

Steroid Sulfatase Inhibition *via* Aryl Sulfamates: Clinical Progress, Mechanism and Future Prospects

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Abstract

Steroid sulfatase is an emerging drug target for the endocrine therapy of hormone-dependent diseases, catalyzing estrogen sulfate hydrolysis to estrogen. Drug discovery, developing the core aryl *O*-sulfamate pharmacophore, has led to steroidal and non-steroidal drugs entering numerous clinical trials, with promising results in oncology and women's health. Steroidal estrogen sulfamate derivatives were the first irreversible active-site-directed inhibitors and one was developed clinically as an oral estradiol pro-drug and for endometriosis applications. This review summarizes work leading to the therapeutic concept of sulfatase inhibition, clinical trials executed to date and new insights into the mechanism of inhibition of steroid sulfatase. To date the non-steroidal sulfatase inhibitor Irosustat has been evaluated clinically in breast cancer, alone and in combination, in endometrial cancer and in prostate cancer. The versatile core pharmacophore both imbues attractive pharmaceutical properties and functions *via* three distinct mechanisms of action, as a pro-drug, an enzyme active site-modifying motif, likely through direct sulfamoyl group transfer, and as a structural component augmenting activity, for example by enhancing interactions at the colchicine binding site of tubulin. Preliminary new structural data on the *Pseudomonas aeruginosa* arylsulfatase enzyme suggest two possible sulfamate-based adducts with active site formylglycine as candidates for the inhibition end product *via* sulfamoyl group or sulfonylamine transfer, and a speculative choice is suggested. The clinical status of sulfatase inhibition is surveyed and how it might develop in the future.

Also discussed are dual-targeting approaches, development of 2-substituted steroidal sulfamates and nonsteroidal derivatives as multi-targeting agents for hormone-independent tumours with other emerging directions.

Keywords: Cancer, Steroid Hormone, Oncology, Breast Cancer, Estrogen Receptors

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Abbreviations

STS – Steroid sulfatase. DHEA – Dehydroepiandrosterone. DHEAS – Dehydroepiandrosterone-3-*O*-sulfate. E1 – Estrone. E2 – Estradiol. E1S – Estrone-3-*O*-sulfate. E2S – Estradiol-3-*O*-sulfate. EMATE – Estrone 3-*O*-sulfamate. E2MATE – Estradiol-3-*O*-sulfamate. ER – Estrogen receptor. ESR1 – Estrogen receptor α gene. SERD – Selective estrogen receptor down-regulator. SERM – Selective estrogen receptor modulator. 17 β -HSD1 – 17 β -hydroxysteroid dehydrogenase type 1. PET – positron emission tomography. FLT - 3'-deoxy-3'-[^{18}F] fluorothymidine. DHT – dihydrotestosterone. FGly – formylglycine. AI - aromatase inhibitor. ITT - intent to treat. CBR – clinical benefit rate. NETA – norethindrone acetate. CRC – colorectal cancer. CA II – carbonic anhydrase II. CAIX – carbonic anhydrase IX. MA - megestrol acetate. (*PaAtsA*) - *Pseudomonas aeruginosa* arylsulfatase.

Introduction

Many hormone-sensitive tumours depend upon the steroid 17 β -estradiol (E2) for growth (Dixon 2014) and about two thirds of such breast tumours are estrogen receptor α (ER) positive (ER+ status). Endocrine therapy in the postmenopausal breast cancer setting, with most ovarian estrogen production having ceased, aims to target E2 reduction and is critical to underpin modern targeted cancer therapy (Rugo *et al.* 2016). For example, the newer mTOR and CDK4/6 inhibitors in breast cancer, such as everolimus and palbociclib respectively, are generally administered upon a background of endocrine therapy (Baselga *et al.* 2012, Finn *et al.* 2016). Access of estrogens to the ER can be blocked clinically through a selective estrogen receptor modulator (SERM) or down-regulator (SERD), e.g. raloxifene, ospemifene, fulvestrant, etc (Patel & Bihani 2018). The SERM tamoxifen has been the drug of choice for many years and enormously successful (Davies *et al.* 2013). In recent decades, however, inhibitors of estrogen biosynthesis targeting the aromatase enzyme, aromatase inhibitors (AIs), have become the new gold standard for treatment of hormone-dependent breast cancer and the so-called “third generation aromatase inhibitors” anastrozole (Arimidex), letrozole (Femara) or exemestane (Aromasin) are supplanting tamoxifen as first line adjuvant endocrine therapy. AIs can reduce systemic estrogen levels by as much as 98% and have been found to be superior to tamoxifen both in terms of disease-free survival and adverse side effects (Early Breast Cancer Trialists' Collaborative Group 2015), although resistance will develop and ultimately tumour progression will occur (Dixon 2014, Reinert *et al.* 2017, Augusto *et al.* 2018). Although the use of AIs is clinically successful, and recent recommendations are to extend therapy for 5-10 years (Goss *et al.* 2016), such endocrine therapy is still not fully optimized and there is a need for new approaches and agents.

Dysregulation of sulfation and desulfation processes is associated with numerous pathologies (Reed *et al.* 2005, Mueller *et al.* 2015). The structural biology and enzymology of the enzymes of estrogen metabolism have been recently reviewed (Thomas & Potter 2013). The *in situ* hydrolysis of the sulfated estrone conjugate estrone 3-*O*-sulfate (E1S) by steroid sulfatase (STS) has been proposed as an unexploited source of postmenopausal tumour estrogen and was judged in the mid-1990s to be an area ripe for new drug discovery (Reed *et al.* 2005). In this setting the half-life of E1S in plasma is long and levels of E1S formed *via* a sulfotransferase (SULT2A1) are much higher than those of E1 (Ruder *et al.* 1972, Noel *et al.* 1981, Reed *et al.* 2005). Circulatory E1S may thus act as a reservoir of E1 and can enter tumour cells *via* organic anion transporters (Mueller *et al.* 2015). STS converts E1S *in situ* in tumour cells to E1, which can then be converted to E2 by 17 β -hydroxysteroid dehydrogenase type 1 (17 β -HSD1), thus producing the hormone in an intracrine fashion (Labrie 2015, McNamara & Sasano 2015). This may be the main route of estrogen production in tumour cells and levels of STS and 17 β -HSD1 are reported to be higher in such tissues than in normal tissues (McNamara & Sasano 2015).

STS mRNA expression is significantly higher in malignant breast tissue and has prognostic significance with high levels a predictor of reduced relapse-free survival (Utsumi *et al.* 1999, Miyoshi *et al.* 2003). Moreover androstenediol, a steroidal androgen with estrogenic activity, is formed mostly *via* STS from androstenediol sulfate in postmenopausal women, itself derived from dehydroepiandrosterone sulfate (DHEAS). Although not an aromatic estrogen the high level of androstenediol synthesis in the postmenopausal setting nevertheless means that it can provide an additional estrogenic stimulus at the ER (Poulin & Labrie 1986, Dauvois & Labrie 1989). Although the ER affinity of androstenediol is lower than that of E2, due to its higher serum and tissue concentrations it may be of high importance in comparison to E2 in postmenopausal women (Spinola *et al.* 1986). Importantly, as there is only one STS enzyme

(Purohit *et al.* 1994) STS inhibition, as a new therapeutic modality, should address both of these aromatase-independent pathways (Billich *et al.* 2000) and lead to a reduction in both the circulating androstenediol level and in *in situ* tumour cell E2 production, thus offering considerable new promise. Importantly, the use of AIs in postmenopausal women at risk of breast cancer, but not diagnosed, has been reported to have significant preventive action if given prophylactically (Cuzick *et al.* 2014). STS inhibitors might eventually also be found to exhibit such an effect.

A landmark 1994 paper (Howarth *et al.* 1994) reported the first, and picomolar-potent, steroidal STS inhibitor, one which possessed an aryl sulfamate pharmacophore. The overall strategic aim was to design a substituent group at the steroidal 3-phenolic position that resembled a sulfate group in structure, shape, electronics etc, but which would not be hydrolyzed by the enzyme. Although early work explored such surrogates and indeed successfully generated useful prototype inhibitors the result was, not surprisingly, generation primarily of compounds with low micromolar and reversible inhibitory activity such as estrone 3-*O*-methyphosphonothioate (Figure 1(a)) (Thomas & Potter 2015a). The key advance was the derivatization of the phenolic hydroxyl group of estrone by a neutral sulfamate moiety and this produced the first active site-directed irreversible inhibitors estrone 3-*O*-sulfamate (EMATE) (Howarth *et al.* 1994, Purohit *et al.* 1995a) and estradiol 3-*O*-sulfamate (E2MATE) (Figure 1) that were highly potent both *in vitro* and *in vivo* against STS (Purohit *et al.* 1995b). The unique activity of this new compound class has since spawned hundreds of such sulfamate-based inhibitors based around the aryl sulfamate pharmacophore (Figure 2) and founded new academic, industrial and clinical research areas. Estrogen sulfamates also bind to carbonic anhydrase II (CA II) in red blood cells and transit the liver without first-pass metabolism (Ho *et al.* 2003). The underpinning pharmacophore has attractive properties, widely exploitable in

drug discovery, imbuing excellent oral activity, bioavailability and pharmacokinetics (Ireson *et al.* 2004). A 2.6 Å X-ray crystal structure of human STS first became available in 2003 (Hernandez-Guzman *et al.* 2003, Ghosh 2007) illustrating a “mushroom-like” structure (Figure 1b) and confirming the presence of an active site hydrated formylglycine residue (FGly) that is known to be conserved across the STS family (Ghosh 2005, 2007). Mechanistically, while such phenol sulfamate ester drugs have been known to be time- and concentration-dependent inactivators of STS for some time now, and are thought to chemically modify the active site of the enzyme irreversibly (see Figure 1(b) for a pre-inhibition docking model of the clinical inhibitor Irosustat), the actual mechanism and site of inhibition have remained the subject of speculation (Thomas & Potter 2015a). This review aims to survey briefly the developmental history of the STS inhibition concept and the clinical translation of the first STS inhibitors, summarize the most recent published clinical findings with future prospects and also illuminate new mechanistic pointers that throw some preliminary light on how such sulfamate-based inhibitors function as irreversible STS active site blockers.

Clinical STS Inhibitor Development

With the discovery of EMATE and its 17-keto group-reduced form estradiol 3-*O*-sulfamate (E2MATE, also known as J995) (Figure 1) as the first potent and irreversible STS inhibitors the scene was apparently quickly set for the translation of such a steroidal sulfamate ester drug into clinical trials in hormone-dependent breast cancer. However, surprisingly, this class of compound was found to be highly estrogenic in rodents and thus unsuitable as an anticancer therapeutic. This was thought to be because of its activity as a pro-drug for E1 or E2 respectively, with STS cleaving the sulfamate group (Elger *et al.* 1995) and stimulated the active search for a non-steroidal, non-estrogenic STS inhibitor. Because this estrogenicity of

the steroidal sulfamate series effectively precluded applications in oncology (although it provided the stimulus for other clinical work *vide infra*) a search for an orally active, non-steroidal, non-estrogenic, Lipinski rule-compliant STS inhibitor was undertaken, primarily based upon using steroidal A/B ring surrogates. A first lead was the two-ring coumarin aryl sulfamate, 4-methylcoumarin-7-*O*-sulfamate (Figure 2), which was orally active *in vivo* and also like EMATE a highly potent time- and concentration-dependent STS inhibitor, but with no rodent estrogenic activity (Woo *et al.* 1996, Purohit *et al.* 1996). The related 3,4-dimethylcoumarin-7-*O*-sulfamate (Figure 2) inhibited STS in MCF-7 cells with $IC_{50} = 30$ nM and a series of derived tricyclic compounds was subsequently synthesized that proved even more potent (Woo *et al.* 2011a) (Figure 2). The synthetic route comprising only two steps was very straightforward; a Pechmann reaction between resorcinol and the corresponding cyclic β -ketoester, followed by a sulfamoylation of the coumarin phenol made them attractive candidates for industrial scale-up. The greater activity of these non-steroidal compounds relative to the steroidal parent was attributed to the enhanced “sulfamoyl transfer potential” as a result of the lower phenolic pK_a (Woo *et al.* 2011a), the idea being that STS is irreversibly modified through sulfamoylation of the active site. Of these STX64 (667 COUMATE), also subsequently known as BN83495 and now more commonly known as Irosustat (Figure 3) was selected for extensive *in vivo* studies.

STX64/Irosustat was orally very active *in vivo* with a bioavailability of 95%. Single or multiple oral doses inhibited rat liver STS by >90%, prevented the growth of rodent nitrosomethylurea-induced mammary tumours and were very effective at inhibiting the growth of human breast cancer xenografts implanted in nude mice (Thomas & Potter 2015a). The drug passed pre-clinical toxicological testing (Stanway *et al.* 2006). In plasma *ex vivo* the drug was quickly degraded, but *in vivo* this is prevented by its sequestration inside red blood cells, where it binds

to CA II (Ireson *et al.* 2004). Irosustat was co-crystallized with CA II (Lloyd *et al.* 2005, PDB 1TTM) and the complex was studied by X-ray crystallography. This stability and facilitation of transport to tissues may account for the high bioavailability of the drug. Moreover, the sequestration allows the drug to evade first pass metabolism. Members of the non-steroidal tricyclic STX64/Irosustat drug class can be visualized with the aliphatic third ring in a C/D-like folded fashion to show how they could potentially mimic a steroid structure in binding to STS (Thomas & Potter 2015a) (Figure 3). Sulfamate esters, like sulfonamides, are weak acids with the first pK_a in the 7-9 region and there will thus be a significant amount present in the anionic form at physiological pH, thus making the drug a good mimic of the normally charged STS substrate. Importantly, however, a sulfamate ester can also be present as the neutral form which will aid permeability, tissue penetration and presumably its reversible sequestration into red blood cells. Thus, it is clear how Irosustat has all the structural properties to bind effectively to STS, an enzyme designed to bind a steroidal structure possessing a charged phenolic sulfate group. More extensive details of the background and development of Irosustat and the wider STS inhibitor classes are provided elsewhere (Thomas & Potter 2015a, b).

Clinical studies

STS inhibition has so far been studied using two sulfamate-based drugs in 19 international human clinical trials in 5 distinct pathologies. Positive clinical effects of Irosustat and E2MATE/J995 have been established with the underpinning academic science validated. These trials are summarized below:

Hormone replacement therapy

Despite its unexpected estrogenic activity a steroidal sulfamate was nevertheless the first aryl sulfamate-based drug to enter human clinical trials. Innovatively, the absence of hepatic estrogenicity/production of clotting factors for this class of compound reduces risks of adverse events for applications in hormone replacement therapy/oral contraception. The orally active steroidal super-estrogen E2MATE or J995 (Figure 1), that was five times more estrogenic than ethinylestradiol when administered orally in the rat (Elger *et al.* 1995), thus reached multiple clinical trials with >170 healthy women dosed in six Phase I & II studies in a hormone replacement therapy setting. The drug was very safe and well-tolerated and the role of STS in activating the sulfamate derivative as a pro-drug was firmly established (Chander *et al.* 2004). However, estrogenic activity was subsequently significantly limited in humans by metabolic deactivation at C-17, most likely in the gut, and this issue needs to be overcome for further development of the idea (Elger *et al.* 2017).

Breast cancer

With the overall rationale for STS inhibition established in pre-clinical studies (Reed *et al.* 2005, Thomas & Potter 2105a) and the design of the tricyclic non-steroidal STS inhibitor STX64/Irosustat successfully achieved, the way was clear for the drug to enter a “first in class” clinical trial, performed initially in an academic setting. Irosustat was administered orally to fourteen postmenopausal women with advanced breast cancer (9 patients at 5 mg and 5 at 20 mg dose) as an initial dose followed a week later by three 2 weekly cycles of 5 day dosing and 9 days off treatment. The drug had a pharmacokinetic profile suitable for daily dosing and was established as clinically potent and well tolerated with only minor adverse events. The median inhibition of STS activity was 98% in peripheral blood lymphocytes and 99% in biopsied breast tumour tissue at the end of the 5-day dosing period. Serum concentrations of steroids with

estrogenic properties, *ie* estrone, estradiol, and especially androstenediol, as well as dehydroepiandrosterone (DHEA) all decreased significantly from their pre-treatment levels and, surprisingly, androstenedione and testosterone concentrations also decreased. Four patients, previously progressing on AIs, encouragingly showed evidence of stable disease for 2.75 to 7 months (Stanway *et al.* 2006).

In a subsequent phase I clinical study (Coombes *et al.* 2013) using a new solid tablet formulation to determine the optimal biological dose a three-part, open-label, multicentre, dose escalation study of Irosustat in ER+ breast cancer patients was performed. A single oral dose of the drug was administered with a 7-day observation period, followed by a daily dose for 28 days and an extension phase, in which dosing was continued discretionally if the patient was seen to benefit. Five doses of Irosustat were tested up to 80 mg in fifty patients. After 28 days of daily administration all evaluated patients in the 5, 20, 40, and 80 mg cohorts achieved $\geq 95\%$ STS inhibition in peripheral blood mononuclear cells and corresponding endocrine suppression. The maximum tolerated dose was not reached and the 40 mg dose was established as optimal. The median time to progression in the 40 mg cohort was 11.2 weeks. Biopsy-validated erythematous skin infiltration in one patient was no longer visible after one month of treatment. Disease stabilization is often taken to be a reliable indicator of effectiveness of a novel therapy, particularly endocrine therapy; five patients of this heavily pretreated patient population (10 %) remained progression-free for at least 24 weeks (33.1 weeks in one patient receiving 20 mg, 72.3, 28.4, and 27.1 weeks in three patients receiving 40 mg and 30.7 weeks in one patient receiving 80 mg), potentially indicative of drug activity. Dry skin, the most frequent adverse event, was easily managed. This study provided clinical proof-of-concept that STS is inhibited by Irosustat in patients with an effective suppression of peripheral steroid hormones. It was noted that a larger study is required to define an accurate

response rate to the drug as a single agent and also whether co-administration with an aromatase inhibitor might potentially be more effective (*vide infra*). Further details of other Phase II studies are yet to be reported.

In clinical trials, such as those above, the postmenopausal subjects have normally already been heavily pre-treated and thus may have various resistance pathways activated. One of the most recent clinical studies reported in breast cancer is the “IPET” study, which was a pre-surgical window-of-opportunity study to assess Irosustat for the first time in ER+ early breast cancer patients (Palmieri *et al.* 2017a). The aims of this trial were to assess the effect of Irosustat on tumour cell proliferation in treatment-naïve subjects, as measured by 3'-deoxy-3'-[¹⁸F] fluorothymidine (FLT) uptake monitored *via* positron emission tomography (PET) scanning (FLT-PET) and levels of the tumour proliferation marker Ki67. Postmenopausal women with untreated early breast cancer were recruited and treated orally for at least 2 weeks with Irosustat at 40 mg/d. Tumours were imaged with FLT-PET at baseline and after drug treatment, with the primary endpoint being changes in FLT uptake; secondary endpoints included safety and tolerability, changes in tumoural Ki67, circulating steroid hormone levels and expression of steroidogenic enzymes. Of thirteen women recruited, ten started Irosustat for 2 weeks, followed by repeat FLT-PET scans for eight women. The drug was generally well tolerated with all adverse events < Grade 2. STS decreases were seen in tumours with high basal STS expression and significant decreases were also noted in aromatase and 17β-HSD types 1 and 2. With the definition of a response being decreases of ≥20% in standardized uptake value or ≥30% in Ki67, one and three patients responded respectively. Six out of seven patients had a Ki67 reduction with the median percentage difference being 52.3%. Irosustat treatment thus resulted in significant reductions in FLT uptake and Ki67 and was well tolerated. Baseline expression of STS was judged to be a potential biomarker of sensitivity to Irosustat and may facilitate

future prior patient stratification. Importantly, these data are the first to demonstrate clinical activity of Irosustat in early breast cancer, albeit in a rather small patient population. Encouragingly also, data are broadly comparable to those from tamoxifen in the same setting and to Phase III data for the well-established drugs exemestane and fulvestrant. However, patient recruitment in this single centre pre-surgical population was challenging and larger studies are now required to build upon these results.

Inhibition of aromatase lowers estrogen levels and is an effective endocrine therapy for many breast cancers. However, it is to be expected that chronic treatment with an AI will lead to the development of compensatory mechanisms to increase E2 levels: indeed, a report (Chanplakorn *et al.* 2010) showed that the administration of an aromatase inhibitor to breast cancer patients increased levels of both STS and 17 β -HSD1 leading to increased E2 levels. Moreover, a recent study also demonstrated the contribution of both STS and organic anion transporters in E1S metabolism to the proliferation of AI-resistant breast cancer cells and AI resistance (Higuchi *et al.* 2016). Letrozole-resistant cell lines were established that had higher levels both of STS mRNA and organic anion transporters. Such cells proliferated more in an E1S-supplemented medium and this was effectively inhibited by Irosustat in combination with the AI Letrozole. This model was also supported by analysis of ER⁺ primary breast cancer tissues. The administration of an STS inhibitor should, as above, address the *in situ* intracrine production of estrogen (and importantly including circulating androstenediol) from the sulfate by STS in tumour cells and, moreover, alongside an AI should lower estrogen levels further in a multi-targeting fashion. In addition, the development of ESR1 mutations represent another resistance mechanism to AIs (Wang *et al.* 2016, Reinert *et al.* 2017). In some tumours the rarer ESR1-mutant clones are enriched by endocrine therapy. Implications here for STS inhibition therapy are as yet unknown.

Another recent clinical study, reported in 2017, was the “IRIS” study (Palmieri *et al.* 2017b). This multicentre, open label, phase II trial explored the clinical value of adding an STS inhibitor in addition to a first-line AI in patients with advanced breast cancer. This was both to evaluate the safety of the combination and to test the hypothesis that the addition of Irosustat to an AI may further suppress E2 levels and result in clinical benefit. Postmenopausal women with ER+ locally advanced or metastatic breast cancer who had benefited from a first-line AI but were subsequently progressing were enrolled. The first-line AI was continued and 40 mg/d oral Irosustat was added. The primary endpoint was clinical benefit rate (CBR) and secondary endpoints included safety, tolerability, and pharmacodynamics. The study was judged to have met its pre-defined success criterion by both local and central radiological assessments. Twenty seven women were recruited, although four discontinued treatment. Based on local reporting, the CBR was 18.5% on an intent to treat (ITT) basis, increasing to 21.7% by per-protocol analysis. In those five patients that achieved clinical benefit, the median duration was 9.4 months and the median progression-free survival time was 2.7 months in both the ITT and per-protocol analyses. The most frequent adverse effects were < grade 2, with the most common one being dry skin, as expected. Encouragingly, the addition of Irosustat to standard aromatase inhibitor therapy thus resulted in clinical benefit with an acceptable safety profile.

The objective response and clinical benefit rates of endocrine treatments used second-line are clearly limited and results should be seen in this context. It is also important to stress that the newer targeted mTOR and CDK4/6 inhibitors that are showing current clinical success in breast cancer are used in concert with endocrine therapy (Hortobagyi *et al.* 2016); the efficacy of the former will be dependent upon optimization of the latter that could perhaps be enhanced further through *eg* an STS inhibitor and AI combination. Further studies are, however, needed to explore the clinical activity of targeting both aromatase and STS, ideally as a first-line

metastatic treatment in patients likely to be endocrine sensitive. Moreover, it would also be most desirable to enrich any future patient cohort in those that can be measurably selected for high tumour STS expression. Additionally, another innovative approach of future potential, building on these results is that enshrined in the idea of dual aromatase-sulfatase inhibition, using a single agent (*vide infra*).

Endometrial cancer

Endometrial cancer, developing from the inner uterine lining, is the most common cancer found in the female reproductive system. Malignant endometrial tissue has a high STS activity and advanced/metastatic or recurrent endometrial cancer has a poor prognosis. STS was judged to be a good novel drug target for this indication (Foster *et al.* 2008a). Despite progress in the treatment of early endometrial cancer, the advanced disease remains incurable and there is a strong need for new agents. STS was evaluated as a therapeutic target in patients with endometrial cancer *via* a phase II multicentre (11 European countries), randomized, 2-arm study of Irosustat in women with advanced/metastatic or recurrent estrogen receptor-positive endometrial cancer (Pautier *et al.* 2017). The optimal oral dose of 40 mg/d was used in comparison with the current standard of care, oral megestrol acetate (MA), at 160 mg/d. The primary endpoint was the proportion of patients without progression or death at 6 months and secondary endpoints included progression-free survival, time to progression, overall survival, and safety. Thus, seventy one postmenopausal patients with histologically verified estrogen and/or progesterone receptor-positive endometrial cancer with recurrent/advanced disease, were treated; thirty six received Irosustat and thirty five MA. Results showed clinical activity and a good safety profile for Irosustat with 36% of patients on Irosustat alive without progression at 6 months; 11% showed responses and there was more stable disease noted (47%) compared to the current therapy (32%). However, while overall there were no statistically

significant differences between Irosustat and MA in response and survival rates, the study was terminated early after futility analysis. Overall, 36.1% and 54.1% of patients receiving Irosustat or MA had not progressed or died at 6 months, respectively. Irosustat patients had a median progression-free survival of 16 weeks versus 40 weeks for MA-treated patients. The drug was well tolerated. Mainly grade 1 or 2 treatment-related adverse events occurred in 55.6% and 37.1% patients receiving Irosustat or MA respectively.

Thus, while Irosustat monotherapy did not demonstrate activity sufficient for further commercial development in patients with advanced/recurrent endometrial cancer, the study confirmed the clinical benefit of the drug and further validated the underpinning scientific rationale of targeting STS. However, in a recent study to determine STS expression in endometrial cancer patients and its potential prognostic significance (Lee *et al.* 2016) it was found that STS expression is not significantly associated with disease free survival and overall survival, despite positive STS expression in 27% of endometrial cancer patients. Thus, STS as a prognostic factor in endometrial cancer patients requires more analysis.

Prostate cancer

Androgen-dependent prostate cancer represents a very large unmet medical need. STS activity has been detected in prostate tissue and in LNCaP prostate cancer cells suggesting that, like in the breast, STS could facilitate intracrine production of hormones to stimulate tumour growth from a precursor source (Day *et al.* 2009) and was also explored with the steroidal STS inhibitor EM-213 (Roy *et al.* 2013). DHEAS is present at plasma concentrations up to 500 times higher than testosterone and can potentially be delivered into prostate cancer cells *via* organic anion transporters and converted *via* endogenous STS into desulfated DHEA and then into testosterone by hydroxysteroid dehydrogenases and into dihydrotestosterone (DHT) *via* 5 α

reductase. In the prostate adrenal androgens can be converted into testosterone and DHT, driving tumour growth, analogously to the *in situ* cleavage of E1S in breast tumours. The effect of Irosustat on hormone levels in patients suffering from castration-resistant prostate cancer with on-going androgen deprivation therapy was studied in the first clinical trial of an STS inhibitor in men through a US phase I dose escalation study (Denmeade *et al.* 2011). This was conducted in seventeen chemo-naïve, castration-resistant patients with evidence of disease progression following 28 days of oral Irosustat administration. The aims were evaluation of safety, tolerability, pharmacokinetic, pharmacodynamic and endocrine parameters (plasma DHEA: DHEAS ratio, inhibition of androstenediol, androstenedione and testosterone) consequent upon STS inhibition, with dosing cohorts at 20, 40 and 60 mg. Pharmacodynamic proof of concept was demonstrated and in all patients there was notable suppression of the non-sulfated androgens testosterone, androstenediol and DHEA. The DHEA: DHEAS ratio was significantly decreased. Effects were slightly better at the two higher doses. Importantly, Irosustat effected nearly complete STS inhibition at all three doses and the drug was well tolerated at all doses, with dry skin the most common related adverse event. No peer-reviewed publication has, however, yet appeared from this study. The results also provide a basis for potential future trials in those patients who have demonstrated resistance to the CYP17A1 inhibitor Abiraterone (Zytiga) and who may potentially be employing STS-mediated conversion of sulfated androgens such as DHEAS to contribute to the resistance phenotype and drive tumour growth.

Since prostate cancer may also be associated with high plasma estrogen exposure an *in vivo* rat study examined the effects of STX/64/Irosustat to explore whether E1S uptake in the prostate increases during ageing. If applicability to humans is valid the protective results seen with the

drug support the idea in principle of reducing STS activity in older men with high E1S plasma levels (Giton *et al.* 2015).

Endometriosis

Another clinical target, the poorly-understood gynaecological disease endometriosis, is also hormone-driven and leads to chronic pelvic pain and infertility. This benign disease is characterised by the presence of endometrial tissue outside the uterus. With an estimated 80 million patients worldwide, the disease is still poorly understood, most treatments have unpleasant side effects and current therapies are inadequate (Vercellini *et al.* 2014). Discovery of new drugs is thus a pressing unmet need. In addition to production of estrogens in the ovaries, there is compelling evidence that local synthesis of estrogens in endometriotic lesions promotes progression of the disease and resistance to endocrine therapy (Tosti *et al.* 2016). The first rationale for STS inhibition in endometriosis was devised (Purohit *et al.* 2008) and led directly to a phase I study using the steroidal drug E2MATE (PGL2001) to explore a new treatment option for this disease. In such an application not directed at the postmenopausal setting it is necessary to employ combination of an STS inhibitor with a progestin such as norethindrone acetate (NETA) to reduce ovarian estrogen production and the STS inhibitor would also decrease local estrogen production. A randomized, double-blind, and placebo-controlled phase I study was used to investigate the pharmacodynamics, pharmacokinetics and safety of the E2MATE (PGL2001) and NETA combination (Pohl *et al.* 2014). Twenty four healthy women of reproductive age were treated with weekly doses of the STS inhibitor or daily doses of NETA or a combination of both compounds for 4 weeks. Such treatment reduced STS activity in the endometrium by 91% and 96% respectively, with comparable values observed 1 month after the cessation of treatment. The combined drug treatment led to significantly higher STS inhibition at both times.

This study showed that administration of the sulfatase inhibitor either alone at 4 mg/week or with the progestin to healthy pre-menopausal women led to STS inhibition and changes in functional endometrium STS biomarkers, resulting in synergistic effects of the combination on STS activity. The proof-of-principle combination was thus well-tolerated. Double-blind, multicentre, placebo-controlled, phase II clinical trials have been in progress since 2012 to investigate efficacy, safety, pharmacokinetics and pharmacodynamics in Hungary, Poland and Romania. One potential disadvantage of these current studies is however that, as described earlier, E2MATE possesses residual estrogenic activity which, although reduced in humans, would still seem non-applicable to this indication, with the ideal overriding preference being for use of a pure STS inhibitor such as Irosustat, which should be a future aim. Publication of detailed clinical results is awaited.

Other potential clinical targets for STS inhibition are emerging. For example, estrogens affect the incidence and progression of colorectal cancer (CRC) that exhibits dysregulated estrogen metabolism with E2 synthesis favoured, but molecular mechanisms are still unclear. In a recent study (Gilligan *et al.* 2017) STS activity was shown to be significantly elevated in human CRC and STS over-expression accelerated CRC proliferation *in vitro* and *in vivo*. STS inhibition using Irosustat can block this effectively and future clinical studies may be indicated. Ovarian cancer is also an attractive possibility as a clinical target and Irosustat was shown to inhibit the growth of the ER+ ovarian cancer OVCAR-3 cell line (Day *et al.* 2009). In a study of specimens from thirty seven patients with advanced stage ovarian cancer it was investigated whether a clinical correlation existed between STS activity and survival (Chura *et al.* 2009). STS activity is widely present in specimens of ovarian cancer. Median progression-free survival was 23.5 months for patients with low STS activity compared to 6.9 months for patients with high

STS activity. Increased STS activity is associated with worse progression-free survival in patients with advanced stage disease and the STS pathway is thus a potential therapeutic target in the treatment of ovarian cancer. A recent study has suggested that in the most frequent and lethal type of the disease, advanced high grade serous epithelial ovarian cancer, SULT1E1 expression is significantly associated with better survival and targeting the STS pathway should show clinical benefit (Mungenast *et al.* 2017). Intracrine activation of precursors and the use of more stratified patient population as well as combination therapy, including dual inhibitors (*vide infra*), in *eg* ovarian cancer was stressed in a recent comprehensive review (Lanišnik Rižner *et al.* 2017). There are also interesting preliminary implications for STS involvement in bladder cancer (Eri *et al.* 2014).

Mechanism of action

Sulfatase enzymes are derived from an evolutionarily highly conserved gene family. While they catalyze sulfate group cleavage from a wide variety of substrates including glucosaminoglycans, glycolipids, amino acids and hydroxysteroids, the various enzymes have similar folds, active site architecture, mechanisms of action and bivalent metal ion binding sites (Ghosh 2005, 2007). In 2003 the 3D structure of human STS became available (Hernandez-Guzman *et al.* 2003). Here, as in eukaryotic sulfatases, a critical formylglycine FGly residue is generated by post-translational modification of an active site cysteine residue; in some bacteria this is by modification of a serine residue (Marquardt *et al.* 2003). This FGly, in its hydrated form as a *gem*-diol, is thought to be generally involved in the catalytic mechanism of all such sulfatases and catalytic mechanisms have been developed with the aid of structural biology (Lukatela *et al.* 1998, Boltes *et al.* 2001, von Bülow *et al.* 2001). While a mechanism for STS-mediated cleavage is now generally well established, with nucleophilic attack on the sulfated

phenolic substrate by a hydroxyl of the FGly *gem*-diol leading to an intermediate pro-*R gem*-diol sulfate adduct, that can sometimes be observed crystallographically in the resting state, this does not yet hold for STS inhibition by aryl sulfamates, the mechanism of which is still controversial. It was shown some time ago that aryl sulfamates act as irreversible active-site-directed STS inhibitors, working in a time-and-concentration-dependent fashion (Purohit *et al.* 1995). However, little progress has been made over the years in firming up an inhibitory mechanism, even though proposals have been made and compounds such as Irosustat have now proceeded to many clinical trials (Thomas & Potter 2015a).

A feasible working hypothesis adopted for the mechanism of action of aryl sulfamates in STS inactivation is generally that the sulfamoyl group is transferred to the enzyme active site in some fashion and that this destroys activity irreversibly. Several mechanisms have been postulated, although none is conclusively proven. Early work suggested, given the several active site lysines and histidines flanking the FGly residue that lysine or histidine sulfamide-type adducts might be the end product of inhibition (Woo *et al.* 2000). FGly in its hydrated or aldehydic form is clearly another obvious target for irreversible modification. Using the STS crystal structure docking experiments indicated that the likely orientation of the sulfamate group of Irosustat and related tricyclic sulfamates in the active site is in close proximity to the crucial hydrated FGly residue (see Figure 1(b)), further suggesting that, as a good steric and electronic sulfate mimic, it might be transferred to this residue during inhibition (Woo *et al.* 2011a). Indeed, it has been noted that there is a qualitative similarity in the reaction coordinates for bimolecular sulfamoyl and sulfuryl transfer reactions of sulfamate and sulfate monoesters and thus a close mechanistic relationship for sulfamoyl and sulfuryl group transfer (Denehy *et al.* 2006). The idea that inhibition of STS is facilitated by some kind of sulfamoyl group transfer to the active site is still the currently accepted mode of action for such drugs, even if the precise

transfer mechanism and any subsequent consequences for the active site machinery are as yet unknown.

Working with human STS is difficult for mechanistic purposes; it is membrane bound and can be heterogeneously glycosylated. By contrast, the soluble bacterial *Pseudomonas aeruginosa* arylsulfatase (*PaAtsA*) also catalyzes the cleavage of aryl sulfates and is an excellent model for human estrone sulfatase and the enzyme has been crystallized (Boltes *et al.* 2001, PDB 1HDH). Valuable experimental insight into the mechanism of sulfatase inactivation by sulfamates was provided *via* this model. The inactivation of *Pseudomonas aeruginosa* arylsulfatase A by a range of aryl sulfamates was studied, including the clinical Irosustat (Bojarová *et al.* 2008). Inactivation was found to be similarly time-dependent, irreversible, and active-site directed, consistent with a covalent modification at the active site. The transition state for the first irreversible chemical step of inactivation was shown to involve a high degree of charge transfer and cleavage of the ArO-S bond. Interestingly, however the stoichiometry of inactivation was established as in the range of 3–6, with the highest value found for the most effective inactivators. Thus, it was concluded that multiple sulfamoylation events may occur during the inactivation process. In a recent extensive physical-organic extension of this work (Williams *et al.* 2014) reversible sulfamoylation of the active site lysines and histidines was invoked to explain this surprising stoichiometry. Attempts to shed light on the irreversible adduct(s) using mass spectrometry were reported to be unsuccessful, but an *N*-imine sulfate FGly adduct was nevertheless proposed to be the most likely end product of inhibition.

In more recent work in our group it has proved possible to inactivate *PaAtsA* with Irosustat until there is no more activity measurable and subsequently to crystallize the inactivated

protein, that could then be studied using structural biological techniques (G Cozier, C Martinez-Fleites, G Davies & B V L Potter, unpublished work). The preliminary results are shown in Figure 4. Interestingly, it can be seen that there is new covalent electron density apparently at the pro-*R* hydroxyl of the hydrated FGly *gem*-diol (but see also the figure legend for a stereochemistry caveat). While this density cannot be unambiguously assigned as yet at the resolution achieved it nevertheless seems to be located at the pro-*R* hydrated FGlyS site seen generally to be sulfated in the resting form of sulfatase enzymes (Figure 5), and which provides some encouragement in favour of a sulfate-mimicking sulfamoyl transfer inhibitory mechanism. Importantly, however, there are no covalent His or Lys modifications as previously suggested above, both originally (Woo *et al.* 2000) and in the later inactivation experiments (Bojarová *et al.* 2008), but just an apparently covalently-modified FGly residue. With this result it is possible to tentatively construct theoretical inactivation possibilities with both direct sulfamoyl group transfer leading to either an *O*-linked hydrated FGly product **I** (and possible interference blocking the next catalytic step perhaps as a result of new *H*-bonding possibilities provided by the amino group of the sulfamate or disturbed metal ion interactions) or potentially to an *N*-linked product **II** formed *via* interaction of the sulfamate nitrogen with an unhydrated FGly, analogous to the synthetic reaction noted of a sulfamate ester anion with dimethylformamide (Woo *et al.* 1998, Williams *et al.* 2014)). The involvement of an electrophilic sulfonylamine in this process (Thea *et al.* 1986, McCaw & Spillane 2006), either singly or doubly charged, and “free” or otherwise, could be invoked in both possibilities and is a compelling mechanistic prospect.

The new crystallographic results, although only preliminary, have implications for the recent inactivation model proposed (Williams *et al.* 2014). Here a range of potential end products of STS inactivation by aryl sulfamates was evaluated theoretically, employing physical-organic

considerations. The most favoured irreversible adduct was proposed to be an imine *N*-sulfate **III** (Figure 5). While it is certainly not possible from the present preliminary crystallographic data to distinguish between *N*- and *O*-linked FGly covalent adducts **I** and **II** respectively (Figure 5), it seems nevertheless apparent from the new electron density observed that the steroidal phenol motif is absent, which is not too unexpected, and that the adduct comprising the new electron density is likely linked to the pro-*R* hydroxyl group. The other pro-*S* hydroxyl group, however, is still clearly present and this therefore seems to rule out the possibility that the adduct is the proposed imine *N*-sulfate **III**. Therefore, it is proposed on this basis that the best candidates for the final irreversible adduct are the two *O*- and *N*-linked FGly adducts (Figure 5). One should note, of course, that an *N*-imine sulfate moiety could be reached by dehydration of the *N*-linked FGly adduct proposed. With no evidence for retention of the parent phenolic group in the adduct, such a dead-end sulfonimine, however formed (Hanson *et al.* 2006, Woo *et al.* 2000), or any other putative adducts with the phenolic group still in place (Woo *et al.* 2000, Williams *et al.* 2014), a double-sulfamoylated hydrated FGly (Bojarová *et al.* 2008, Williams *et al.* 2014) or even an irreversible internal Schiff base (Hanson *et al.* 2006) can all now essentially be excluded.

Here is, not the place, however for extensive discussion of the various mechanistic possibilities that are viable to reach either of these irreversible adducts and that have to some extent already been rehearsed elsewhere, but this new emphasis on the two most likely candidates should now at least make it possible to refine and test the possibilities in a more focused fashion experimentally. Nevertheless, given the lack of progress in this area over very many years some outline suggestions are worthy of mention (Figure 6) with brief discussion. In mechanism A the sulfamate drug is deemed to bind to the catalytically non-productive FGly aldehyde form of the enzyme. Potential electrophilic activation of the phenol-sulfur bond by the enzyme leads

to a well-precedented elimination of sulfonylamine (Thea *et al.* 1986, McCaw & Spillane 2006), that is more stable than the corresponding sulphur trioxide in sulfuryl transfer (Williams *et al.* 2014) and that then can attack the FGly keto group, in principle from either of its two faces. The resulting planar and electrophilic adduct is trapped by water ingress to give the *N*-linked FGly sulfamate covalent adduct **II**. Note the ambiguous stereochemistry at the adduct; such a possibility, to the best of our knowledge, has not been discussed before and could potentially contribute to the irreversibility of the process enzymatically. In an alternative pathway B, with ligand binding to the active hydrated form of the enzyme, the sulfamoyl group is transferred from the substrate to FGly, akin to a sulfate group to give the *O*-linked FGly sulfamate adduct **I**, analogous to the crystallographically observed resting pro-*R* sulfate intermediate FGlyS (Figure 5) for sulfatases. Note that this could also occur in principle *via* the simple capture of sulfonylamine in A as above by the hydrated FGly diol.

As discussed above, adduct **I** could in principle also be a dead-end in nature, but such an adduct has been deemed to be potentially reversible from energetic considerations (Williams *et al.* 2014). However, noting that in the normal catalyzed process sulfate is most likely eliminated from the sulfated FGly diol by an energetically favourable E2 process (Williams *et al.* 2014), thus regenerating the FGly aldehyde, it is possible that this next enzymatic step could also proceed with sulfamic acid elimination instead. The sulfamic acid anion thus released could be extruded from the enzyme, parallel to sulfate, or it could potentially tumble and attack the FGly aldehyde generated (in principle also from one of the two faces, although this is likely to be severely restricted within the confines of an active site) to generate again the final adduct **II**, again with potentially indeterminate stereochemistry. The new preliminary crystallographic work cannot explain the surprising stoichiometry reported in the inactivation of *PaAtsA* (Bojarová *et al.* 2008), but perhaps this could be the result of enzyme inactivation not being

the product of a single substrate turnover in principle in either of A or B; especially if, as in Figure 6, this proceeds *via* the non-catalytically active FGly formyl form of the enzyme *via* route A and the sulfonylamine is not always successful at inactivation, sometimes being quenched by *eg* water before it or any resulting sulfamic acid can reach the FGly.

In any case, much higher resolution crystallographic structures are required to move on definitively from these preliminary data and to identify unambiguously the true form of covalent modification. To distinguish between either the *O*- and *N*-linked FGly adducts crystallographically, however, is likely to be a very tough prospect indeed, requiring very high resolution. Most interestingly, although highly preliminary and still speculative in nature, is the observation that the data shown in Figure 4 implying a tetrahedral adduct at FGly were obtained for only one of the two subunits of *Pa*AtsA. While the other subunit was indeed also modified at the same FGly site, this new density seemed to show characteristics of a more “planar-like” adduct (see also legend to Figure 4). These results are to be viewed as guidelines only supporting directions for future work and should not be over-interpreted, but it is nevertheless tantalising to speculate that such data are in principle in line with mechanism A outlined in Figure 6, with an initial planar adduct (at sulphur) being converted to a tetrahedral *N*-linked final structure **II**. Taken together with all other evidence to date it is therefore tempting to de-prioritise adduct **I** of the two possibilities and be most drawn to adduct **II**. Given the well-precedented physical organic mechanistic data for sulfonylamine generation from aryl sulfamates by elimination, together with the preliminary crystallographic observations discussed here, a serious candidate for the inactivation mechanism must thus surely be A of Figure 6, with adduct **II** as the end product. Chemically, adduct **II** might also in principle be reversible, but perhaps this is not feasible within the STS active site because of possibly modified FGly stereochemistry, or simply that the presence of an *N-H* substitution replacing *O*

in the modified but standard stereochemistry of the FGly residue might offer *eg* new active site *H*-bonding possibilities that could interfere with the normal catalytic machinery.

Future developments

Dual aromatase-sulfatase inhibitors (DASIs)

As mentioned already, an STS inhibitor given alongside an AI should, in a multi-targeting fashion possess significant advantages. An alternative to administering two drugs to hit each enzyme is to develop a single molecule to inhibit both aromatase and STS, i.e. a Dual Aromatase-Sulfatase Inhibitor (DASI). Pharmaceutical arguments in favour of using single molecule multi-targeted agents have been made (Morphy & Rankovic 2006). Moreover, there are now numerous reports of dual- and even triple-targeted agents in clinical pipelines, particularly to address the development of resistance. An attractive approach to imbue dual activity in a DASI prototype is to take an advanced or clinically used AI with its existing heterocyclic CYP19 binding motif and to build in the aryl sulfamate pharmacophore (Woo *et al.* 2007) (Figure 7(a)). Lead DASI compounds have been developed possessing IC₅₀ values as AIs that are at least comparable to, or even better than, those AIs in widespread clinical use. Such compounds are effective *in vivo* against both enzymes validating the concept (Foster *et al.* 2008b). Two examples of optimized sulfamate-based compounds with excellent *in vivo* activity against both targets *eg* STX681 and the biphenyl-based STX1983, have been published (Woo *et al.* 2007; Woo *et al.* 2010) (Figure 7) and the overall concept and wider structures have been discussed in detail (Thomas & Potter 2015a). Using a “merged pharmacophore” strategy single compounds were even designed with dual picomolar multi-targeting activities *in vitro* (Woo *et al.* 2011b) *eg* the four-ringed structures (Figure 7(a)). It should be noted that in such a “double warhead” DASI the low nM AI activity is of a reversible nature, whereas the

low nM STS inhibitory activity is of an irreversible nature. Thus, clinically there should only effectively be a need to titrate the dose required for aromatase inhibition, since STS will always be rapidly and irreversibly inhibited in a time-dependent fashion. Since AIs alone have major preventive effects in healthy women with high breast cancer risk and this might also extend eventually to STS inhibitors it is conceivable that DASIs could also prove valuable in this regard in the longer term.

Development of enzyme-based PET cancer imaging agents has also been pursued using the DASI concept. New carbon-11-labeled sulfamate derivatives were designed and synthesized as potential PET radiotracers for imaging of aromatase and STS expression in breast cancer (Wang *et al.* 2009). Also interestingly and expanding on the DASI idea, a recent report has provided the first single molecule dual inhibitors of STS and 17 β -HSD1 (Salah *et al.* 2017) (Figure 7). Since 17 β -HSD1 is also implicated in controlling levels of E2 and is also upregulated in tumour tissue of patients treated with AIs it is also an attractive idea to combine these two activities for exploitation in estrogen-dependent diseases.

Aryl sulfamates for hormone-independent tumours

Hormone receptor–negative subtypes of breast tumours remain an unmet clinical challenge, with a high rate of recurrence and poor survival in patients following treatment. 2-Methoxyestradiol (2ME) is an endogenous non-estrogenic anticancer steroid with multiple activities relevant to hormone receptor–negative tumours (Parada-Bustamante *et al.* 2015) and also anti-inflammatory activity (Duncan *et al.* 2012), but has the big disadvantage of very low oral bioavailability (ca 1%), poor solubility and extensive metabolism *in vivo*. These very poor pharmaceutical properties are a significant barrier to further progression and although numerous oncology clinical trials have been carried out up to Phase II, with the compound

developed under the trade name Panzem, and with some promising results, severe limitations are apparent (Tevaarwerk *et al.* 2009, Parada-Bustamante *et al.* 2015). 2ME has been evaluated in patients with *eg* different refractory solid tumours, ovarian cancer, breast cancer, metastatic prostate cancer, primary peritoneal carcinomatosis, metastatic kidney cancer and advanced carcinoid tumours. For one clinical trial the phase II dosing regimen was 1000 mg orally every 6 hours (Tevaarwerk *et al.* 2009) and one trial of 2ME2 had to be cancelled because plasma drug concentrations after oral administration were lower than the effective dose. Structure activity relationships and synthetic design strategies in this area have been reviewed (Peyrat *et al.* 2012).

A new sulfamate-based drug candidate STX140 was designed (Leese *et al.* 2006) as a derivative of 2ME, an anti-tumour agent with excellent oral activity, improved potency, low metabolism and good pharmacokinetic properties (Thomas & Potter 2015a, b) (Figure 7(b)). As well as being a potent inhibitor of STS it exhibits a range of interesting additional properties, anti-angiogenic activity, induction of cell cycle arrest and apoptosis in human tumour xenografts, and has major clinical potential for the therapy of hormone-independent tumours. Some of this activity is thought to stem from tubulin binding at the colchicine site and disruption of interphase microtubules. STX140 is also highly active in tumours that have become resistant to chemotherapy (Newman *et al.* 2008).

In pre-clinical mouse xenograft models using STX140 complete cures were achieved after oral treatment in models of breast and prostate cancer and drug-resistant tumours also shrank in size after oral treatment (Thomas & Potter 2015b). Conventional treatments for hormone-independent cancers targeting tubulin are associated with major side effects, including neurotoxicity, and can only be given infrequently and intravenously (Bates & Eastman 2017).

STX140 is more effective on the same tumours, blocks metastatic spread and with no signs of the peripheral neuropathy associated with current clinical anticancer drugs (Meyer-Losic *et al.* 2013). STX140 thus offers, in a uniquely competitive fashion, not only a highly innovative solution to the problems faced by 2ME, but a new bioavailable drug with additional activities and properties that enhance its overall potential. While targeted therapies are offering new approaches in oncology, ultimately they rarely overcome acquired resistance mechanisms and they are still most attractive in combination with other drugs. STX140 offers, in one molecule and with highly attractive pharmaceutical properties, two novel “first-in-class” targeted therapeutic approaches: inhibition of STS and also of another novel target carbonic anhydrase IX (CAIX), in concert with other anticancer activities of established clinical potential (Thomas & Potter 2015b). Like the labelled DASI sulfamate derivatives 2-[¹¹C]methoxy-3,17β-O,O-bis(sulfamoyl)estradiol ([¹¹C]-STX140) was designed and synthesized as a new potential imaging agent for the PET imaging of STS in cancers (Wang *et al.* 2012).

Because of its multi-targeting activities STX140 can function as an STS inhibitor in hormone-dependent settings, with its extra activities, or it can be utilized for the more intractable hormone-independent solid tumours. Importantly, with its aryl sulfamate pharmacophore it also benefits from the red blood cell transport mechanisms of other STS inhibitors and enhanced stability. Moreover, a range of small molecule non-steroidal STX140 mimics have been developed (Leese *et al.* 2010) such as STX 2484 (Figure 7(b)) that exhibit similar activities and development potential, but are pharmaceutically even more versatile and synthetically more tractable. These show a range of interesting biological activities (Thomas & Potter 2015a, b, Stengel *et al.* 2014, Shen *et al.* 2015) and are now ripe for wider development. Interestingly, some of these analogues, despite possessing an aryl sulfamate pharmacophore, are only very weak STS inhibitors. Very recently an example of such a non-

steroidal sulfamate analogue from a quinazolinone-based series was shown in atomic detail to bind to the colchicine binding site on tubulin, as the first example of any sulfamate-based ligand (Dohle *et al.* 2018). Unlike the previous pro-drug and sulfamoyl transfer activities discussed, in this setting the sulfamate group is simply acting as an additional binding element to enhance interaction of ligand with target. These classes of compounds continue to illustrate the potential of the aryl sulfamate pharmacophore.

Wnt signalling pathway activity

While STS is now well established as a therapeutic target for estrogen-dependent diseases potential wider cellular functions of STS are still unclear. A recent report (Shin *et al.* 2017) showed that STS induces Wnt/ β -catenin signalling in PC-3 and HeLa cells and STS knockdown results in downregulation of Wnt/ β -catenin signalling. Treatment with an STS inhibitor prevented STS-mediated Wnt/ β -catenin signalling and Twist1 expression. Importantly, cancer cell migration, invasion, and matrix metalloproteinase expression induced by STS could also be inhibited by an STS inhibitor. The ability of STS to induce the Wnt/ β -catenin signalling and an epithelial-mesenchymal transition may have interesting implications for estrogen-mediated carcinogenesis in the human cancer cell and opens up a whole new area of interest.

Conclusions

AIs are clinically successful and their prophylactic properties in breast cancer add to their utility. The recent striking clinical success exemplified by combining androgen deprivation therapy with the 17 α -hydroxy lyase/C17, 20 lyase inhibitor Abiraterone (Zytiga) in prostate cancer (James *et al.* 2017) is also highly notable. Application of new mechanisms for endocrine therapy involving intracrine biosynthetic principles are both attractive academic and clinical

pursuits. Pharmacological intervention in the emerging STS-mediated pathways is clearly worthy of clinical exploration and the last 10-15 years have seen that come gradually to greater fruition in the clinic. There is now a greater appreciation of the intracrine mechanisms whereby circulating precursors can be converted *in situ* in tumour cells, for example *via* mechanisms involving the import of E1S locally into tumours *via* organic anion transporters followed by further *in situ* enzymatic transformations. Of the various enzymes implicated in such processes the STS-mediated pathway has emerged as a significant player, not only in breast cancer but also in other hormone-dependent disease states.

The aryl-*O*-sulfamate pharmacophore of the most successful STS inhibitors seems to function in three different and distinct roles: it can be a prodrug with the sulfamate group cleavable by STS; an STS inhibitor where the sulfamate moiety is almost certainly transferred in some fashion in a similar way to sulfonyl transfer to a residue in the STS active site – this now at last appears to be clarified tentatively as the catalytic FGly, with the end product not a previously proposed *N*-imine sulfate, but a sulfamoylated adduct with the linking atom being either *O*- or *N*- and most likely *N*; finally, the sulfamate group can be viewed simply as supplying structural augmentation to aid potency in *eg* the multi-targeting series of steroidal and non-steroidal microtubule disruptors. In this latter case it has been shown to augment ligand binding to the colchicine site at the $\alpha\beta$ dimer interface of tubulin. The pharmaceutical properties intrinsic to such sulfamate esters could possibly be widely exploited more generally in drug design. The sulfamate group not only offers versatility and high potency, but by virtue of facilitating sequestration in red blood cells to CA II also confers *in vivo* drug stabilization, resistance to first pass metabolism and excellent pharmacokinetic and bioavailability properties. Sulfamate-based STS inhibitors have yet to be surpassed in terms of potency and their other attractive pharmaceutical properties.

The steroidal E2MATE was originally developed as the estradiol prodrug J995 and reached phase II trials. Now, as PGL2001, it is being actively developed in endometriosis employing its STS inhibitory properties. Such aryl-*O*-sulfamates, as exemplifiers of the first potent and irreversible inhibitors of STS have stimulated a large body of preclinical and clinical work through the “first-in-class” drugs E2MATE (J995, PGL2001) and Irosustat and early clinical studies have substantially validated the scientific and therapeutic concepts originally proposed. While only one non-steroidal sulfamate-based drug Irosustat has been explored clinically in three types of hormone-dependent cancer to date, there are wider emerging targets becoming available. Some, such as ovarian cancer, are already well predicated while others, such as colorectal cancer and bladder cancer, are just emerging as possibilities. Measurement of tumour STS expression can be a helpful prognostic biomarker and this should almost certainly be targeted for patient stratification in future clinical studies.

No aryl sulfamate-based drug targeting hormone-dependent disease has yet progressed beyond phase II clinical trials. It remains to be seen whether this will be achieved in some indication as a monotherapy and the limited patient cohort of the recent IPET clinical trial offers the attraction to pursue this in early breast cancer with a larger patient cohort; or whether it might better be achieved *via* a combination approach, eg with an AI, where the first such potential has been demonstrated recently in the IRIS clinical trial. In terms of such a multi-targeting strategy it seems clear that the DASI approach also offers great potential in cases where there is expected to be a synergy between STS and aromatase inhibition and that such an approach could address some acquired resistance mechanisms that have now been established. Additionally, compounds such as STX140 or their non-steroidal counterparts offer huge potential as multi-targeting agents for either hormone-independent tumours, where the pre-

clinical potential is already strongly proven, or perhaps in a hormone-dependent tumour setting even as a still highly potent STS inhibitor with added cytotoxic/anti-angiogenic activity and activity against CAIX. Compared with the parent endogenous 2ME that is very poorly bioavailable and extensively metabolized, the bis-sulfamate STX140 is highly bioavailable, protected against metabolism, whilst maintaining and even enhancing the core activities of the parent steroid. However, it must be noted that the STS inhibitory activity of the non-steroidal variants cannot always be taken for granted.

After some 20 years of discovery and development it seems that while the clinical promise of aryl-*O*-sulfamates as STS inhibitors are now established they have yet to demonstrate their full potential. The optimal setting for use of an STS inhibitor still needs to be found, both in terms of pathology and whether as monotherapy, classical combination therapy with an AI or, in certain relevant applications, using a single molecule dual agent such as a DASI or even as a combined STS-17 β -HSD1 inhibitor. Exploitation in hormone-independent settings is predicated by excellent pre-clinical data, for example for 2-substituted estradiol derivatives such as STX140 and non-steroidal relatives, but clinical entry has yet to be achieved. In the context of this particular review support for the continuing development and optimisation of new types of endocrine therapy is also provided by a recently reported study (Roswall *et al.* 2018) that used resistant ER α -negative breast tumours and showed that they can be sensitized to hormone therapy. Genetic or pharmacological targeting of platelet-derived growth factor-CC isoform activity converted basal-like tumours in mouse cancer models into a hormone receptor-positive state. This and the emergence of new roles for STS, for example in Wnt/ β -catenin signalling will be sure to maintain levels of excitement and motivation to apply further the concepts and the related drugs discussed here, both in endocrine therapy applications and beyond.

Declaration of interests

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review. He is a co-inventor on patents for some of the agents reported here, was a co-founder of Sterix Ltd and holds stock in EstryX Pharma Ltd.

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Figure legends

Figure 1: (a) Structures of the first reversible steroidal STS inhibitor estrone 3-*O*-methylthiophosphonate and the first irreversible inhibitors EMATE, estrone 3-*O*-sulfamate and E2MATE estradiol 3-*O*-sulfamate, (b) Structure of human steroid sulfatase showing its mushroom-like shape (adapted from PDB.1P49); the arrow indicates the region of the active site and the expansion shows Irosustat (orange) docked into the active site, showing the Ca^{2+} ion depicted as a yellow sphere, FG75 as the *gem*-diol form of the catalytic FGly residue and illustrating the proximity of the sulfamate group to the FGly residue. Dotted line is a putative *H*-bond.

Figure 2: Structures of the aryl *O*-sulfamate pharmacophore (top, X, Y & Z are substituent groups), 4-methylcoumarin 7-*O*-sulfamate (R = H), 3,4-dimethylcoumarin 7-*O*-sulfamate (R = CH₃) and the tricyclic series of coumarin-based STS inhibitors; n=5 was chosen as optimal for the clinical candidate.

Figure 3: Structures of the non-steroidal STS inhibitor Irosustat (top) and an example of a member of the the tricyclic series in folded and ionized format (bottom right), showing its similarity to the steroidal estrone 3-*O*-sulfate (left).

Figure 4: The X-Ray crystal structure of the active site region of inactivated *Pa*AtsA. Active *Pseudomonas aeruginosa* arylsulfatase A (*Pa*AtsA) was inactivated by incubating the enzyme at room temperature for *ca.* 10min with a *ca.*10-fold molar excess of Irosustat/STX64, as monitored by using a 4-nitrocatechol sulphate assay. The enzyme was shown to be completely inactivated. The hanging drop vapour diffusion method was used for protein crystallization. Inactivated enzyme was mixed with well buffer and after *ca.* 2 weeks at room temperature

diamond-shaped crystals in the C2 space group formed, which diffracted to 2.0 Å. Two subunits were observed in the unit cell with differences at FGly between them. The structure for the Irosustat-inactivated enzyme showed only a single, apparently covalent, modification at FGly in both subunits, with no modification of nearby flanking Lys or His residues. The structure showed an area of new density (marked by the yellow arrow) in the active site close to the hydrated FGly and calcium in one subunit that was best modelled as a tetrahedral covalent attachment to the *pro-R* hydroxyl group of FGly (possibly with a small degree of dual occupancy); and in the other subunit (not shown) the density seemed to be more planar in character, perhaps suggestive of a potential two-step inactivation process (see Figure 6A). In the former case while both a *pro-R* and *pro-S* stereochemistry for FGly can be modelled into the density, the *pro-R* stereochemistry had the best fit, as illustrated.

Figure 5: Top, STS active site hydrated formylglycine FGly residue (left) and proposed *N*-imine sulfate end product **III** (right); bottom, the sulfated hydrated FGlyS resting form (left), and the suggested *pro-R* *O*- **I** and *N*-linked FGly **II** modifications (middle and right respectively) derived from the preliminary crystallographic study of *Pseudomonas aeruginosa* arylsulfatase inactivated by Irosustat.

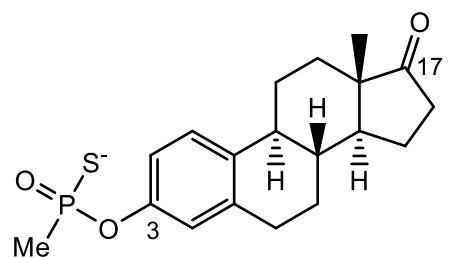
Figure 6: Putative mechanisms to produce the two candidates for irreversibly inhibited enzyme adduct. *Route A:* Sulfamate ester binds to the catalytically non-productive formyl form of STS and the phenol-S bond is possibly electrophilically activated (by group X) to facilitate elimination of a reactive sulfonylamine that then attacks FGly (note in principle this can be from either face of the keto group so the stereochemistry of the adduct is not fixed). The planar highly electrophilic adduct is then trapped by water ingress to generate adduct II (note again indeterminate stereochemistry). *Route B:* The *pro-R* hydroxyl of hydrated FGly of STS attacks

the sulfamate ester analogously to normal attack on a sulfate ester to generate adduct I, a candidate for an irreversible adduct if the rest of the catalytic mechanism leading normally to sulfate extrusion is impeded by the presence of the amino group. If not, however, and the next stage of the reaction proceeds then sulfamic acid rather than sulfate is released, as in the normal catalytic cycle for a pro-*R* sulfated intermediate, and can then either be extruded like sulfate or could tumble and rearrange internally with its nitrogen moiety attacking the FGly formyl form (note in principle this can be from either face of the keto group so the stereochemistry of the adduct is again not fixed) to generate adduct II.

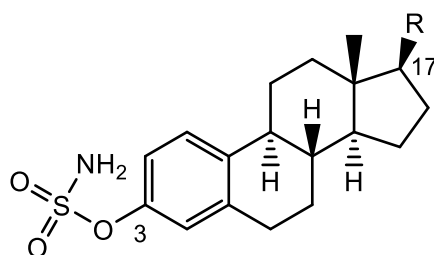
Figure 7: (a) Dual inhibitors: top, dual inhibitory DASI compounds *eg* STX681 and STX 1983 (showing two pharmacophoric components) and bottom (left), the merged pharmacophore template of the pM-active dual inhibitors and (right) the first example of a dual STS and 17 β -HSD1 inhibitor; (b) multi-targeting agents: structures of 2-methoxyestradiol bis-sulfamate STX140 and a typical non-steroidal derivative STX2484.

Figure 1

(a)



Estrone 3-O-methylphosphonothioate



R= =O, Estrone 3-O-sulfamate, EMATE
R= -OH Estradiol 3-O-sulfamate, E2MATE

(b)

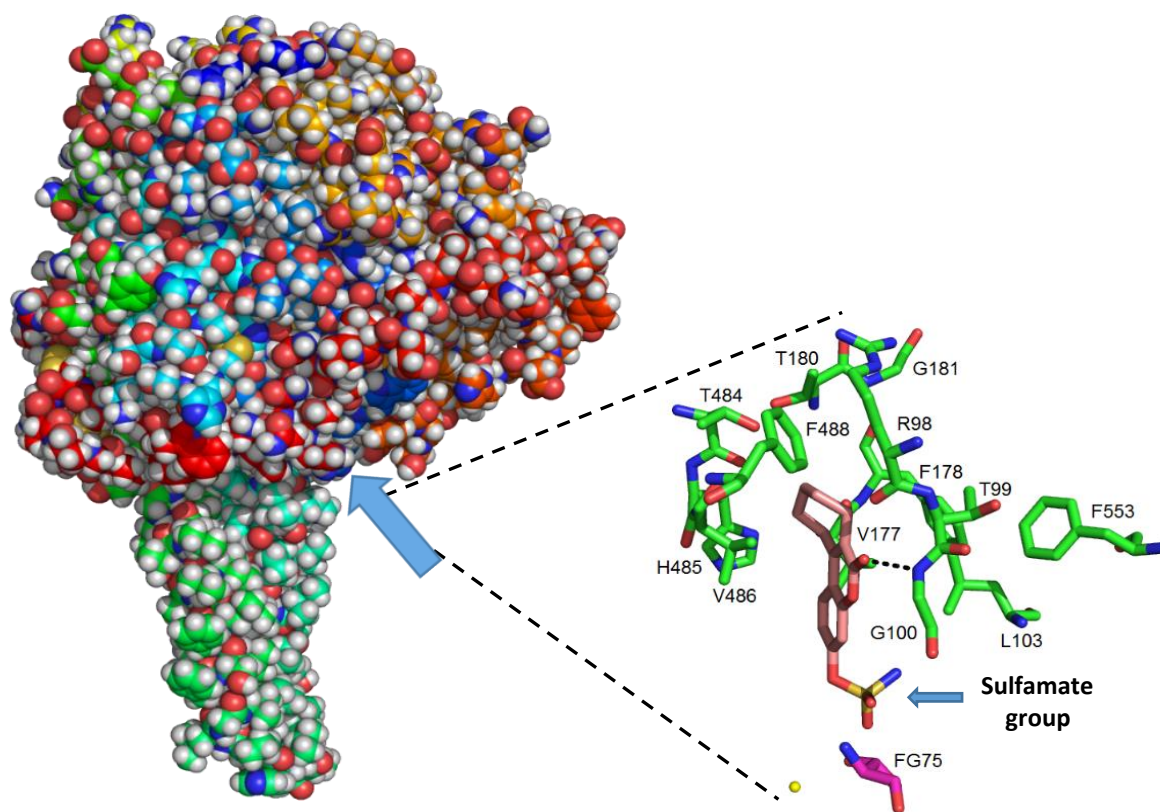
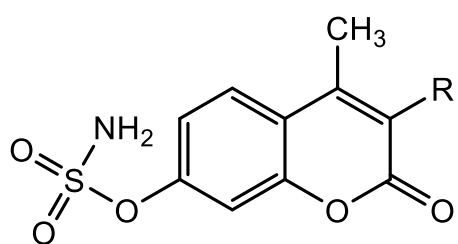
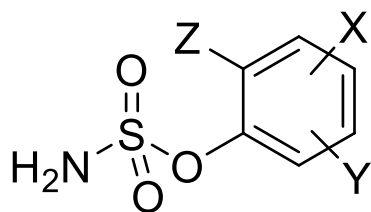
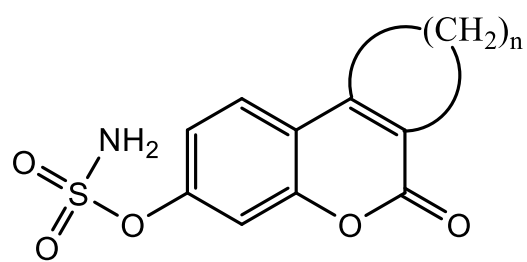


Figure 2

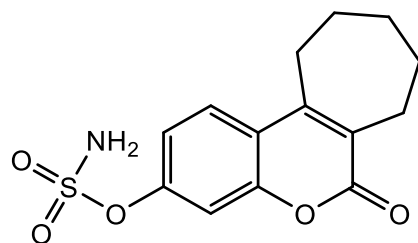


$\text{R} = \text{H}, \text{CH}_3$

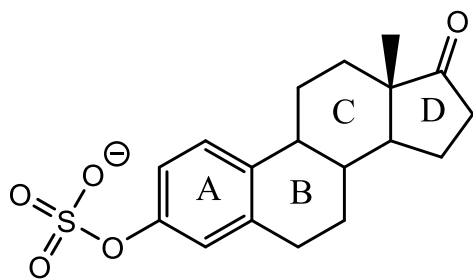


Tricyclic drug series
 $n = 5$ 667 COUMATE

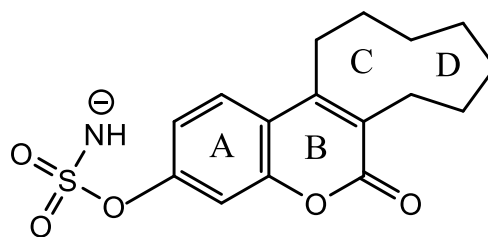
Figure 3



Irosustat
STX64, 667 COUMATE, BN83495



Estrone 3-O-sulfate, E1S



Tricyclic coumarin with n=7
and ionized sulfamate

Figure 4

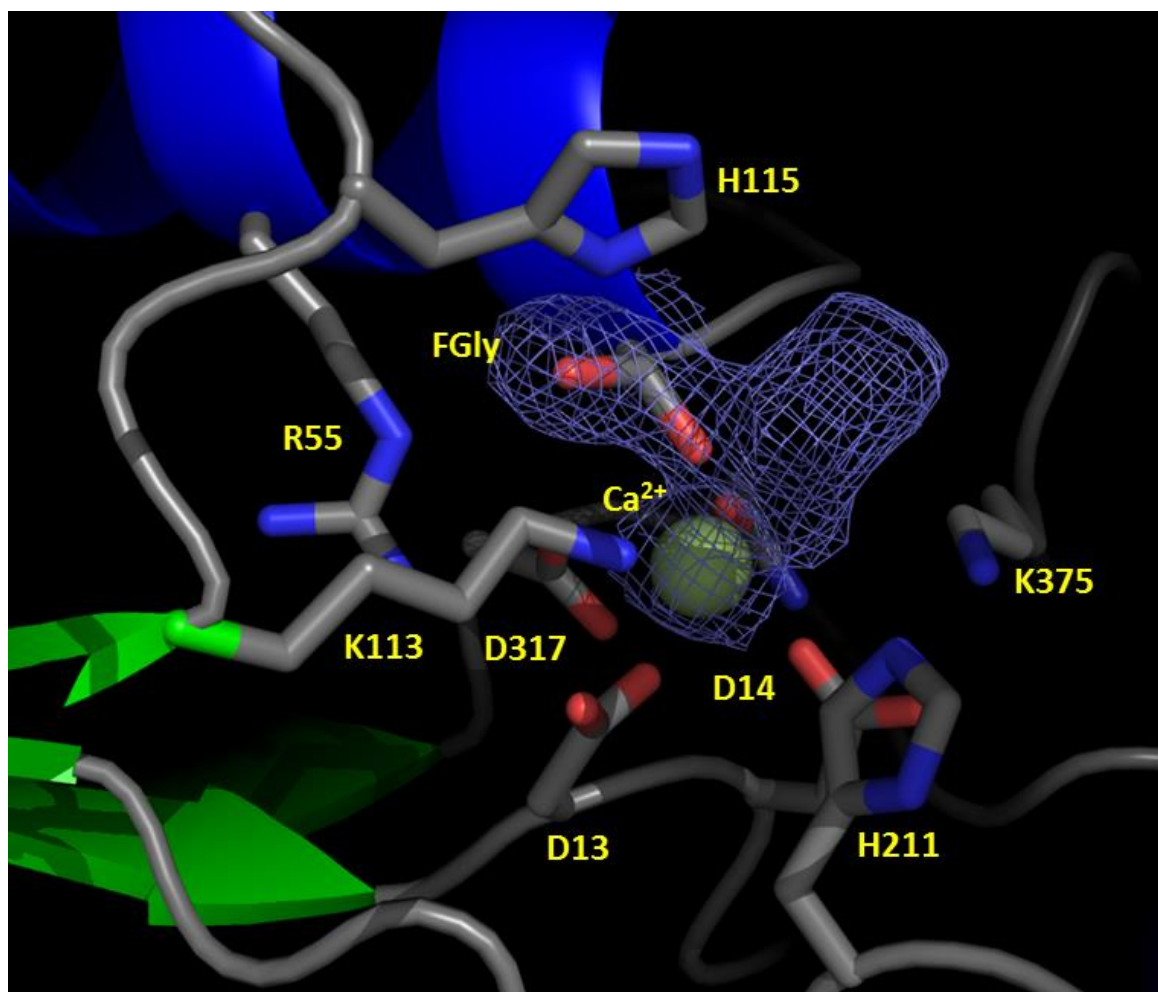


Figure 5

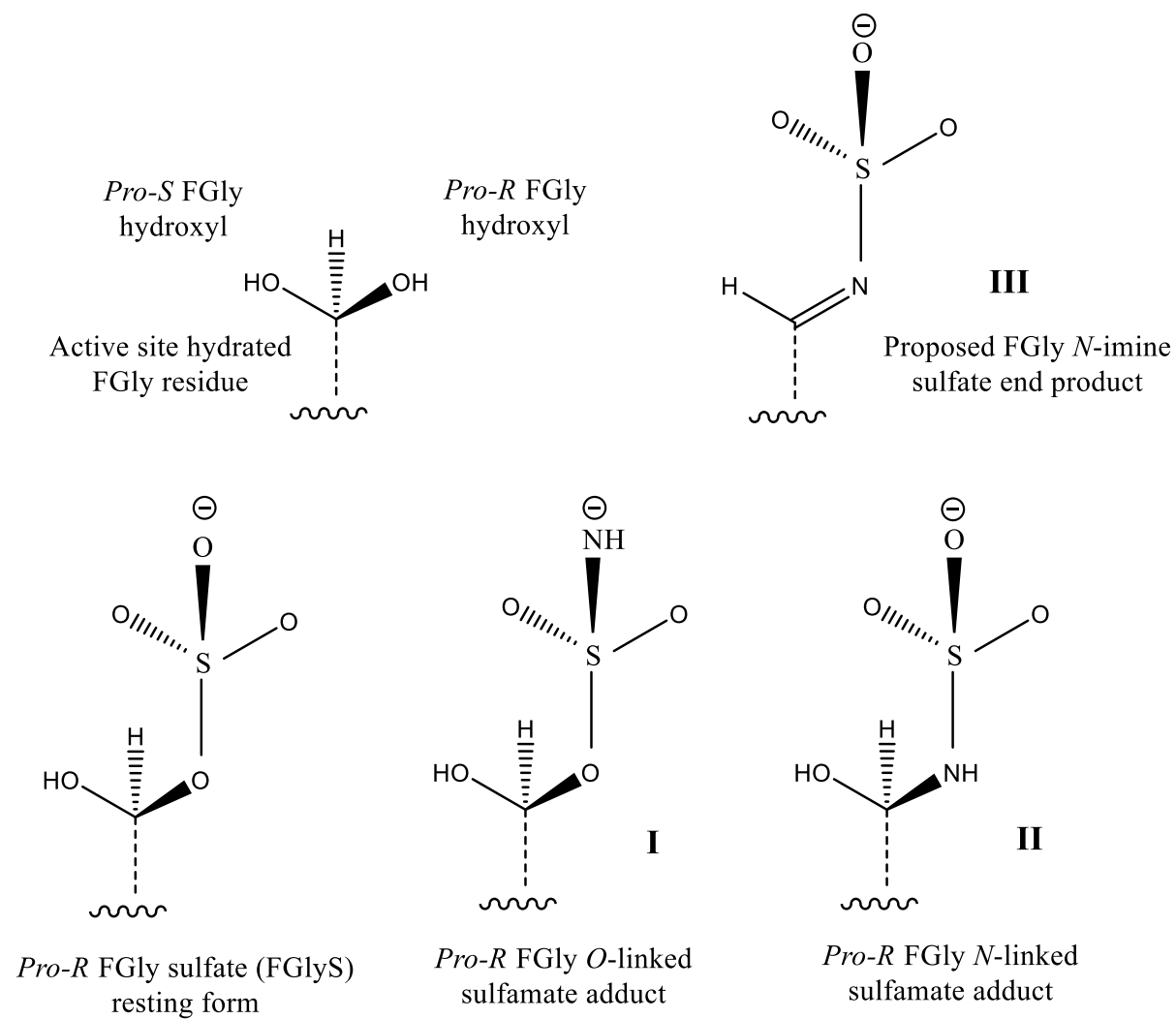
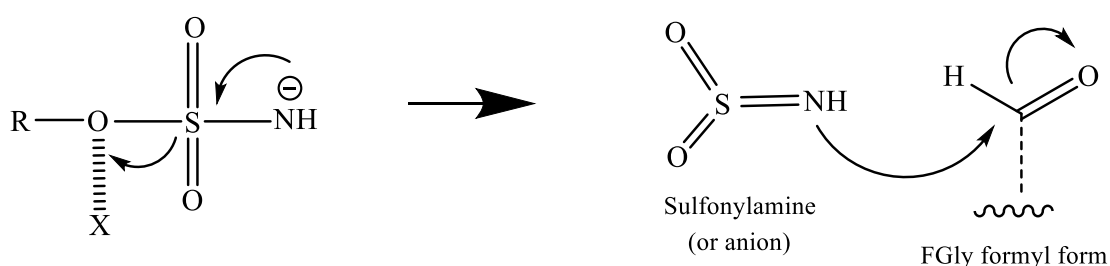
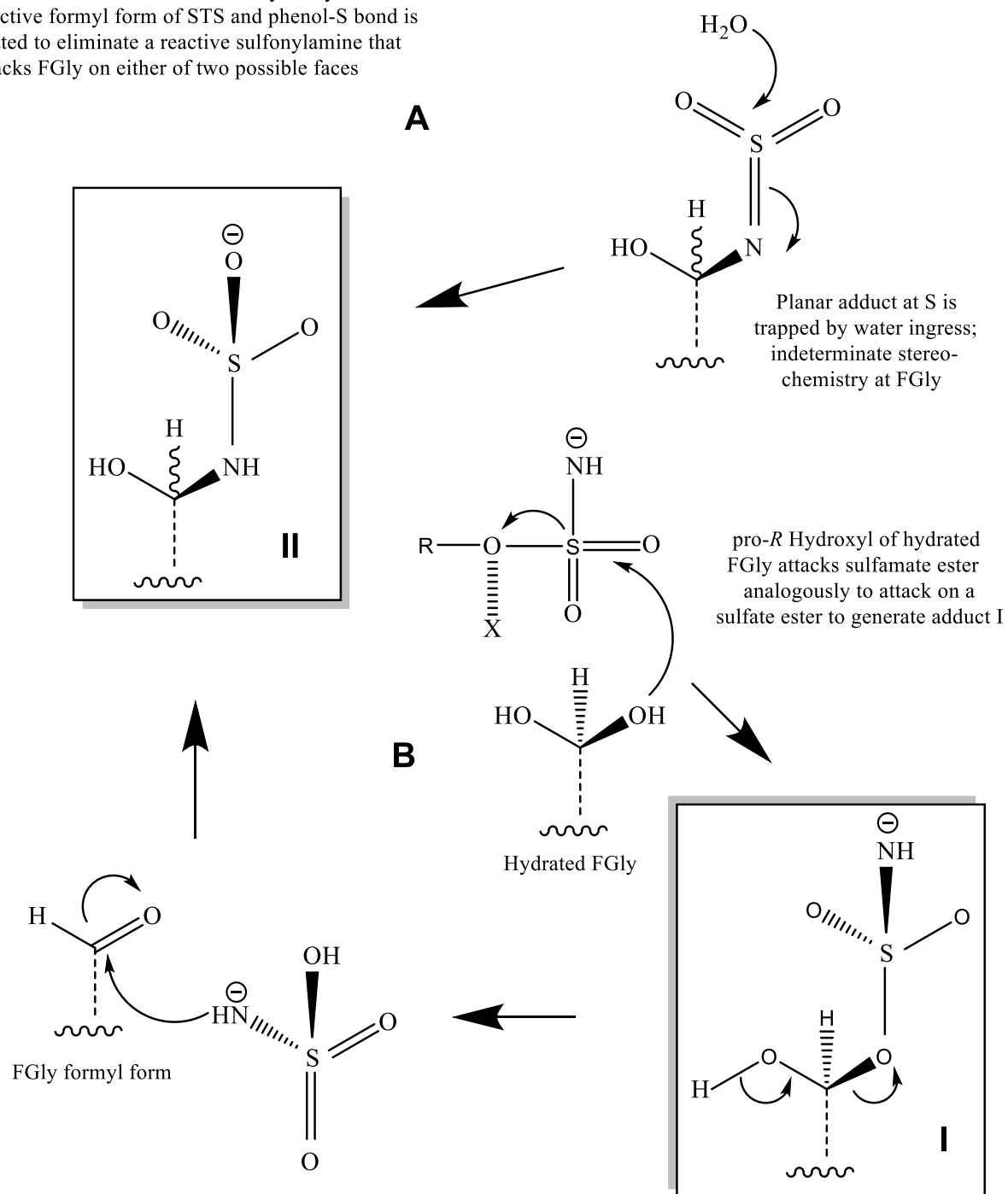


Figure 6



Sulfamate ester binds to the catalytically non-productive formyl form of STS and phenol-S bond is activated to eliminate a reactive sulfonamide that attacks FGly on either of two possible faces



Sulfamic acid is released as in the normal catalytic cycle for a pro-*R* sulfated intermediate and can be extruded or can tumble and rearrange with nitrogen attacking the FGly formyl form on either of two possible faces to generate adduct II

Figure 7

