthesis may play an important role in the growth of breast tumors. The role of aromatase mRNA expression analysis as a prognostic marker was also examined in view of the pivotal role that the enzyme is considered to have in regulating tumor estrogen synthesis. Aromatase mRNA expression was found to have no prognostic value, a finding consistent with previous studies which examined aromatase activity as a prognostic indicator (96, 97). The lack of prognostic value of aromatase mRNA determination led the authors to speculate that the sulfatase pathway may be more important than the aromatase route for intra-tumoral estrogen synthesis. STS mRNA expression was also found to correlate with tumor size and to be significantly higher in tumors with lymph node metastasis than in those without lymph node metastasis (24, 95). An examination of the intra-tumoral expression of genes from the estradiol metabolic pathway has provided further confirmation of the high expression and prognostic significance of STS mRNA expression (98).

Immunohistochemistry and STS mRNA expression of laser captured microdissected samples were also used to examine the location of STS within breast tumors (24). STS immunoreactivity was detected in the cytoplasm of cancer cells (Fig 3) with STS mRNA expression being detected in microdisected carcinoma cells but not in stromal cells. This contrasts with reports as to the localization of the aromatase enzyme.
Biochemical studies have consistently revealed higher aromatase activity in the stromal rather than the epithelial component of breast tumors (99). Immunohistochemical studies, however, have provided evidence for both an epithelial or stromal location for the aromatase enzyme complex (100-102).

As previously discussed when estrogen sulfates were first isolated it was thought that they represented the end products of metabolism with sulfation rendering them water soluble. Many in vitro studies have now convincingly shown that E1S can be hydrolysed by breast cancer cells, induce the production of estrogen sensitive proteins such as pS2 and cathepsin D, and induce cell proliferation (103, 104). To examine if E1S could support the growth of tumors in vivo it was infused into rats bearing nitrosomethyl-urea (NMU) – induced mammary tumors (105). This animal model has been widely used to examine the effects of hormones on tumor growth. The tumors are hormone-dependent and regress after ovariectomy but can be stimulated to regrow with estrogens. These tumors contain high levels of STS activity but are devoid of aromatase activity. Infusions of E1S at 300 pmol/h inhibited ovariectomy induced tumor regression while 3000 pmol/h stimulated tumor growth.

While this infusion study clearly demonstrated that NMU-induced mammary tumors in rats can be stimulated to grow by E1S it did not differentiate between hydrolysis of E1S occurring in peripheral tissues, such as liver, and that occurring within the tumor. Two elegant studies have addressed this question, using the NMU-induced mammary tumor model or inoculation of MCF-7 breast cancer cells transfected with the STS cDNA. In the NMU model a double isotope infusion technique was used to determine the extent of in situ E1 formation from E1S in the tumor (106), based on a method that had previously been employed to measure the extent of formation of E1 from Adione in human breast tumors (107). For this, $^{14}$C E1 was infused into animals over a 3 day period to ensure that an isotopic steady state had been achieved. By measuring $^{14}$C E1 levels in tumor tissue and blood an index of the uptake of unconjugated E1 into the tumor can be calculated. By simultaneously infusing $^{3}$H E1S it is possible to calculate how much E1 is being formed within the tumor. Some of the infused $^{3}$H E1S will be hydrolysed in peripheral tissues, with some of the released $^{3}$H E1 being taken up by the tumor. As uptake from the circulation can be calculated from the infusion of $^{14}$C E1, any $^{3}$H E1 in the tumor above that expected to
be present due to uptake is considered to be formed by \textit{in situ} synthesis. Using this technique it was found that as much as 50% of the E1 formed within the tumor could originate from E1S.

As an alternative approach to investigate the importance of \textit{in situ} formation of unconjugated estrogen from estrogen sulfates, MCF-7 cells transfected with either a vector (MCF-7\textsubscript{v}) or vector containing the STS cDNA (MCF-7\textsubscript{STS}) were inoculated into the flanks of ovariectomised nude mice (108). The incidence of proliferating tumors in mice bearing MCF-7\textsubscript{STS} cells, supplemented with E2S (71%), was significantly higher than in animals bearing this cell line but not supplemented with E2S (22%). Supplementation with E2S and subsequent hepatic hydrolysis was not sufficient to stimulate the growth of MCF-7\textsubscript{v} cells. This finding demonstrates the importance of \textit{in situ} estrogen synthesis, compared with that occurring in peripheral tissues, in supporting tumor growth. E2S was used for these studies as, unlike E1S, it does not require the liberated steroid to be reduced by estradiol dehydrogenase (Type 1) before being biologically active.

Interestingly, results from both \textit{in vitro} and \textit{in vivo} experiments have suggested the possibility that estrogen sulfates may have different biological activity than their unconjugated counterparts in cells expressing high STS activity. E2S was found to be more mitogenic than E2 \textit{in vitro} producing a greater increase in anchorage – independent colony formation in the MCF-7\textsubscript{STS} clones (108). \textit{In vivo} the volumes of tumors of animals supplemented with E2S (138mm\textsuperscript{3}) were greater than those in animals supplemented with E2 (51mm\textsuperscript{3}). One possible explanation for this observation is that some STS activity may reside in the nucleus (46). Evidence for a nuclear STS isozyme has been obtained and it is possible that the formation of active estrogen by STS within the nucleus may not be subjected to the same degree of inactivation by 17βHSD type II or sulfotransferase prior to exerting their action.

ii Hydrolysis of dehydroepiandrosterone sulfate
Evidence for the role that DHEAS, and its unconjugated metabolite DHEA, may have in breast cancer stems from two sources. Firstly, steroid dynamic studies have revealed that these steroids can act as precursors for the formation of steroids with
estrogenic properties, such as 5-androstenediol (Adiol). Secondly, studies in cells and animals have revealed that DHEAS, DHEA and Adiol can stimulate the proliferation of breast cancer cells in vitro and induced mammary tumors in vivo. DHEAS is the most abundant steroid secreted by the adrenal cortex and, like estrogen sulfates, its half-life in plasma (10-20h) is considerably longer than that of unconjugated DHEA (1-3h) (109, 110). Isotopic infusion studies have revealed that in women as much as 75% of the daily production rate of DHEAS is converted to DHEA in peripheral tissues (111). After removal of the sulfate group by STS the resulting DHEA can undergo reduction to Adiol, a steroid of particular importance with regard to breast cancer development. In postmenopausal women the major proportion of Adiol formed is derived in peripheral tissues from DHEAS and DHEA (112). DHEAS can also be converted to Adiol-sulfate but the contribution that this pathway makes to Adiol production remains to be resolved. Adiol, although an androgen can bind to the ER with a somewhat lower affinity than that of E2. However, as the plasma concentrations of Adiol is at least 100-fold higher than that of E2 in postmenopausal women it is considered to be equipotent with E2 as an estrogen in this group of women (113).

It has been known for many years that Adiol can stimulate the growth of ER+ breast cancer cells in vitro (114, 115). In addition, in vivo studies employing 7, 12 – dimethylbenz [9] anthracene (DMBA) induced mammary tumors in rats revealed that Adiol could stimulate tumor growth (116). Importantly the aromatase inhibitor 4-hydroxyandrostenedione did not block the ability of Adiol to stimulate tumor growth. This finding showed that Adiol did not need to be converted to an estrogen in order to be able to stimulate tumor growth. More recent studies have revealed that DHEA and Adiol can directly activate the ER and stimulate the proliferation of breast cancer cells (117). Co-incubation of these steroids with an aromatase inhibitor did not block their ability to activate the ER. Using a physiological concentration of DHEAS, mass spectrometry analysis has revealed that it can be converted to estrogens and Adiol in MCF-7 breast cancer cells (118).

Further evidence for an important role of adrenal androgens and the sulfatase pathway in breast cancer was obtained from a study in which their effects on MCF-7 breast cancer cell proliferation was examined (119). DHEAS, DHEA and Adiol were all
found to stimulate cell proliferation but their ability to do so was blocked by the ER antagonist nafoxidene, but not by aromatase inhibitors. In contrast, a potent STS inhibitor completely blocked that ability of DHEAS to stimulate cell growth. These results provide strong evidence that the stimulation of cell growth by DHEAS occurs via an aromatase–independent pathway which can be blocked by a STS inhibitor.

There is, therefore, convincing evidence that adrenal androgens and their metabolites can stimulate breast cancer cell growth \textit{in vitro} and induced mammary tumors in rodents. Recently convincing clinical evidence was obtained in support of a role for DHEAS in stimulating breast tumor growth in humans (120). In a study carried out to monitor serum DHEAS concentrations in women being treated with third-generation aromatase inhibitors the important observation was made that, while those with stable disease had low (0.6µM) levels of DHEAS, levels were elevated (3.8µM) in women in whom tumor progression occurred. Serum levels of E1 and E2 in all subjects remained suppressed to minimal detectable levels. It was concluded from this study, that in patients with progressive disease DHEAS appeared to stimulate tumor progression and led to the suggestion that this finding had serious implications for the use of aromatase inhibitors on their own. A likely explanation for this observation is that DHEAS is converted to DHEA by STS. The subsequent reduction of DHEA will yield a steroid, Adiol, for which there is now convincing evidence that it can stimulate breast cancer cell growth. Inhibition of STS, in addition to blocking the formation of E1 from E1S, should also reduce the production of Adiol, by blocking the conversion of DHEAS to DHEA (Fig 2).

B. Steroid sulfatase in skin

STS is also found in the epidermis and there is increasing evidence that its action within skin may make an important contribution to androgen production in this tissue. It has been known for sometime that STS has an important role in skin function since the description of a deficiency of STS in X-LI (121, 122). Clinically X-LI is characterized by scaling of the skin with large, dark brown scales and an increase in \textit{stratum corneum} thickness (123). Lipids are important for normal \textit{stratum corneum} structure and function and may be important for the process of normal desquamation. Concentration of cholesterol sulfate in \textit{stratum corneum}, and the scales associated
with X-LI, are increased (up to 5-fold) compared with levels in *stratum corneum* from normal subjects (124). As STS inhibitors currently in development could severely reduce STS activity in skin it is reassuring to note that ichthyosis can be readily treated by the topical applications of keratolytic agents, such as ammonium lactate or cholesterol cream (125).

Plasma concentrations of DHEAS can be increased in subjects with androgenic alopecia or hirsutism (126, 127). It is therefore possible that this steroid sulfate may be an important precursor for the formation of more active steroids within the skin. DHEAS can be converted to 5α-dihydrotestosterone, the androgen that activates the androgen receptor, in axillary hair follicles (128). Using an immunohistochemical technique STS was found to be predominantly expressed in the dermal papilla of hair follicles (129, 130). STS activity was also highest in the dermal papilla fraction of hair follicles. Its activity could be effectively inhibited with 1nM of the potent STS inhibitor estrone-3-O-sulphamate (EMATE) (130). In patients with *acne vulgaris* there is some evidence of increased STS immunoreactivity in affected skin areas (131). Thus, STS inhibitors may be of value in treating skin and/or hair conditions where the action of the enzyme may be increasing local production of biologically active androgens.

C. Steroid sulfatase and the immune system

Although DHEAS is secreted in large amounts by the adrenal cortex it has remained controversial as to whether it has a specific biological role apart from serving as a precursor for the formation of active androgens and estrogens. Studies by Daynes (132, 133) and Rook (134) have suggested that DHEAS/DHEA may have an important role in regulating T-helper (Th) cell maturation. Th cells can progress to either a Th1 or Th2 phenotype, each of which secrete a characteristic profile of cytokines (e.g. Th1 cells secrete IL-2 and interferon γ (IFNγ); Th2 cells secrete IL-6 and IL-10). The response of Th cells is mutually exclusive with IFNγ inhibiting the formation of Th2 cells and IL-10 the formation of Th1 cells (135, 136).

Plasma IL-6 concentrations were found to be elevated in elderly human subjects reflecting the increased production of this cytokine by Th2 cells that occurs with
aging. In aged mice, where IL-6 plasma concentrations were also increased, it was possible to correct the elevated levels by the acute or chronic administration of DHEA or DHEAS (137). These studies also revealed that in vitro DHEA, but not DHEAS, was able to suppress the release of Th2 cytokines. Thus, STS which is present in macrophages within the lymphoid tissues where Th cell maturation occurs and which converts DHEAS to DHEA, has a crucial role in regulating part of the immune response. From such investigations it has emerged that the balance of DHEA to glucocorticoid determines whether Th cells progress to either a Th1 or Th2 phenotype i.e. DHEA favors development to Th1 cells while cortisol promotes a Th2 response.

Using a contact sensitisation model convincing evidence has been obtained that in vivo DHEA and DHEAS have an immunostimulatory role (138). However, the ability of DHEAS, but not DHEA, to act as an immunostimulant was completely blocked by the co-administration of the potent STS inhibitor EMATE. As a number of pathological conditions, such as rheumatoid arthritis, may result from an inappropriate immune response, and increased production of Th1 cytokines, inhibition of STS could be of therapeutic benefit in such conditions. Using a collagen-induced model of arthritis, evidence has been obtained showing that the progression of arthritis was markedly altered by the STS inhibitor EMATE (139).

The finding that DHEA has a role in regulating the Th1/Th2 immune response has provided an important insight into the regulation of estrogen synthesis in women (140, 141). IL-6 has a major role in regulating peripheral aromatase activity (142). It has been known for many years that the peripheral aromatase activity increases upon aging and is also higher in obese subjects (143). It is also known that plasma IL-6 levels increase with aging and its production is increased in obese subjects (137, 144). It is well established that the production of DHEAS starts to decrease from the mid – 20s (145). This reduction in the production of DHEAS will favour a Th2-type cytokine response with increased production of IL-6. Thus, increased production of IL-6 is the most likely explanation to account for the increase in aromatase activity detected in aging and obese subjects.

D. Steroid sulfatase, neuro-function and memory
In addition to being synthesized in the adrenal cortex, steroids such as DHEAS and DHEA, are also formed in parts of the central nervous system and are therefore classified as neurosteroids (146, 147). These neurosteroids have important roles in regulating brain function. Sulfated steroids, e.g. DHEAS and pregnenolone sulfate, are considered to act as GABA<sub>A</sub> receptor antagonists whereas their unconjugated analogs act as GABA<sub>A</sub> receptor agonists (148). In addition, both the sulfated and unsulfated forms of these steroids act positively to modulate N-methyl-D-aspartate receptor function (149).

As blood levels of DHEAS and DHEA decrease on aging, experiments were performed to examine the possibility that administration of these neurosteroids to rodents could improve memory. Intra-cerebroventricular or subcutaneous administration of DHEAS produced significant memory-enhancing effects in mice when tested using a foot-shock active avoidance training method (150, 151). While there is convincing evidence that increasing blood levels of DHEAS and DHEA in rodents can result in memory-enhancing effects, it was not known if such effects resulted from the sulfated or non-sulfated form of the neurosteroid. As STS activity is present in brain tissues it is possible that DHEAS could be hydrolysed to DHEA by the action of this enzyme (152). With the advent of potent STS inhibitors, such as EMATE, it became possible to test whether the sulfated or unsulfated form of DHEA was responsible for the memory-enhancing effects of these neurosteroids (153). DHEAS is known to reverse scopolamine-induced amnesia in rodents. Blocking the hydrolysis of DHEAS with EMATE potentiated the ability of this sulfated neurosteroid to reverse scopolamine-induced amnesia. Similar results were obtained in this model using the non-steroidal STS inhibitor (p-O-sulfamoyl)-N-tetradecanoyl tyramine (154). These findings strongly suggest that it is the sulfated form of DHEA which is responsible for the memory-enhancing effects of this steroid in rodents. While results from these studies suggest that STS inhibitors may have a role in modulating the neuroexcitory effects of steroid sulfates in rodents there is, as yet, no information as to their possible affects in humans. In subjects with sulfatase deficiency there is no evidence to suggest any abnormality in cognitive function. This suggests that the long-term therapeutic use of STS inhibitors should not have any adverse neurological effects. While decreases in blood levels of DHEA and DHEAS occur in humans with aging, there is no evidence for a decrease in rodents. Therefore
experiments in rodents employing DHEA or DHEAS must be interpreted with caution.

Inhibition of STS has recently been shown to increase aggressive behaviour in CBA/H mice (155). Experimental evidence had previously indicated a possible link between attack behaviour and the pseudoautosomal region of the Y chromosome which contains the sulfatase gene (156). The finding of a correlation between the initiation of aggressive behaviour and liver STS activity in mice also suggest that the sulfatase gene could be a candidate for attack behaviour in mice (157). Using a non-steroidal inhibitor a single oral dose was found to significantly inhibit brain STS activity and increase the effect of DHEAS on aggressive behaviour in CBA/H mice (155).

E. Steroid sulfatase in reproductive tract tissues
   i  Female
   STS activity has been detected in most tissues of the female reproductive tract. It is present in ovarian tissues from pre- and postmenopausal women suggesting that in the ovary sulfated precursors, such as DHEAS, could be used as precursors for the formation of androgens and estrogens (158). Support for this concept was obtained from the finding that relatively high STS activity was detected in ovarian follicles, stroma and corpus luteum which were capable of utilising DHEAS as a substrate for the production of DHEA, androstenedione and testosterone (159). DHEAS is present in high concentrations in follicular fluid in close proximity to the ovarian cells involved in steroidogenesis (160). Using human granulosa cells, obtained from women undergoing treatment for in vitro fertility, significant conversion of DHEAS to DHEA was detected, confirming the presence of STS activity in these cells (32). Such conversion was effectively inhibited by the STS inhibitor EMATE. Addition of DHEAS to cultured granulosa cells stimulated estrogen production, in a dose-dependent manner, demonstrating that granulosa cells can utilize DHEAS as a substrate for estrogen production. STS is also expressed in human fallopian tubes which are involved in gamete transport and fertilization (22). Expression of STS was higher in fallopian tubes obtained from the early luteal phase than from the follicular phase of the menstrual cycle.
In addition to the role that steroid sulfates may have in breast cancer development it is also likely that they may support the growth of hormone-dependent tumors in the reproductive tract of women. STS activity has been detected in normal and hyperplastic endometrial tissues (161). It has been suggested that uterine STS activity may have an important role in regulating the uterotrophic activity of E2S (162). In a comparison of STS activities in malignant and normal endometrial tissues, activity was found to be 12-fold higher in malignant endometrial tissue (87). Sulfotransferase activity was also measured in this study and found to be significantly lower than STS activity, with no difference being detected between normal and malignant tissues. STS activity has also been detected in cultured cells derived from carcinomas of the ovary and vagina (163). Using an immunohistochemical technique positive STS expression was detected in 70% of ovarian clear cell adenocarcinoma tissue samples (164). Evidence showing that STS activity is present in hormone-sensitive tissues from the reproductive tract of women suggests that this enzyme may have an important role in regulating estrogen production in these tissues. With the development of potent STS inhibitors it will be possible to explore their therapeutic potential for the treatment of malignancies in the female reproductive tract.

ii Male, including the prostate gland

STS is present in the testes of mammals and it is likely that hydrolysis of steroid sulfates contribute to overall androgen production in this gland (165). An important role for STS has been postulated in the biochemical process of sperm maturation and capacitation (166). High levels of radiolabeled cholesterol sulfate are taken up by spermatozoa and this was mainly localized within the plasma membrane of the acrosome region. It is thought that the cholesterol sulfate may act as a stabilizing factor that is associated with sperm membranes during transit or storage, inhibiting the release of acrosomal enzymes while sperm remain in the male reproductive tract. STS is present in the female reproductive tract and hydrolysis of cholesterol sulfate by the STS may allow the release of acrosomal enzymes which facilitate penetration of the ovum by spermatozoa.

In males the prostate gland is likely to be the major peripheral site where STS activity makes an important contribution to the production of biologically active androgens. It has been known for many years that men who have been castrated as part of their
treatment for prostate cancer can have a further period of remission after adrenalectomy (167). The reason for this is thought to be due to the production of weak androgens, such as DHEAS, by the adrenal cortex which can be converted to testosterone and dihydrotestosterone in prostatic tissues (168). More recently the combination of castration or LHRH agonist with an anti-androgen has been shown to result in an improved therapeutic response in subjects with prostate cancer (169). While castration/LHRH agonist treatment removes the testicular source of androgen the use of an anti-androgen is thought to block the action of androgen derived from the adrenal cortex.

STS activity has been detected in prostatic tissue (170). In studies where the epithelial and stromal components of the prostate were separated the highest STS activity was found to reside in the epithelial compartment (171, 172). LNCaP cells, which are derived from prostatic cancer, also possess STS activity although at a somewhat lower level than that found in breast cancer cells (173). DHEAS was efficiently converted to DHEA in LNCaP cells and hydrolysis of this steroid sulfate was almost completely blocked by the STS inhibitor, EMATE. The non-steroidal inhibitor (p-O-sulfamoyl)-tetradecanoyl tyramine also inhibited the hydrolysis of DHEAS by these cells but was considerably less potent than EMATE. In view of the evidence supporting a role for STS in transforming weak androgens into biologically active androgen in the prostate, STS inhibitors could have considerable therapeutic potential for the treatment of prostate cancer.

F. Steroid sulfatase activity in osteoblast cells
The reduction in ovarian estrogen production that occurs at the menopause has been implicated as an important factor in the development of osteoporosis. Since studies have generally failed to detect any consistent reduction in plasma estrogen concentrations in women with osteoporosis compared with women of similar age without fractures, the possibility of in situ estrogen synthesis by bone cells was considered (174). In three human osteoblast cell lines, HOS, MG 63 and U2 OS, the main enzyme activities for estrogen synthesis, i.e. aromatase, 17βHSD type 1 and STS, were all detected (29). STS activity in the MG 63 osteoblasts was 1000-fold higher than aromatase activity, suggesting that the local formation of E1 from E1S
could be an important source of estrogen for regulating bone formation. In similar investigations HOS and MG 63 osteoblasts were shown to express STS mRNA and to be capable of utilizing both E1S and DHEAS as substrates for STS activity (30).

In view of the potential importance of local formation of estrogens by osteoblasts for the maintenance of bone structure, it is possible that inhibition of STS could result in an increased rate of bone loss in treated subjects. However, studies with tibolone, which is used for hormone replacement therapy, have suggested that tibolone or its metabolites may have tissue-specific inhibitory effects on STS activity (175). After ingestion, tibolone is rapidly converted to metabolites which exert estrogenic effects or have progestogenic/androgenic properties (176). While tibolone, or its metabolites, have estrogenic effects on bone and the central nervous system no estrogenic stimulation of breast tissue occurs (177). In an attempt to find an explanation for these important effects the ability of tibolone, its metabolites or EMATE to inhibit STS activity in breast cancer cells, endometrial cells or osteoblast cells was compared (175). All the compounds tested inhibited STS activity strongly in breast cancer cells and moderately in endometrial cells. In contrast, no significant inhibition of STS activity was detected in osteoblast cells. This study therefore raises the intriguing possibility that different tissues may express different isoforms of STS or may be subjected to different modes of regulation. As STS inhibitors have now entered clinical trials it will be important to confirm that compounds such as EMATE can act to inhibit STS activity in a tissue-specific manner.

G. Steroid sulfatase activity in leukocytes and thrombocytes

In addition to the widespread distribution of STS in body tissues the enzyme is also found in peripheral blood leukocytes (PBLs) and thrombocytes (26, 178). PBLs are capable of metabolising steroid sulfates and STS activity measurements, using these cells, have been used for the detection of the STS deficiency related to X-LI (179). Using $^3$H E1S as a substrate, STS activity in PBLs from women in the follicular phase of their menstrual cycles was found to be almost twice as high as in cells collected from luteal phase subjects (26). This finding suggests that the high levels of progesterone present during the luteal phase may be involved in regulating STS activity. STS activity in PBLs obtained from men is lower than that in cells from female subjects (26, 180). As many breast tumors are infiltrated by macrophages and
lymphocytes (181), it is possible that the STS activity of these cells may make an important contribution to estrogen synthesis within tumors.

The presence of STS activity in a readily available tissue, such as PBLs, suggested that these cells could be used to provide a relatively simple method to monitor the extent and duration of STS inhibition when these drugs became available. This contrasts with the complex double – isotope infusion technique that is currently used to monitor aromatase inhibition in postmenopausal women (182). The discovery of the first potent STS inhibitor, EMATE, led to a pre-clinical study to evaluate the use of measuring STS activity in PBLs to determine the effectiveness of this inhibitor (183). Two hours after the oral administration of EMATE to rats the extent of STS inhibition was similar in PBLs and liver. STS activity measurements in PBLs were also used to monitor inhibition of this enzyme in a preliminary male volunteer study in two subjects receiving 0.5mg/kg EMATE (183). Assays of STS activity in PBLs from these subjects revealed that inhibition was almost complete by 4h post-dosing and was maintained for at least 1 week. The ability to monitor the extent and duration of STS inhibition should be of considerable value when carrying out clinical trials to test the efficacy of this new form of therapy.

6. Tissue availability of steroid sulfates

A central question with regards to the ability of steroid sulfates to exert physiological or pathological effects is whether they are taken up by cells, as such, or whether hydrolysis is a pre-requisite for their entry into cells. It has generally been considered that while lipophilic unconjugated steroids are able to diffuse across cell membranes, polar hydrophilic steroid conjugates are unable to do so. Several studies have been carried out to investigate the uptake of steroid sulfates by cells and tissues using radiolabeled substrates but these results have been difficult to interpret (103, 184).

In the last few years convincing evidence has emerged for the existence of a super family of membrane transporter proteins (185). Some of these proteins appear to be involved in the specific uptake of organic anions, such as steroid sulfates, which have a negative charge and hydrophobic backbone. These transporters include the organic anion transporter and organic anion transporter polypeptide (OATP) proteins. Oatp1 was first identified in rats as a multispecific, sodium-ion-independent, transporter for
a range of xenobiotics, bile acids and conjugated metabolites (186). Subsequently other structurally related homologs of Oatp1, Oatp2 and Oatp3, were isolated (187, 188). In humans OATP was originally cloned from a human liver-derived cDNA library as a homolog of rat Oatp1 (189). A series of related homologs was subsequently identified in humans (OATP-B, OATP-C, OATP-D and OATP-E) which were expressed at varying levels in a wide range of tissues (190). OATP-E was expressed in several different cancer cell lines whereas OATP-D was not expressed in G1-101 breast cancer cells. Functional studies with HEK293 cells transfected with the cDNAs for the different transporters revealed that while OATP-B, -C, -D and –E all transported E1S, the highest activity was observed for OATP-B and OATP-C. Results from these studies show that OATPs are active transporters for E1S.

Using an immunohistochemical technique OATP-B was recently found to be highly expressed in the human mammary gland (191). As the main substrate for OATP-B is E1S it was suggested that the major physiological function of this carrier in the mammary gland is the uptake of E1S. In the normal mammary gland OATP-B expression was confined to myoepithelial cells. As these cells have been found to possess STS activity (192) it was postulated that the myoepithelial cells may be responsible for supplying non-sulfated estrogen to the adjacent epithelial cells. The major finding to emerge from this study was that OATP-B is strongly expressed in the majority of epithelial cells in invasive ductal carcinomas. In related in vitro studies uptake of E1S and DHEAS by OATP-B was found to be stimulated by prostaglandin A1 (PGA1) and PGA2, suggesting that the uptake of steroid sulfates could be regulated locally at the plasma membrane.

As the plasma concentrations of E1S are much higher than those of unconjugated E1 or E2 the finding of a specific transporter for steroid sulfates in malignant breast tissues is of considerable importance. STS activity and expression, as previously discussed, are elevated in breast tumors. Thus, all the elements are in place in breast tumors to ensure the efficient uptake of E1S from the circulation and its rapid hydrolysis to a biologically active estrogen. This combination of an effective transporter for E1S, and high STS activity, offers a likely explanation for the high concentrations of E1 and E2 that are found in breast tumors.
7. Steroid sulfatase inhibitors

In view of the important roles of STS in physiological and pathological conditions considerable research has been carried out to develop potent inhibitors of this enzyme (78-81, 193, 194).

i Alternative substrates
This type of compound, which contains at least one sulfate group in the structure, is designed to compete with E1S for binding to the STS enzyme active site and impede the hydrolysis of the natural substrate to E1 as a consequence. These inhibitors, in principle, are alternative substrates for STS whose sulfate group(s) are expected to be hydrolysed by the enzyme. The very first example of such a class of STS inhibitor was a series of 2-(hydroxyphenyl) indole sulfates, one of which (Fig 4, 1) showed an IC$_{50}$ value of 80 $\mu$M (196). Several synthetic and naturally occurring steroids were also investigated for STS inhibitory activity, of which 5-androstene-3$\beta$,17$\beta$-dial-3-sulfate (Fig 4, 2) was found to be the most potent ($K_i = 2.0 \mu$M) (197). Flavonoids daidzein 4$'$-O-sulfate (Fig 4, 4) and daidzein 4$'$,7-di-O-sulfate (Fig 4, 5) were synthesised and found to inhibit STS competitively with $K_i$ values of 5.9 and 1 $\mu$M respectively (198). However, inhibitors such as 2, 4 and 5 could potentially be problematic since their corresponding metabolites, androstenediol (Fig 4, 3) and daidzein (Fig 4, 6), are known estrogens rendering them of little value clinically for the treatment of hormone-dependent breast cancer.

ii Reversible inhibitors
The initial strategy employed for generating a lead STS inhibitor involved the replacement of the sulfate group (OSO$_3^-$) of E1S with surrogates or mimics such as phosphate, (199), phosphonates [-OP(=X)(OH)Me], (200-203), sulfonates (-OSO$_2$R), (204, 205) sodium methylenesulfonate (-CH$_2$SO$_2$Na$^-$), (205) sulfonyl halides (-SO$_2$Cl and -SO$_2$F), (206) sulfonamide (-SO$_2$NH$_2$), (206, 207) and the methylsulfonyl group (-SO$_2$CH$_3$) (206, 207). Most of these E1 derivatives were designed to compete with
E1S for the enzyme active site but remain metabolically stable by not acting as substrates. Phosphate esters of \( p \)-acylphenols and \( p \)-alkylphenols were also prepared and one derivative, \( n \)-lauroyl tyramine phosphate (Fig 5, 7) inhibited STS with \( K_i \) values of 3.6 \( \mu \)M and 520 nM at pH 7.5 and 7.0 respectively (208).

After the discovery of EMATE and as a result of the subsequent synthetic efforts that followed, its \( N \)-monomethyl- (Fig 5, 8) and \( N,N \)-dimethyl (9) derivatives were found to be weak reversible STS inhibitors (209, 210). Replacing the 3-O-atom of EMATE with other heteroatoms (Fig 5, S, 10 and N, 11) gave analogs that were also weak reversible inhibitors of STS (211). Several estrone 3-amino derivatives (e.g. CF\(_3\)CONH-E1) were prepared but these were only weak inhibitors (212).

Derivatives of 17\( \alpha \)-benzylestradiol (Fig 5, 12) bearing either a 4\(^{\prime}\)-t-butyl (Fig 5, 13), 3\(^{\prime}\)-bromo (Fig 5, 14) or 4\(^{\prime}\)-benzyloxy (Fig 5, 15) substituent were amongst the most potent reversible inhibitors reported to date showing IC\(_{50}\) values (JEG-3 cells) between 22 nM and 28 nM (213, 214). It was found that 13 was about 7-fold weaker than EMATE as an STS inhibitor when tested in a transfected HEK-293 cells preparation (214). A series of 17\( \alpha \)-alkan- or alkynamide derivatives of E2 were prepared and the propanamide 16 (Fig 5) gave an IC\(_{50}\) of 80 nM in JEG-3 cells (215). The relatively high potency against STS observed for 16 is evident of exploitation by the hydrophobic substituent of the hydrophobic binding area(s) that have been postulated to be in the vicinity of the D-ring of EMATE.

In an attempt to overcome the unwanted estrogenicity of some 17\( \alpha \)-substituted derivatives of EMATE (Fig 6, 36 and 37) (see Section 7, iii), several sulframates of C19 (androstene) or C21 (pregnene) derivative were prepared (Fig 5, 17, 18 and 19) (216). 17\( \alpha \)-t-Butylbenzyl-5-androsten-17\( \beta \)-ol (19) was the best reversible inhibitor (IC\(_{50}\) = 46 nM) in a homogenate preparation of HEK-293 cells and showed no estrogenic or androgenic activities in vitro (216).
The sulfamate derivatives of (E)- and (Z)-4-hydroxytamoxifen (Fig 5, 20 and 21 respectively) were prepared and found to competitively inhibit STS in a rat liver microsomes preparation with an apparent $K_i$ of 35.9 $\mu$M for the (E)-isomer (20) and $>500$ $\mu$M for the (Z)-isomer (21) (217). It appears that their sulfamate group was not activated to inhibit the enzyme in an EMATE-like manner.

Sulfamoyloxy-substituted 2-phenylindoles have recently been synthesised as anti-estrogen-based STS inhibitors (218). Compounds (Fig 5, 22 and 23) inhibited the conversion of E1S to E1 in MCF-7 breast cancer cells with IC$_{50}$ values of 0.3 and 0.2 $\mu$M respectively. Despite bearing a sulfamate moiety, the mechanism of action was not reported for this class of inhibitor.

Recently, a series of thiosemicarbazone derivatives of madurahydroxylactone were studied and the best agent, the cyclohexylthiosemicarbazone derivative (Fig 5, 24) inhibited STS non-competitively with a $K_i$ value of 0.35 $\mu$M and an IC$_{50}$ value of 460 nM in a placental microsomes preparation (219). A series of nortropinyl-arylsulfonylurea derivatives were prepared of which (Fig 5, 25) inhibited STS in a purified enzyme assay with an IC$_{50}$ value of 0.084 $\mu$M (c.f. EMATE 0.056 $\mu$M) (220).

Bayer identified from their compound library aryl piperazines 26 and 27 (Fig. 5) which inhibited STS in a STS protein preparation with a respective IC$_{50}$ value of 48 nM and 78 nM (221).

In addition to those agents specifically designed to inhibit STS, other experimental or clinically used endocrine agents including danazol, (61, 222) nomegestrol acetate (223), demegestone and chlormadinone acetate (224), ethinylestradiol (61), tibolone (Org OD14) and its metabolites (225), the ‘pure’ anti-estrogen ICI 164384 (226), tamoxifen and its metabolites (226), and pregnenolone 16$\alpha$-carbonitrile (227) have all been shown to exhibit STS inhibitory activities.

iii Irreversible inhibitors
The bulk of STS inhibitors reported to date belong to this class of inhibitor. Estrone-3-\(O\)-sulfamate (EMATE, Fig. 6), the very first inhibitor displaying such a mechanism of action, was originally designed to act as a surrogate of E1S. However, it was found to inhibit STS not only potently but also uniquely, in a time- and concentration-dependent manner indicating that EMATE differed mechanistically from its contemporary E1S surrogates and acted as an irreversible active site-directed inhibitor (78, 209). Although EMATE is orally active and highly potent, it is not suitable for use as a therapeutic agent in the treatment of hormone-dependent breast cancer because it has been shown subsequently to be five times more estrogenic than ethinylestradiol when administered orally in the rat (228).

a) Irreversible steroidal steroid sulfatase inhibitors

After the discovery of EMATE, all steroidal irreversible inhibitors that followed were analogs of EMATE designed to be less estrogenic than the parent, but to still possess similar or superior inhibitory activity, to that of EMATE.

The initial strategy was to introduce substituents such as 2-propenyl, \(n\)-propyl, nitro, methoxy, cyano and halogens to the A-ring of EMATE at the 2- and/or 4-positions (229). Analogs of EMATE with electron-withdrawing substituents on the A-ring showed comparable or higher potency than EMATE \textit{in vitro} (e.g. the 2-nitro derivative, 28, Fig. 6; IC\textsubscript{50} in a placental microsomes preparation = 30 nM). In comparison, those analogs with bulkier aliphatic substituents were found to be weaker STS inhibitors. Overall, the most successful A-ring modified analogs of EMATE were 2-methoxyestrone 3-\(O\)-sulfamate (2-MeOEMATE, Fig. 6) and 2-methoxyestradiol-3,17\(\beta\)-bis-\(O\),\(O\)-sulfamate (2-MeOE2bisMATE, Fig. 6) whose IC\textsubscript{50} values from placental microsomes were 30 nM (230) and 39 nM (231) respectively, indicating that these two derivatives were equipotent to EMATE in inhibiting STS \textit{in vitro}.

A recent paper described the synthesis of a series of steroidal 2',3'- and 3',4'-oxathiazines as inhibitors of estrone sulfatase (232). The most active compound in the series and with reduced estrogenic activity was (Fig 6, 29) which showed an IC\textsubscript{50}
value of 9 nM against the STS activity in an intact MCF-7 human breast cancer cells preparation. *In vivo*, this agent showed moderate antitumor activity against MCF-7 breast cancer xenografts in Balb/c athymic nude mice. The mechanism of action for compound (29) has not been reported. While it could simply act as a reversible inhibitor of STS, it is possible that compound (29), a Schiff base, could be hydrolysed/metabolised *in situ* to 2-formyl-EMATE (Fig 6, 30) which then acts as the active species inactivating the enzyme in a similar manner to EMATE.

In an attempt to understand the structure-activity relationships (SARs) for the sulfamate group of EMATE, two N-acylated derivatives were prepared of which N-acetyl-EMATE (Fig 6, 31), but not the benzoyl derivative (Fig 6, 32), inhibited STS irreversibly, albeit less potently and efficiently than EMATE (210).

Since it is accessible to synthetic modifications, the D-ring of EMATE has also been targeted for reducing the estrogenicity of the inhibitor. Early work had seen the reduction of the 17-carbonyl of EMATE to the methylene (CH₂) derivative, NOMATE (Fig. 6) which was as potent as EMATE but less estrogenic (230). In contrast, the (E)-17-oximino derivative (Fig 6, 33), inhibited STS equipotently to EMATE *in vitro* (> 99% inhibition at 0.1 µM in MCF-7 breast cancer cells) and *in vivo* but stimulated uterine growth in ovariectomized rats about 1.5-fold greater than that achieved by EMATE, suggesting that this agent had enhanced estrogenicity (233).

Introduction of hydrophobic substituents to the D-ring of EMATE was shown to increase its potency and significantly reduce its estrogenicity. Therefore, 17β-(N-alkylcarbamoyl)estradiol-3-O-sulfamates and 17β-(N-alkanoyl)estradiol-3-O-sulfamates were highly potent STS inhibitors with optimal inhibitory activities shown by their respective *n*-heptyl derivatives (Fig 6, 34 and 35) both showing IC₅₀ values of 0.4 nM in MDA-MB-231 cells (234). Using the estrogen-sensitive MCF-7 cell line which proliferates upon stimulation by estrogens, no significant estrogenic potential of both inhibitors was observed at a concentration of 1 µM, a dose which was about 2000-fold higher than their IC₅₀ values against STS (234). The hydrophobic side-
chains of these inhibitors were designed as membrane insertion region and to help anchor the inhibitors in cellular membranes, where STS resides.

It has already been illustrated that the introduction of hydrophobic substituents at the 17α-position of estradiol led to derivatives that exhibited potent reversible STS inhibition (see Section 7, ii). When two of these derivatives, namely 17α-benzyl-E2 (Fig. 5 12) and 17α-4′-t-butylbenzyl-E2 (Fig 5, 13) were sulfamoylated, their corresponding sulfamates (Fig 6, 36 and 37 respectively) were found to inhibit STS irreversibly in the same manner as EMATE. They were 5- to 14-fold more potent than EMATE in vitro at inhibiting the conversion of E1S to E1 in homogenates of HEK-293 cells transfected with STS (235). More significantly, sulfamates 36 and 37 were found to be nearly 600 and 55 times respectively more potent than their corresponding phenolic parent compounds (Fig 5, 12 and 13) as STS inhibitors in the same enzyme preparation. These results clearly demonstrate that a sulfamate group is crucial for potent inactivation of STS. Because of the potency exhibited by compound (13) against STS in vitro, it has been reasoned that this phenolic steroid released after the inactivation of enzyme by the irreversible STS inhibitor (37) would still exert reversible inhibition against any unreacted STS. Unfortunately, 17α-4′-t-butylbenzyl-E2MATE (37) was subsequently shown to be estrogenic in vivo rendering this inhibitor unsuitable for further therapeutic exploitation (216, 236) . On recognition that substitution at the 2-position of EMATE with a methoxy group abolished estrogenicity, but retained potency against STS, (230) 2-methoxy-17α-benzylestradiol-3-O-sulfamate (Fig 6, 38) and 2-methoxy-17α-4′-t-butylbenzylestradiol-3-O-sulfamate (Fig 6, 39) were prepared (237). As anticipated, both agents were found to be as potent as their corresponding 2-unsubstituted counterparts in the inhibition of STS activity in homogenates of transfected HEK-293 cells. In vivo, the 2-methoxy-17α-benzyl-derivative (38) showed no estrogenic activity in ovariectomised mice and efficiently blocked uterine growth induced by E1S.

Continual investigation into 17α-substituted analogs of estradiol as STS inhibitors gave two separate libraries of the N-derivatized 17α-piperazinomethyl derivatives of
estradiol and estradiol 3-O-sulfamate (238). The best STS inhibitors in both the phenol and sulfamate series (Fig 6, 40 and 41 respectively), as assayed in homogenates of HEK-293 cells transfected with STS, shared the same N-derivatization sequence, i.e. the secondary piperazino N-atom was acylated first with the amino acid phenylalanine whose primary amine was then amidated with 3-cyclopentyl propionic acid. At a 1 nM test concentration, sulfamate (41) inhibited STS by 94% which was close to that obtained with 17α-benzyl-E2MATE (Fig 6, 36) and 17α-4′-t-butylbenzyl-E2MATE (Fig 6, 37). The corresponding 3-hydroxy analog (40) inhibited STS by 50% at 1 µM and hence was a weaker STS inhibitor than 17α-benzyl-E2 (Fig 5, 12) and 17α-4′-t-butylbenzyl-E2 (Fig 5, 13) which inhibited STS by 71% and 98% respectively at the same concentration.

A novel D-ring enlargement of EMATE led to a series of N-substituted piperidinedione derivatives. Two compounds, the N-(propyl)- (Fig 6, 42) and N-(1-pyridin-3-ylmethyl)- (Fig 6, 43) derivatives, showed exceptional high potency with both sharing the same IC₅₀ value of 1 nM in a human placental microsomes preparation (c.f. EMATE, 18 nM) (256). The N-unsubstituted derivative (Fig 6, 44) showed similar potency (IC₅₀ = 20 nM) to EMATE indicating that the six-membered piperidinedione ring is a good mimic of the D-ring of EMATE. After an oral dose of 10mg/kg per day for 5 days, compounds (42) and (43) were found to inhibit rat liver STS by 99% (239). Both compounds were devoid of estrogenic activity in the rat uterine weight gain assay.

b) Irreversible non-steroidal steroid sulfatase inhibitors

It has long been recognised that non-steroidal agents themselves and their metabolites are less likely to exhibit unwanted endocrine effects in vivo than their steroidal counterparts. The initial development of non-steroidal irreversible STS inhibitors resulted in the preparation of the A/B ring mimic of EMATE, tetrahydronaphthalene (THN) 2-O-sulfamate (Fig 7, 45), and the mono- (Fig 7, 46) and bis-sulfamate (Fig 7, 47) derivatives of diethylstilbestrol (DES). While the bicyclic sulfamate (45) was a much weaker inhibitor than EMATE, (209), DES bis-sulfamate (47) was a moderate
STS inhibitor (IC\textsubscript{50} = 10 nM, MCF-7 cells) (79, 230). This finding indicated that it is not necessary to have a fused ring system for STS inhibition.

The finding that \textit{n}-lauroyl tyramine phosphate (Fig 5, 7; see Section 7, ii) was a significantly reversible STS inhibitor led to the development of a series of (\textit{p}-\textit{O}-sulfamoyl)-\textit{N}-alkanoyl tyramines, of which the \textit{N}-tetradecanoyl derivative (Fig 7, 48) was found to have an IC\textsubscript{50} value of 55.8 nM against STS derived from human placental microsomes (240). The IC\textsubscript{50} value for this agent in MDA-MB-231 human breast cancer cells was found to be 350 nM and the inhibition of STS was irreversible (241). When this agent was studied for inhibition of the proliferation of MCF-7 human breast cancer cells in the presence of E1S at 1 \(\mu\text{M}\), the IC\textsubscript{50} value obtained was 38 nM. It was postulated that the phenyl ring of these tyramine sulfamates mimics the A-ring of EMATE, while the amido functionality, which was shown to be crucial for inhibition by this class of inhibitors, participates in essential hydrogen bonding(s) (242). The optimal distance between the \textit{p}-sulfamoyloxyphenyl ring and the amido group was found to be 1 to 2 methylene units (243). Analogs of this class of STS inhibitor (Fig 7, 49 - 52) were prepared but they were only weak inhibitors with IC\textsubscript{50} values against the STS activity in placental microsomes ranging from 10-40 \(\mu\text{M}\) (230). \textit{In vivo}, while \textit{E}-capsaicin sulfamate (52) showed a modest degree of STS inhibition in rat uterine and liver tissues, other analogs were inactive (230).

Simple \textit{p}-sulfamates of benzoic acid esters, substituted benzene and phenyl ketones have also been pursued as STS inhibitors. One compound, the cyclo-octyl derivative (Fig 7, 53) was claimed to be the most active with an IC\textsubscript{50} value of 170 nM in a placental microsomes preparation (c.f. 500 nM for EMATE) (244). However, this IC\textsubscript{50} value of EMATE reported is much higher than values (18 – 80 nM) obtained by other groups using similar placental microsomes preparations (209, 219, 245).

A series of bicyclic coumarin sulfamates was synthesized as alternative A/B-ring mimics of EMATE, of which 4-methylcoumarin 7-\textit{O}-sulfamate (COUMATE, Fig. 7) showed an IC\textsubscript{50} of 380 nM in an MCF-7 cell preparation, (246) about 3-fold more potent than the bicyclic (Fig. 7, 45), but was still much weaker than EMATE (IC\textsubscript{50} =
65 pM in the same assay). This finding, for the first time, highlighted a relationship between the pKₐ value (or leaving group ability) of a parent phenol and the inhibitory activity of its sulfamate. For coumarin sulfamates, it was reasoned that the extended conjugation of the coumarin core structure as a result of its α,β-unsaturated lactone over the saturated cyclic hydrocarbon of THN enhances the overall potency of a coumarin sulfamate by virtue of lowering the relative pKₐ of the leaving phenol released during enzyme inactivation (246). This SAR was supported by the weaker inhibition exhibited by analogs of COUMATE in which i) the sulfamate group was relocated to the 6-position of the ring or ii) the extended conjugation in the coumarin motif was disrupted by either the reduction of the double bond or replacement of the carbonyl group with a methylene group (247).

Further extension of the coumarin sulfamate series has established that derivatives with hydrophobic substituents introduced at the 3- and/or 4-positions of COUMATE were more potent STS inhibitors (245, 247). Hence, a series of tricyclic coumarin sulfamates was developed, of which 667COUMATE (Fig. 7) was found to inhibit STS in a placental microsomes preparation with an IC₅₀ value of 8 nM, some 3-fold more potent than EMATE (245, 248). The apparent Kᵢ value for 667COUMATE was found to be 40 nM which was significantly lower than that for EMATE (670 nM) (245). This finding suggests that the lower IC₅₀ value observed for this non-steroidal inhibitor in comparison with EMATE could be attributed to a higher affinity of 667COUMATE for the enzyme active site in addition to an enhanced “sulfamoylation potential” of 667COUMATE as a result of the better leaving ability of its phenolic coumarin precursor (Fig 7, 54) [pKₐ ca. 8.5 for (54) vs ca. 10 for E1].

Upon extension of the tricyclic coumarin sulfamates series, it was shown that the in vitro inhibitory activity was the highest with 6610COUMATE (Fig. 7) (IC₅₀ = 1 nM, placental microsomes) (249). In vivo, this compound was found to be marginally more potent than 667COUMATE in inhibiting the STS activity in rat liver tissues (89% vs 86%, at 1 mg/kg, p.o.). Surprisingly, the analog 6615COUMATE (Fig. 7), whose IC₅₀ value in vitro was 370-fold higher than that of 6610COUMATE, was the most potent compound of the series in vivo (94%, at 1 mg/kg, p.o.) (249).
Because of the structural resemblance of the naturally occurring flavonoids to estrogens, sulfamates of flavones, isoflavones and flavanones were prepared and compounds such as (Fig 7, 55 - 57) were shown to be moderate STS inhibitors (230, 250). Further exploitation of this class of compounds led to a series of chromenone- and thiochromenone-based sulfamates, (118, 251) of which compound (58) showed an IC\textsubscript{50} value of 0.34 nM in purified STS, rendering this compound about 170-fold superior to EMATE (IC\textsubscript{50} = 56 nM from the same assay). However, compound (58) was estrogenic, stimulating the growth of MCF-7 breast cancer cells by 99% at 100 nM (252).

Since 17\textalpha-4′-t-butylbenzyl-E2MATE (Fig 6, 37) was a highly potent STS inhibitor, a series of structurally related 4-substituted monoaryl sulfamates was designed as non-steroidal mimics of sulfamate (37). The optimal inhibitor of the series, compound (59) (IC\textsubscript{50} = 0.4 nM) was more potent than EMATE (IC\textsubscript{50} = 0.9 nM) in homogenates of HEK-293 cells transfected with STS (236).

It has been observed that 4,4′-benzophenone-\textit{O,O′-bis}-sulfamate (BENZOMATE, Fig 7, 60) was a potent inhibitor (IC\textsubscript{50} = 190 nM, c.f. 56 nM for EMATE) against recombinant human STS (253). SAR studies have shown that the \textit{bis}-sulfamate motif is crucial to the high potency since the monosulfamate derivative, i.e. benzophenone 4-sulfamate (Fig 7, 61) was about 25 times less active. These results have been confirmed independently and expanded in a recent report (254).

Some sulfamic acid biphenyl esters were also reported to be STS inhibitors. For example, propyl 4′-sulfamoyloxy-biphenyl-4-carboxylate (Fig 7, 62) (255) inhibited STS in a human placental microsomes preparation with an IC\textsubscript{50} value of 3.5 \textmu M, suggesting that this class of inhibitor inhibited STS weakly.

8. Active pharmacophore required for potent inhibition
All irreversible STS inhibitors reported to date share a common pharmacophore, that is, a phenol sulfamate ester (246). The potency of this class of inhibitor is apparently increased by substituent(s) that exploit favorable hydrophobic interactions with the enzyme active site, (234, 235, 240, 245, 246, 256) and/or enhance the “sulfamoylation potential” of the sulfamate group (229, 246, 257). However, it is important to note that the presence of a phenol sulfamate ester moiety does not always result in an irreversible inhibitor. Despite the higher affinity of the sulfamate derivative of (E)-4-hydroxytamoxifen (Fig 5, 20) for STS, this compound only inhibited the enzyme competitively (217), suggesting that its sulfamate group was not activated to inhibit the enzyme in an EMATE-like manner.

The phenolic component of the pharmacophore is crucial for high potency since sulfamate ester of aliphatic alcohols were weak STS inhibitors. Hence, alkyl O-sulfamates (258), and 3β-sulfamate derivatives of the aliphatic C19 and C21 steroids such as dehydroepiandrosterone 3β-O-sulphamate (79) and compounds (Fig 5, 17 – 19) (216) were significantly weaker STS inhibitors than EMATE and its congeners. The lack of potent inhibitory activity observed for these aliphatic compounds, despite the presence of a sulphamate group, could be attributed to the fact that their sulfamate group is not activated in the same manner as those of EMATE-like phenol sulfamate esters. The poorer leaving ability of their parent aliphatic alcohols (pKₐ > 16 for most primary and secondary alcohols vs pKₐ ~ 10 for phenol) in effect precludes the cleavage of the S-O bond of the sulfamate group which is thought to be crucial mechanistically for the inactivation of the enzyme by EMATE-like inhibitors via sulfamoylation (i.e. enzyme-SO₂NH₂).

Although non-sulfamoylated phenolic compounds such as (Fig 5, 12 and 13) were good reversible inhibitors of STS in vitro, the sulfamate (Fig 6, 37) was a time-dependent inactivator with superior potency to its phenolic counterpart (13) (see Section 7, iii) (235). The N-monomethyl (e.g. Fig 5, 8) and N,N-dimethyl derivatives of EMATE (e.g. Fig 5, 9) were weak reversible inhibitors of STS although N-acetyl-EMATE (Fig 6, 31), but not the benzoyl derivative (Fig 6, 32), (209, 210) inhibited the enzyme irreversibly, albeit less potently than EMATE. Analogs of EMATE in
which the 3-O-atom was replaced by other heteroatoms (Fig 5, S, 10 and NH, 11) were only weak reversible inhibitors of STS (211). All these findings demonstrate that a phenol sulphamate ester with no substitutions at the N-atom (i.e. H2NSO2O-Ar) is the prerequisite for highly potent irreversible STS inhibition.

The pharmacophore for reversible STS inhibitors has not yet been established. It is anticipated that the SAR studies of several new classes of reversible inhibitors will provide this information in the future. The activity of this class of inhibitor is presumably dependent upon the inhibitor acting so as to mimic the steroid structure, in a similar way to the prototype danazol.

9. *In vivo* activity of steroid sulfatase inhibitors and efficacy in tumor models

Danazol, a synthetic derivative of 17α-ethinyl testosterone, was one of the first compounds shown to be active *in vivo* as a STS inhibitor. This compound is used in the treatment of endometriosis and was found to increase the ratio of DHEAS: DHEA in plasma suggesting that it was acting as a STS inhibitor (222). Studies with the first compound that was specifically designed and synthesized as a STS inhibitor, estrone-methylthiophosphonate (E1-MPT, see Section 7, ii), revealed that it could reduce plasma E2 levels in rats, indicating that it was active *in vivo* (79). As danazol and E1-MPT are both reversible inhibitors their effects on *in vivo* STS activity can only be measured indirectly via changes in circulating hormone levels.

The identification of EMATE as the first potent STS inhibitor in *in vitro* screening assays was rapidly followed with studies to test its efficacy *in vivo* as an inhibitor. Daily subcutaneous (s.c.) administration of EMATE (10 mg/kg) to rats was found to almost completely (>99%) inhibit the ability of liver STS to hydrolyse both E1S and DHEAS (259). To examine the duration of STS inhibition, EMATE was administered as a single 10 mg/kg s.c. dose or daily at this level for 7 days. A single dose was found to inhibit STS activity in liver, brain, adrenal, uterine and ovarian tissues for up to 3 days with only 10-15% recovery being detected 7 days after dosing. Multiple dosing with EMATE resulted in complete inhibition of STS activity in
tissues for up to 10 days after the end of dosing. EMATE was also found to be active as a STS inhibitor after oral dosing. The reason for the long duration of steroid-based STS inhibitors, such as EMATE, remains unclear. The half-life of STS has been reported to be of the order of 4 days, but as STS activity continues to be inhibited by EMATE for a much greater length of time, it is likely that EMATE forms a depot in tissues, with its sustained release contributing to its prolonged inhibition of STS activity. Alternatively, EMATE and related sulfamate-based inhibitors, may have a long half-life in blood. The NMU-induced mammary tumor model in rats was used to test the ability of EMATE to inhibit the growth of E1S-stimualted tumors in ovariectomized rats. Over a 12 day period EMATE (10 mg/kg/day) induced significant tumor regression and also completely inhibited tumor STS activity (259). Recently, the 2-methoxy derivatives of EMATE have also been shown to be equipotent to EMATE, as STS inhibitors, in in vivo studies (231).

Similar studies have also been carried out to assess the in vivo potency of COUMATE and a series of tricyclic coumarin sulphamates, including 667 COUMATE. Daily dosing with the 2-ring coumarin sulfamate, COUMATE, at 10 mg/kg, only resulted in 85% inhibition of STS activity with full restoration of STS activity occurring by 7 days (260). Thus, COUMATE was less active in vivo than EMATE. The development of a series of tricyclic coumarin sulfamates, including 667 - and 6615 COUMATE, resulted in the identification of non-steroidal STS inhibitors that were equipotent in vivo with EMATE (248, 249, 261). In the NMU-induced mammary tumor model 667 COUMATE caused significant regression of E1S-stimulated tumor growth at 10 mg/kg (85 ± 5%) and at 2 mg/kg (56 ± 13%). These in vivo studies in ovariectomized rats confirmed that 667 COUMATE was not estrogenic and together with its ability to inhibit E1S-stimulated mammary tumor growth in rats, led to this compound being selected for therapeutic development.

10. Dual function inhibitors
i. Dual aromatase – sulfatase inhibitors
As noted earlier, there are two pathways by which estrogens can be synthesized in postmenopausal women i.e. the aromatase and sulfatase routes. If STS inhibitors prove to have clinical efficacy in women with breast cancer it would be logical, as a
next step, to test them in combination with an aromatase inhibitor. Such an approach would maximise the deprivation of estrone, and steroids such as Adiol, and could enhance the response rates to this form of endocrine inhibitor therapy. While it would be possible to administer aromatase and STS inhibitors as separate agents an alternative approach would be to develop single molecule compounds with dual aromatase-sulfatase inhibitor (DASI) properties.

Initial studies in this area took advantage of the fact that a number of flavonoids can inhibit aromatase activity (262, 263). It was reasoned that sulfamoylation of this class of compound could give rise to molecules with DASI properties. Sulfamoylation of 4’-hydroxy and 4’,7- dihydroxyisoflavone to give the 4’-mono- and 4’,7-bis-sulfamates revealed that these compounds could inhibit STS activity in vitro. At 1 µM, when tested using intact MCF-7 breast cancer cells, the mono- and bis-sulfamate derivatives inhibited STS activity by 83% and 90% respectively (230). Both compounds were active in vivo as STS inhibitors but were considerably less potent than EMATE. After a single 10mg/kg, p.o., dose to adult female Wistar rats, liver STS activity was inhibited by 62% and 81% by the mono- and bis-sulfamate derivatives respectively. While this study indicated that it should be possible to employ a compound with aromatase inhibitory properties to develop a DASI, it was apparent that if this research was to lead to a therapeutic agent, it would be essential to use a molecule with more potent aromatase inhibitory properties than the isoflavones.

A more recent approach has been to sulfamoylate a number of third generation, non-steroidal, aromatase inhibitors (257). These inhibitors contain a triazole ring which co-ordinates reversibly to the heme iron of the aromatase. This class of aromatase inhibitors is reversible, in contrast to the steroid - based inhibitors, such as exemestane, which act as irreversible inactivators. Thus, the incorporation of the active pharmacophore required for STS inhibition i.e. a phenol sulfamate ester, to a triazole-containing aromatase inhibitor would give rise to an irreversible STS inhibitor, but reversible aromatase inhibitor.

To test the validity of this concept the third generation aromatase inhibitor YM 511 was chosen for initial sulfamoylation (265). YM 511 is a selective, potent aromatase
inhibitor which was recently reported to give an objective response rate of 20.4% when tested in a Phase II trial (266). Using JEG-3 choriocarcinoma cells, which possess both STS and aromatase activities, the IC$_{50}$ value for the inhibition of aromatase activity by YM 511 was 0.5 nM, while this compound was inactive against the STS enzyme (257). Synthesis of a $p$-sulfamoyloxybenzyl derivative of YM 511 yielded a compound which had both moderate aromatase and STS moderate inhibitory properties (IC$_{50}$ values; arom = 100 nM; STS = 227 nM). The incorporation of a halogen to give the $m$-bromo derivative of this compound significantly increased both its aromatase and STS inhibitory properties (IC$_{50}$ values: arom = 0.82 nM; STS = 39 nM) with a potency against aromatase in the same order of magnitude as YM 511. In vivo in intact rats, using the pregnant mares serum gonadotropins stimulated ovarian aromatase model, the $m$-bromo-$p$-sulfamoyloxybenzyl derivative of YM 511 gave 68% inhibition of aromatase and almost complete (>98%) inhibition of STS activity.

Thus, these studies have revealed that it will be possible to engineer single molecules which possess both potent aromatase and STS inhibitory properties. The development of this class of DASI could offer considerable therapeutic advantage for the treatment of hormone-dependent breast cancer over the use of either an aromatase or STS inhibitor alone.

ii Steroid sulfatase and anti-angiogenic microtubule disruptors

The finding that EMATE, as discussed previously, proved to be a potent estrogen in rodents made it unsuitable for development as an anti-cancer agent for postmenopausal women. In an attempt to reduce the estrogenicity of EMATE, whilst retaining the potent STS inhibitory properties associated with this type of molecule, a number of modifications were made to the A-ring of the steroid nucleus (229). It had previously been shown that substitution of the aromatic A-ring at C-2 and/or C-4 of the steroid nucleus by nitro-, n-propyl or allyl groups greatly reduces their estrogenicity compared with the parent compound (267, 268). While the addition of a 4-allyl, 4-n-propyl or 4-nitro group to EMATE resulted in derivatives that were active as STS inhibitors in vivo, the 4-nitro EMATE retained some estrogenicity.

A series of sulfamoylated derivatives of 2-methoxyestrone (2-MeOE1) and 2-methoxyestradiol (2-MeOE2) was also synthesized and tested (229, 269). 2-MeOE2, a
natural endogenous estrogen metabolite, had previously been shown to be cytotoxic to MCF-7 breast cancer cells when tested at relatively high concentrations (270). There is currently considerable interest in the use of 2-MeOE2 for cancer therapy (271). Production of 2-MeOE2 appears to be increased in women at low risk of breast cancer and it has been suggested that it acts as the body’s natural anti-mitotic metabolite (272, 273). 2-MeOE2 inhibits the proliferation of a wide range of ER+/ER- breast cancer cells. At relatively high doses it is also active in vivo against transplanted Meth-A sarcomas and B16 melanomas in C3H mice and human MDA-MB-435 (ER-) melanoma cells in mice (274, 275). In addition to its anti-proliferative effects, 2-MeOE2 is also a potent inhibitor of angiogenesis in vitro and in vivo (274, 275)

The 2-methoxyestrogen sulfamate derivatives retained potent STS inhibitory properties. A single oral dose of 10 mg/kg of 2-MeOEMATE or 2-MeOE2-bisMATE inhibited rat liver STS by >90% (229, 264). Further investigations with the 2-methoxyestrogen sulfamate derivatives revealed that, like 2-MeOE2, they inhibited the proliferation of ER+/ER- breast cancer cells, being considerably more potent than 2-MeOE2 (276, 277). They induced a G2-M cell cycle arrest and induced cells to undergo apoptosis. In contrast to 2-MeOE2, which induces a reversible G2-M arrest, the cell cycle arrest induced by 2-methoxyestrogen sulfamate derivatives was irreversible. In vivo 2-MeOE2-bisMATE (20 mg/kg/day, p.o) for 28 days almost completely inhibited the growth of tumor xenografts derived from MDA-MB-435 (ER-) melanoma cells in nude mice (278). The sulfamoylated derivatives of 2-MeOE2 have a superior bioavailability and pharmacokinetic profile to that of 2-MeOE2 itself (278). This most likely results from their ability to bind to carbonic anhydrase II (CAII) in red blood cells (rbcs) (see Section 10 iii) and undergo liver transit without first pass metabolism (279).

Like 2-MeOE2, the sulfamoylated derivatives are also thought to act by binding to the colchicine site on tubulin. They also effectively inhibit the paclitaxel induced polymerisation of tubulin, suggesting that they act to inhibit microtubule dynamics (277). 2-MeOE2 has a very low affinity for ERα/ERβ and it is most likely that it acts via a receptor-independent mechanism to inhibit cell proliferation (280). Recently,
MeOE2 was shown to inhibit tumor growth and angiogenesis by reducing the expression of hypoxia-inducible factor-1α (281).

Thus, this class of 2-methoxyestrogen sulfamates are potent STS inhibitors. In addition, they also act to disrupt microtubules, inhibit glucose uptake (282) and are potent angiogenesis inhibitors (283). Attacking tumor growth at multiple points may offer considerable therapeutic advantage over drugs that are only active against a single target.

iii Steroid sulfatase and carbonic anhydrase
During early pre-clinical evaluation, EMATE was found to inhibit STS activity in rats after oral administration (259). This was unexpected, as derivatives of natural estrogens are usually rapidly inactivated after oral ingestion during their first transit through the liver (284). To date, this problem has been overcome either by preventing metabolic inactivation, for example by the introduction of a 17α-ethyl group, or by administering large doses of estrogen. Both approaches result in an increase in the exposure of liver tissues to estrogens which can have a number of adverse effects, including an increase in the production of clotting factors (285). The pre-clinical development of EMATE, as a STS inhibitor, was halted when it was discovered that its estradiol analog was five times more potent than ethinylestradiol on oral application to rats (228). This finding appeared to render EMATE unsuitable for development for breast cancer therapy where complete deprivation of estrogen is required. It was subsequently revealed that earlier studies with N,N-dimethylated sulfamoylated derivatives of estrogens had also shown potent estrogenic properties in rodents but that metabolites of such compounds could accumulate in rbcs. Recent studies have confirmed that estradiol-3-O-sulfamate, the C17-reduced form of EMATE, is also taken up by rbcs and 30 min after administration to rats 98% of dose in blood is present in rbcs (286). While some aryl sulfamates, such as 667 COUMATE, are relatively unstable when added to plasma *ex vivo*, they are stabilized *in vivo* by sequestration into rbcs and binding to CAII (279,287). In general aryl sulphamates, especially those of a steroidal nature, are stable.
Estradiol-3-O-sulfamate per se does not bind to the ER and therefore acts as a pro-drug for the natural estrogen, estradiol (286). On oral application it does not have an estrogenic effect on the liver indicating that after absorption it must rapidly enter rbcs and transit the liver without undergoing metabolic inactivation. The finding that sulfamates are able to transit the liver in rbcs without being degraded therefore offers an explanation as to why EMATE is active as a STS inhibitor when administered orally.

Many sulfonamide drugs, such as acetazolamide, which are structurally similar to the sulfamate-based STS inhibitors, are also transported in rbcs (288). Their transit in rbcs is facilitated by binding to CAII which is present in the cytosol of rbcs. It was therefore reasoned that the sulfamate – bearing steroidal and non-steroidal STS inhibitors may also interact with CAII.

The ability of a number of STS inhibitors, including EMATE and 667 COUMATE, to dock into the active site of CAII was initially examined using the known crystal structure for this enzyme (289, 290). Both compounds were found to dock into the active site of CAII. Subsequent studies, using hCAII derived from rbcs, revealed that EMATE and 667 COUMATE are both good inhibitors of CAII activity (IC$_{50}$ values 42 nM and 25 nM respectively). They are equipotent with the established CAII inhibitor, acetazolamide (IC$_{50}$ 25 nM), which is widely used for the treatment of a number of pathological conditions, including glaucoma. EMATE has been co-crystallized with CAII and the ligand - protein complex studied by X-ray crystallography (291), showing a good correlation with the structure predicted by docking studies (Fig 8).

CAII is a member of a family of 14 CAs which catalyse the reversible hydration of CO$_2$ to HCO$_3^-$ (292). While CAII has an intracellular location other CAs, e.g. CAIX and CAXII, have their active site domains located extracellularly. There is convincing evidence that the expression of these CAs is increased in many tumors, where their action to acidify the extracellular milieu, may give tumors a growth advantage over normal tissues (293-295). Expression of CAIX and CAXII is increased under hypoxic condition through the HIF1-α pathway (296, 297). In squamous cell head and neck
cancer over expression of CAIX was found to be associated with resistance to radiation and chemotherapy (298). Therefore, in addition to CA inhibitors having a potential therapeutic role in the treatment of some cancers, inhibition of CAIX may render some tumors sensitive to the use of medication or chemotherapy.

Although the crystal structure of hCAIX has not yet been reported it has a 41% homology with hCAXII for which the crystal structure is known (299). Docking studies were therefore carried out, with a series of sulfamate-based STS inhibitors, into the extracellular domain of hCAXII in order to predict whether these compounds might also inhibit the activities of hCAIX and hCAXII (279). These studies revealed that the sulfamate-based STS inhibitors could dock into the active site of hCAXII and should be able to inhibit hCAIX and hCAXII. It was recently confirmed that EMATE, and its bis-sulfamate derivative, are potent inhibitors of hCAIX (300).

There is evidence that some CA inhibitors, such as acetazolamide, can inhibit the \textit{in vitro} invasion of renal cancer cells (301). They can also produce additive delays in tumor growth \textit{in vivo} when used with other cytotoxic agents (302). The finding that both steroidal and non-steroidal sulfamates, originally developed as STS inhibitors, are also potent CAII and CAIX inhibitors, raises the intriguing possibility that inhibition of CAs may contribute to the overall anti-cancer efficacy of this class of drug.

11. Mechanism of steroid sulfate hydrolysis and steroid sulfatase inhibition

STS is bound to the membrane of the endoplasmic reticulum. The highly hydrophobic nature of the enzyme has hampered its purification to homogeneity in quantities sufficient for crystallisation. To obtain STS in a pure form, attempts were made i) to express the protein, using STS cDNA, in the pGEX2T expression system; ii) to express a mutant form of the protein, in which the putative membrane-spanning domain was deleted, in CHO cells; and iii) to isolate a soluble STS from the snail \textit{Helix pomatia} (303). Recently, through exploitation of optimal solubilisation and detergent conditions to protect the structural and functional integrity of the molecule, thereby preventing non-specific aggregation and other instabilities, human STS was successfully purified and crystallized (304) and its crystal structure (Fig. 9) was reported subsequently (305).
The overall shape of the protein is “mushroom-like” with the crown protruding towards the lumen side of, and the stalk traversing through, the lipid bilayer of the endoplasmic reticulum (Fig. 9). Similar to its closely related soluble enzymes arylsulfatase A (ARSA) and arylsulfatase B (ARSB), whose crystal structures were published a few years ago, (2, 306) STS shares a similar catalytic site topology and a unique, but universal for all sulfatases, post-translational modification of a conserved cysteine residue to a formylglycine (FGly, •−CHO) residue (307-309). As observed for ARSB, the resting state of human STS at the catalytic site consists of a sulfated gem-diol form of FGly, i.e. [FGlyS, •−CH(OH)OSO₃] which is, in all likelihood, coordinated to a bivalent Ca²⁺ cation (Fig. 10). The catalytic site of STS active site is highly homologous to those in ARSA and ARSB. All three enzymes share nine identical catalytically important residues, namely three aspartic acid residues, two histidine residues, two lysine residues, one arginine residue and the FGlyS residue. In STS, these residues are Arg35, Arg36, Arg342, His136, His290, Lys134, Lys368, Arg79 and Fgly75 (Fig. 10). The only difference apparently is the 10th residue in STS which is assigned to be Gln343 instead of an asparagine as in both ASA and ARSB. When the sulfate group of E1S is superimposed with FGlyS in the crystal structure, amino acid residues Leu74, Arg98, Thr99, Val101, Leu103, Leu167, Val177, Phe178, Thr180, Gly181, Thr484, His485, Val486, and Phe488 surround and interact favorably with the steroid scaffold suggesting that some of these amino acid residues could be involved in substrate recognition via hydrophobic contacts.

It has been demonstrated that the gem-diol form of FGly [•−CH(OH)OH] is crucial to the hydrolysis of sulfate substrates by ARSA and ARSB (310). Hence, the putative mechanism of STS for the hydrolysis of E1S to E1 is depicted in Fig. 11. The first step involves the regeneration of the gem-diol form of FGly from FGlyS via i) desulfation, catalysed by the non-esterified hydroxyl group, followed by the attack of a molecule of water on the FGly intermediate, or ii) a direct attack of a molecule of water on the sulfur atom of FGlyS. One of the hydroxyl groups of •−CH(OH)OH then attacks the sulfur atom of E1S, releasing E1 and regenerating FGlyS as a consequence.
Since the sulfamate group is acting as a mimic of the sulfate group of E1S, it is reasonable to expect that the mechanism of action for sulfamate-based STS inhibitors such as EMATE would also involve the FGlyS residue. One proposed mechanism of STS inhibition by EMATE is shown in Fig. 12 which involves a nucleophilic attack on the sulfamoyl group by $\bullet=\text{CH(OH)OH}$ in the enzyme active site. It is not clear if structure I could be a ‘dead-end’ product or will undergo further modifications to yield a species that irreversibly inactivates the enzyme. The fact that the sulfamoyloxy group of EMATE could exist in an anionic form ($\sim\text{OSO}_2\text{NH}^-$, vide infra) would suggest the existence of a mono-anionic form of I which might deactivate its sulfur atom and hence hamper desulfamoylation of I in a manner similar to that proposed for the regeneration of $\bullet=\text{CH(OH)OH}$ from FGlyS in Fig. 11.

Bond and colleagues (2) proposed that the resting FGlyS observed in the crystals of ASB could be in equilibrium with the free form, i.e. FGly, which could be an intermediate in the regeneration of $\bullet=\text{CH(OH)OH}$ from FGlyS as depicted in Fig. 11. For these reasons, a potential nucleophilic attack on the formyl group by the lone-pair electrons of the N-atom of the sulfamate group of EMATE-like compounds could be envisaged. However, there is now strong evidence to suggest that the nucleophilic attack on the carbonyl group of FGly could well be initiated not by a neutral sulfamate but by its mono-anionic form ($\sim\text{OSO}_2\text{NH}^-$). Since the sulfamate moiety is presumably acting as a sulfate surrogate, its anionic form should presumably interact more favorably with the enzyme active site and hence compete more effectively against E1S for binding than the neutral form.

Anderson and colleagues demonstrated that the mono-anionic form of the phosphate group of estrone phosphate bound most tightly to STS (199). It has been shown that EMATE binds to the crystal structure of carbonic anhydrase II using a coordination of the sulfamate anion to the active site zinc atom (291). Previous studies on various sulfamates have shown that the N-proton is fairly acidic with a $pK_a$ value in the range of 7–11, e.g. ca. 9.5 for EMATE (in 70% aqueous MeOH) (311) and ca. 9.1 for 667COUMATE (in 50% aqueous MeOH) (245). This implies that at physiological
pH a significant proportion of the weakly acidic EMATE and 667COUMATE could be in their conjugate base form. Given that several lysine and histidine residues are amongst the essential amino acids lining the catalytic site of STS (Fig. 10), it is conceivable that N-deprotonation of EMATE-like compounds by these basic amino acid residues takes place and the resulting anionic species then acts as a nucleophile attacking the FGly. Two putative pathways of such an attack by EMATE are depicted in Fig. 13. The hemiaminal-type intermediate (I, Fig. 13) so formed could then be hydrolysed to give estrone and the intermediate II, which upon dehydration gives an imino structure IV (Fig. 13, path A). Alternatively, IV could be formed via hydrolysis of the ester III after the dehydration of the hemiaminal intermediate I (Fig. 13, path B). It has also been suggested that IV could be formed via an attack on FGly by sulfamic acid which is released upon the hydrolysis of the sulfamate group of EMATE-like compounds (255). Structures II, III and IV and possibly even I are proposed to be ‘dead-end’ products and no regeneration of FGly, as depicted in Fig. 11, is therefore anticipated. The formation of an azomethine adduct similar to III or IV is certainly not unprecedented for the non-enzymatic chemical reaction. When a solution of 2-nitrophenol in N,N-dimethylformamide (DMF, HCONMe₂) was treated with sodium hydride followed by sulfamoyl chloride, an azomethine adduct of 2-nitrophenol-O-sulfamate and DMF (i.e. Me₂N-CH=N-SO₂O-Ph-2-NO₂), was isolated as a minor product which was shown to be stable and resistance to hydrolysis (247).

While the putative mechanisms of action depicted in Figures 12 and 13 suggest the involvement of •−CH(OH)OH and FGly, it is also quite possible that irreversibly inhibiting sulfamate esters, like EMATE, could inhibit STS in a more random manner by a specific or non-specific sulfamoylation of amino acid residues in the active site. Such proposed mechanisms are shown in Fig. 14. Path A involves an attack by a nucleophilic amino acid residue in the active site. This mechanism is analogous to Fig. 12 except that the attacking species on the sulfamate group of EMATE is a nucleophilic amino acid residue other than •−CH(OH)OH. Path B involves the generation of a highly electrophilic sulfonylamine species via an E1cB process, either from the bound mono-anion or possibly initiated by an enzyme catalysed N-proton abstraction and stimulated by hydrogen bonding to the bridging O-atom or
coordination of the bridging $O$-atom to $Ca^{2+}$ ion. Such collapse of a sulfamate ester is well precedented in their non-enzymatic chemistry (312, 313).

Before the publication of the crystal structure of STS, evidence for the involvement of two amino acid residues in the inactivation process was provided by the biphasic nature of inactivation exhibited by EMATE and that two ionisable groups with $pK_a$ values of 7.2 and 9.8 were identified upon analysis of the pH dependence of enzyme activity and of enzyme inactivation by EMATE (314). The finding that Rose Bengal inhibited STS activity in a dose-dependent manner strongly suggested that a histidine was involved in the catalytic mechanism. It was originally suggested that the second residue was a tyrosine but the involvement of this amino acid in the catalytic mechanism of STS was subsequenly ruled out by the absence of a tyrosine amongst the conserved active site residues throughout the entire family of sulfatases, on publication of the first crystal structure of a sulfatase (2). When it was reported that the two lysine residues and a histidine residue are important, *inter alia*, for sulfate group binding and catalysis respectively in ARSA, (315) the identity of the second residue was considered to be a lysine, whose $pK_a$ value (as a conjugate acid) of ~10 in proteins (316) matches the experimental finding. With what is now known about the topology of the catalytic site of STS as a result of its crystal structure, the attacking nucleophile in mechanism A and the N-proton abstracting amino acid residue in mechanism B of Fig. 10 is most likely to be either $\text{Lys}^{134}$, $\text{Lys}^{368}$, $\text{His}^{136}$ or $\text{His}^{290}$. It is highly unlikely that the reactive sulfonylamine released, as proposed in mechanism B, would attack an immediate nucleophile within the catalytic site, and not one more remotely located from the enzyme active site. With the presence of $\text{Lys}^{134}$, $\text{Lys}^{368}$, $\text{His}^{136}$ and $\text{His}^{290}$ in the catalytic site of STS, it could be envisaged that the products of inactivation would most likely be sulfamoylated enzyme intermediates or lysine- or histidine-derived sulfamides ($\sim$NHSO$_2$NH$_2$ or $\sim$NSO$_2$NH$_2$), which are proposed to be ‘dead-end’ products. From literature precedent and also our own experience in the handling of this type of compounds, sulfamides such as estrone 3-$N$-sulfamide (211) are inactive and stable entities, rendering them excellent candidates for a ‘dead-end’ product of enzyme inactivation.
The proposed mechanisms above represent the most likely possibilities for the inactivation of STS by an active site-directed EMATE-like inhibitor. However, all of them remain hypothetical since there are no experimental data available yet to support any of these mechanisms. Nonetheless, these hypotheses represent viable models for understanding how inhibition of STS by sulfamate esters may proceed, and should promote the design of experiments to test such models. Since technology is now available for isolating and crystallizing the membrane-bound STS, one challenge ahead is the crystallization of an inactivated STS after the enzyme has been incubated with an irreversible inhibitor such as EMATE. Such a crystal structure should help defining the role of the sulfamate group in the mechanism of action of EMATE-like sulfamate-based STS inhibitors and provide information on the participating essential amino acid(s) in the inactivation process.

12. Future perspectives
The discovery that steroidal and non-steroidal aryl sulfamates are potent STS inhibitors will enable the roles that this enzyme has in physiological processes to be explored. While the enzyme is known to be widely distributed the lack of potent inhibitors has previously made it difficult to determine its role in the body. More importantly, evidence to support a major role for the enzyme in regulating the growth of some breast cancers has been accumulating for many years. With the advent of potent inhibitors it will now be possible to explore their therapeutic potential. Indeed, one of the inhibitors detailed in this review, 667 COUMATE, an irreversible sulfatase inhibitor, has now entered a Phase I trial in postmenopausal women with breast cancer. Preliminary results obtained from a small number of patients are encouraging and have revealed that >90% inhibition of STS in PBLs can be achieved with low doses of the drug (317). Further Phase II/III trials will be required to confirm if such STS inhibitors are to have a place in the armoury against breast cancer. There is also growing interest in the pursuit of reversible sulfatase inhibitors and this is an area that will undoubtedly develop over the next few years as novel templates are synthesised. If the sulfatase inhibitor concept is successful, future trials of STS inhibitors in combination with aromatase inhibitors, or possibly DASI-type compounds, will be required to determine if such combinations offer any advantages over the use of single
agent therapy. Also, there are many other potential disease targets beyond oncology that could receive attention following an initial clinical success.

An important property of this class of aryl sulfamates is their ability to be transported in red blood cells and to avoid first pass inactivation during transit through the liver. This property results from their ability to interact with, and inhibit, a number of CAs which may contribute to the overall efficacy of this class of drug. With the publication of several crystal structures for sulfamates interacting with carbonic anhydrase the molecular features that influence potency are becoming clearer and will support more rational drug design strategies to exploit this idea. It will thus be possible to test if other classes of drugs can also be delivered by this mechanisms upon sulphamoylation. There is already evidence that a sulfamoylated anti-estrogen possesses dual anti-estrogen/STS-inhibitory properties, suggesting that such an approach should be feasible (318).

An unexpected outcome of the research to reduce the estrogenicity of EMATE, the first potent STS inhibitor, was the discovery that the 2-substituted estrogen sulphamate derivatives are also potent anti-tumor/anti-angiogenic agents. These derivatives have increased potency compared with their non-sulfamoylated analogs but, in addition, their metabolic stability \textit{in vivo} is considerably enhanced. The next few years should hold considerable promise for exploring the potential of this new class of sulfamoylated drug which, in addition to exhibiting potent STS inhibition, targets other key steps in the malignant process.

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Legends:

1. The exon-intron structure of the human steroid sulfatase gene [Derived from (4)].


4. Structures of alternative substrates acting as inhibitors of steroid sulfatase.

5. Examples of reversible steroid sulfatase inhibitors.

6. Examples of steroidal irreversible steroid sulfatase inhibitors.

7. Examples of non-steroidal irreversible steroid sulfatase inhibitors.

8. X-ray crystal structure of estrone-3-O-sulfamate (EMATE) complexed with carbonic anhydrase II (CAII). Panel (a) shows the topology of EMATE – CAII interaction; panel (b) shows the formal interactions of the ligand with the binding site, especially the active zinc ion. [Reproduced with permission from Abbate et al., Bioorg Med Chem Lett 14: 231-234, 2004 (291)].
9. Ribbon diagram of the crystal structure of human steroid sulfatase (PDB code: 1P49.pdb) in association with the lipid bilayer. The crown of the ‘mushroom-like’ structure resides on the lumen face of the endoplasmic reticulum while the ‘stalk-like’ hydrophobic helices traverse the membrane. EMATE was docked as a ligand prior to inactivation of the enzyme using Gold® v2.1, running on SGI Octane2. The docking results indicate that the sulfamate group of EMATE interacts with the catalytic site of STS and its Ca$^{2+}$ atom in a similar manner to the sulfate group of E1S. The steroid backbone of EMATE resides within the proposed binding site of the enzyme which is surrounded mostly by hydrophobic amino acid residues. MOE was used to construct the diagram. [Derived from (305)].

10. Sketch view of essential steroid sulfatase catalytic site residues and the coordination of Ca$^{2+}$ in the crystal structure of steroid sulfatase. A number of charges and double bonds are omitted from the figure for clarity. The oval-shaped object denotes the proposed binding site for the steroid scaffold of estrone sulfate which is surrounded mostly by hydrophobic amino acid residues. This site extends towards the lipid bilayer. [Derived from (305)].


12. Proposed mechanism of STS inhibition by estrone-3-O-sulfamate (EMATE) via a nucleophilic attack on the sulfamoyl group by the gem-diol form of formylglycine residue in the enzyme active site. Structure I may be a ‘dead-end’ product which might exist in its anionic form [•−CH(OH)OSO$_2$NH−, not shown].

13. Proposed mechanism of STS inhibition by estrone-3-O-sulfamate (EMATE) involving the conserved formylglycine (FGly) residue in the enzyme active site. It
is possible that ROSO₂NH⁻ could be the predominant attacking species. Structures I – IV are proposed to be ‘dead-end’ products.

14. Proposed random specific or non-specific sulfamoylation by estrone-3-O-sulfamate (EMATE) of an essential amino acid residue in the STS active site. Path A: via an attack by a nucleophilic amino acid residue (:Nu-H) in the active site other than the gem-diol residue. Path B: via the generation of an sulfonlamine species. No regeneration of the enzyme active form from the sulfamoylated intermediate is expected. :B, a proton abstracting amino acid residue; X, a hydrogen-bond donating amino acid residue or Ca²⁺. Dashed line: hydrogen-bonding or co-ordination.

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Reed et al., Fig 1
Reed et al., Fig 2
Reed et al., Fig 3
Reed et al., Fig 4
Reed et al., Fig 5
Reed et al., Fig 6
Reed et al., Fig 7
Reed et al., Fig 9
Proposed binding site for steroid backbone

Reed et al., Fig 10
Reed et al., Fig 11
Reed et al., Fig 12
Reed et al., Fig 13
Sulfamoylated enzyme

Sulfonylamine

Reed et al., Fig 14