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Inhibition of steroid sulphatase activity by tricyclic coumarin sulphamates

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Abstract

The identification of the active pharmacophore required for potent inhibition of steroid sulphatase activity, i.e. an aryl-*O*-sulphamate structure, has led to the synthesis and testing of a large number of 1–4 ring-based inhibitors. 4-Methylcoumarin-7-*O*-sulphamate (COUMATE) was one of the first non-steroid based inhibitors identified. In an attempt to increase the potency of this class of inhibitor a series of tricyclic COUMATEs (665–6615 COUMATEs) have been synthesised and evaluated. Using placental microsomes as a source of oestrone sulphatase (E1-STS) the size of the third ring of the tricyclic COUMATEs was found to have a marked effect on inhibitor potency. Whereas 665- and 6615-COUMATEs had IC_{50}s of 200 and 370 nM, respectively, the most potent inhibitor in vitro in this series was 6610 COUMATE with an IC_{50} of 1 nM. Selected inhibitors were tested for their in vivo potency by administration of a single dose (0.1 or 1 mg/kg, p.o.) to female rats. Surprisingly, in vivo 6615 COUMATE proved to be the most active drug, inhibiting rat liver E1-STS activity by 23 and 94% when assayed 24 h after administration of the 0.1 and 1 mg/kg doses. E1-STS activity in brain tissue and white blood cells was also found to be inhibited when selected drugs were tested. These studies have identified a number of tricyclic COUMATEs with therapeutic potential.

Keywords: Steroid sulphatase activity; Pharmacophore; Inhibitor

1. Introduction

Inhibition of steroid sulphatase activity (STS) is an important target for the development of new drugs for oncology and immunology [1,2]. The sulphatase pathway is a major route for the synthesis of steroids with oestrogenic activity in post-menopausal women [3]. Such steroids include oestrone, formed from oestrone sulphate (E1S) by oestrone sulphatase (E1-STS) but also 5-androstenediol (Adiol) [4,5]. Adiol is synthesised from dehydroepiandrosterone (DHA) after its hydrolysis from DHA-sulphate. Only one STS is thought to be responsible for the hydrolysis of aryl and alkyl steroid sulphates [6]. Steroid sulphatase is widely distributed in tissues throughout the body and the same enzyme is thought to be present in liver, brain and white blood cells (WBCs). The formation of oestrogenic steroids via the sulphatase route is thought to have an important role in supporting the growth of hormone-dependent breast tumours in post-menopausal women [7].

In addition to its oncological role there is now evidence that STS may regulate part of the immune response. Whether T-helper (Th) cells progress to a Th1 or Th2 phenotype, each of which secretes a characteristic profile of cytokines, is thought to be regulated by the balance of the adrenal androgen, DHA, to that of glucocorticoid [8,9]. STS in macrophages, within the lymphoid environment, regulates the hydrolysis of DHA-sulphate to DHA. This enzyme therefore has a crucial role in regulating the DHA: glucocorticoid balance, and thus Th cell maturation. Inhibition of STS may reduce the level of Th1 cells and Th1-type cytoki-

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nes. In an animal model, there is evidence that inhibition of STS can modulate the immune response and have a beneficial effect in collagen-induced rheumatoid arthritis [10].

Since the identification of the active pharmacophore required for potent inhibition of STS, an aryl-*O*-sulphamate structure [11], many steroid and non-steroid-based inhibitors have been identified [12–15]. The lead compound, oestrone-3-*O*-sulphamate (EMATE), proved unexpectedly, to be oestrogenic [16,17] resulting in a search for potent inhibitors that were devoid of oestrogenicity. A number of different approaches have been used in attempts to identify non-oestrogenic inhibitors, including the linking of the sulphamate group to single or polycyclic ring systems or modifications to the A and/or D ring of the oestrone nucleus [13,18]. This research led to the identification of a number of tricyclic coumarin sulphamates [19] which are active in vitro and in vivo [20,21]. In this study we have continued to explore the structure-activity relationship of a number of tricyclic coumarin sulphamates and compared their in vitro and in vivo activities.

2. Materials and methods

2.1. Synthesis

A series of 665–6615 tricyclic coumarins was prepared by reacting resorcinol with the corresponding 5–15-membered cyclic β-ketoester (e.g. methyl 2-oxocycloheptane carboxylate for 667 COUMARIN) in the presence of an equimolar mixture of concentrated sulphuric acid and trifluoroacetic acid (a Pechmann synthesis).

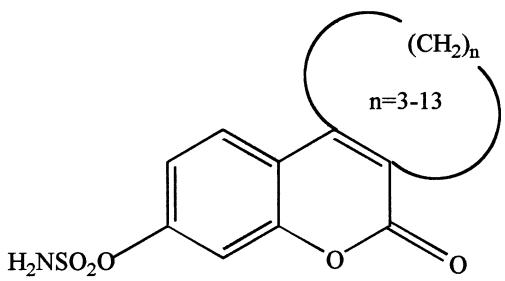
The resulting phenolic compounds were sulphamoylated [11] to give the 665–6615 COUMATEs (Fig. 1). All compounds exhibited spectroscopic and analytical properties consistent with their structure. Full details of the synthesis and characterisation of these inhibitors will be reported elsewhere.

2.2. Inhibition of steroid sulphatase activity

The ability of the tricyclic COUMATEs to inhibit STS activity was examined using placental microsomes [22]. To determine the IC_{50}s for the inhibition of STS, activity was measured in the presence of inhibitor (10 pm–10 μM) using [6,7-^{3}H] E1S (4×10^5 dpm, NEN-Du Pont, Boston, MA) adjusted to 20 μM with unlabelled substrate [22]. Supernatants prepared from homogenised rat liver tissues were also used to test the potency of selected inhibitors in vitro [22].

2.3. In vivo studies

Selected drugs were tested in vivo for their ability to inhibit STS activity. Female Wistar rats (200–250 g) were obtained from Harlan Olac (Bicester, Oxon, UK). Groups of rats, with three rats in each group for each experiment, were treated p.o. with vehicle (propylene glycol) or drug (0.1 and 1 mg/kg) with animals receiving a single dose. Animals were killed, using an approved procedure, 24 h after drug administration to assess the extent of STS inhibition. For this, samples of liver tissue were removed and immediately frozen on solid carbon dioxide and stored at −20°C until assayed. For some animals samples of brain tissue were also collected to assess the extent of inhibition of STS



Tricyclic Coumarin Sulphamates

Compound	Placental IC_{50} (nM)
665COUMATE	200
666COUMATE	70
667COUMATE	8
668COUMATE	30
669COUMATE	2.4
6610COUMATE	1
6611COUMATE	13
6612COUMATE	60
6613COUMATE	75
6615COUMATE	370

Fig. 1. Structures and IC_{50}s, determined using placental microsomes of: 665 COUMATE, 4-oxo-2,3-dihydro-1*H*-cyclopenta-[c][1]benzopyran-7-*O*-sulphamate; 666 COUMATE, 6-oxo-7,8,9,10-tetrahydrodibenzo[b,d]pyran-3-*O*-sulphamate; 667 COUMATE, 6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta-[c][1]benzopyran-3-*O*-sulphamate; 668 COUMATE, 6-oxo-7,8,9,10,11,12-hexahydro-cycloocta-[c][1]benzopyran-3-*O*-sulphamate; 669 COUMATE, 6-oxo-8,9,10,11,12,13-hexahydro-7*H*-cyclonona-[c][1]benzopyran-3-*O*-sulphamate; 6610 COUMATE, 6-oxo-7,8,9,10,11,12,13,14-octahydro-cyclodeca-[c][1]benzopyran-3-*O*-sulphamate; 6611 COUMATE, 6-oxo-8,9,10,11,12,13,14,15-octahydro-7*H*-cycloundeca-[c][1]benzo-pyran-3-*O*-sulphamate; 6612 COUMATE, 6-oxo-7,8,9,10,11,12,13,14,15,16-decahydro-cyclododeca-[c][1]benzopyran-3-*O*-sulp-hamate; 6613 COUMATE, 6-oxo-8,9,10,11,12,13,14,15,16,17-decahydro-7*H*-cyclotrideca-[c][1]benzopyran-3-*O*-sulphamate; 6615 COUMATE, 6-oxo-8,9,10,11,12,13,14,15,16,17,18,19-dodecahydro-7*H*-cyclopentadeca-[c][1] benzopyran-3-*O*-sulphamate.

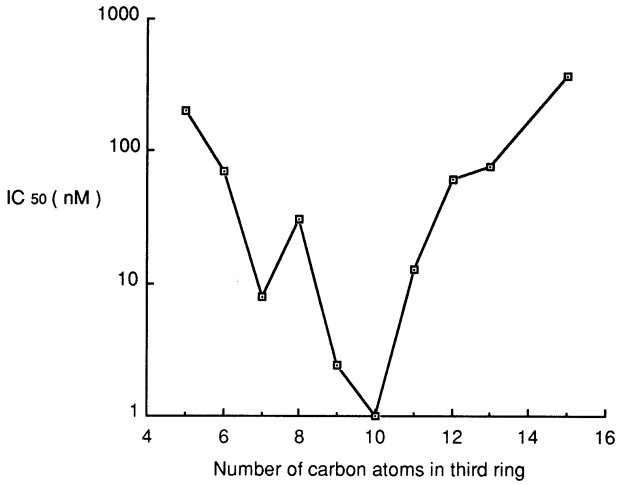


Fig. 2. Effect of the number of carbon atoms in the third ring on E1-STS inhibitor potency of tricyclic coumarin sulphamates (665–6615 COUMATES). IC_{50} values were determined using placental microsomes.

in this tissue. Tissues were homogenised and, after centrifugation to remove cell debris, aliquots of the supernatant were used for the STS assay [23]. For some animals blood was also collected by cardiac puncture under anaesthesia. WBCs were obtained by centrifugation with Histopaque 1077 (Sigma, Poole, Dorset, UK) and used to assay STS activity [24].

2.4. Statistics

Student's t-test was used to assess the significance of the effects of drugs on STS activity.

3. Results

3.1. IC_{50}s for tricyclic COUMATES

The IC_{50}s for the inhibition of placental microsome STS by the tricyclic COUMATEs are shown in Fig. 1. Of the 665–6615 COUMATE series, 6610 COUMATE proved to be the most potent STS inhibitor in vitro with an IC_{50} of 1 nM. It was apparent from this series that the size of the third ring had a marked effect on the ability of the compounds to inhibit STS activity (Fig. 2). While 665 and 6615 COUMATEs were the least potent inhibitors in this series in vitro with IC_{50}s of >200 nM, 669, 6610 and 6611 COUMATEs were all potent inhibitors with IC_{50}s of 2.4, 1 and 13 nM, respectively.

3.2. In vivo activity

From the results obtained after testing inhibitors in vitro a number of compounds were selected for in vivo testing. Animals received a single oral dose of inhibitor at 0.1 or 1 mg/kg with tissue samples being collected 24 h later for the assay of E1-STS activity. Of the series of drugs tested at 0.1 mg/kg (Fig. 3(a)) only 6615 COUMATE produced a small, but statistically significant ($P < 0.05$) decrease (23%) in liver E1-STS activity. However, at 1 mg/kg all of the compounds tested significantly inhibited liver E1-STS activity (Fig. 3(b)). 667 COUMATE resulted in a 86% inhibition of E1-STS activity compared with that for animals receiving vehicle only. 665 COUMATE was less potent, achieving 75% inhibition with 6610 COUMATE having a similar inhibitory potency to that of 667 COUMATE. Surprisingly, in view of its in vitro activity, the most potent compound in vivo in this series was 6615 COUMATE

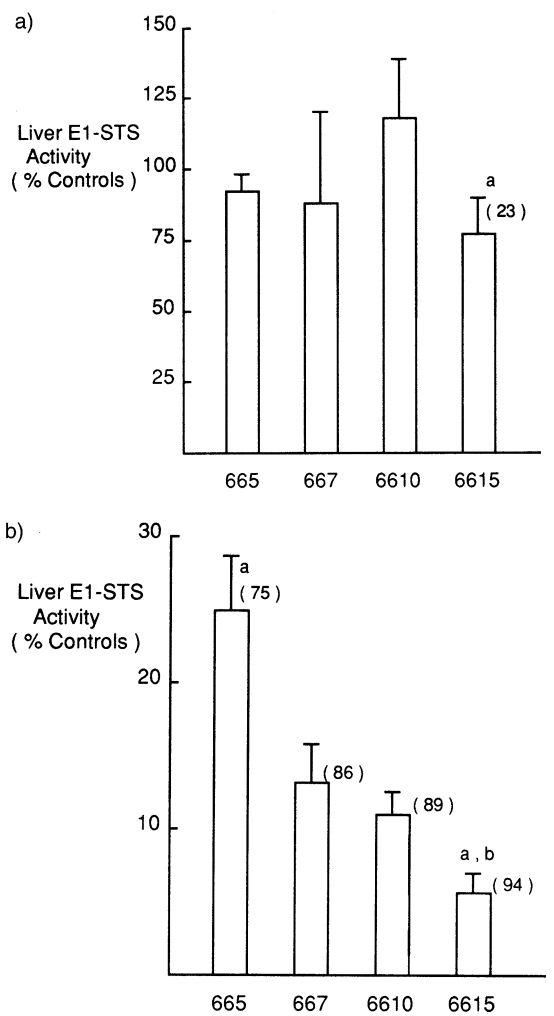


Fig. 3. In vivo effects of selected inhibitors on E1-STS activity in rat liver tissue preparations. Rats were treated with vehicle (propylene glycol) or inhibitors at 0.1 or 1 mg/kg with animals receiving a single dose p.o. Samples of liver tissue were collected for assay of E1-STS activity 24 h after administration of the dose. Results are expressed as the percentage of remaining E1-STS activity compared with that in control animals (mean $\pm$ S.D., $n = 3$). (a) 0.1 mg/kg; a, $P < 0.05$ compared with controls. (b) 1 mg/kg; a, $P < 0.001$ versus 667 COUMATE; b, $P < 0.001$ versus 6610 COUMATE.

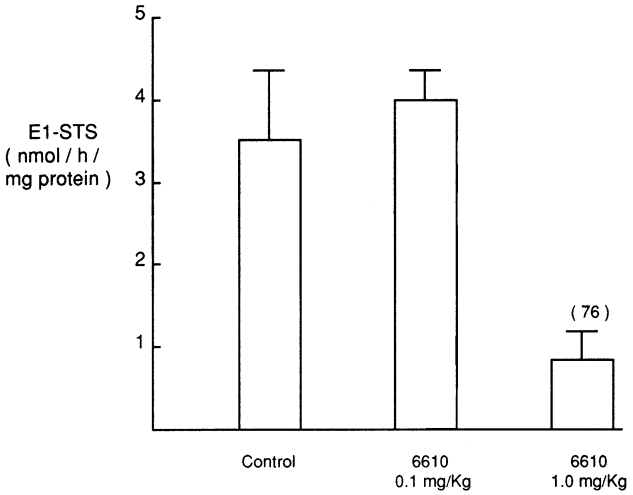


Fig. 4. Effect of 6610 COUMATE on brain E1-STS activity in samples collected 24 h after administration (p.o.) of a single 0.1 or 1 mg/kg dose. At the higher dose brain E1-STS activity was inhibited by 76% (mean ± S.D., $n = 3$).

inhibiting E1-STS activity by 94%. This is significantly ($P < 0.001$) greater than the 86% inhibition achieved by 667 COUMATE at 1 mg/kg.

On the basis of its in vitro potency the effect of 6610 COUMATE on brain STS activity was also examined (Fig. 4). Whereas no significant inhibition of brain STS was detected after dosing animals at 0.1 mg/kg, the higher 1 mg/kg does resulted in a marked 76% inhibition of activity. For 6610- and 6615-COUMATEs inhibition of STS in WBCs was also monitored (Fig. 5). At 0.1 mg/kg 6615 COUMATE inhibited WBCs STS activity by 45% whereas 6610 COUMATE was without effect. However, at the higher dose both compounds almost completely inhibited WBCs STS activity.

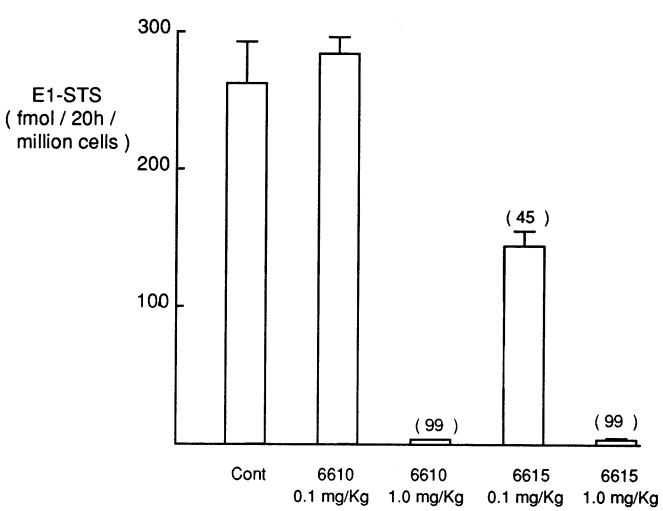


Fig. 5. Effect of 6610 or 6615 COUMATE on E1-STS activity in WBCs 24 h after administration (p.o.) of a single 0.1 or 1 mg/kg dose (mean ± S.D., $n = 3$). Figures in parentheses represent the percent inhibition compared with control animals.

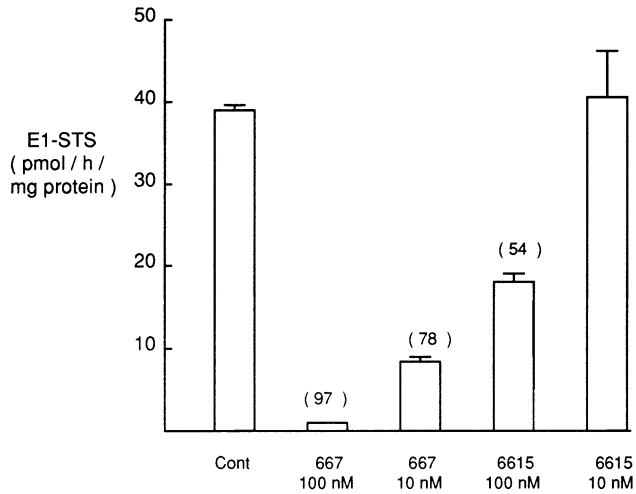


Fig. 6. In vitro inhibition of E1-STS activity in a rat liver preparation by 667- and 6615-COUMATEs (mean ± S.D., $n = 3$). Figures in parentheses represent the percent inhibition compared with controls.

3.3. Inhibition of rat liver steroid sulphatase activity in vitro

Because of the in vivo finding that 6615 COUMATE was a more potent inhibitor of STS activity than 6610 COUMATE, in contrast to their in vitro potencies, the ability of these compounds to inhibit STS in a rat liver supernatant preparation was examined. As previously described IC_{50} values for these compounds were determined using human placental microsomes and it is possible that a species difference in STS could influence the potencies of the tricyclic COUMATEs in vivo. As shown in Fig. 6, however, using a rat liver preparation 667 COUMATE, at both 10 and 100 nM, was significantly more potent than 6615 COUMATE at these concentrations.

4. Discussion

The identification of the active pharmacophore required for potent inhibition of STS activity has enabled a large number of 1–4 ring sulphamoylated compounds to be tested as inhibitors [12–15]. Based upon the possibility that the 2-ringed COUMATE might mimic the A and B rings of steroid based inhibitors, such as EMATE, a series of tricyclic COUMATEs were designed, synthesised and tested [19]. It was reasoned that such compounds might also act as mimics of the C and D rings of the steroid nucleus and might thus enhance inhibitor potency. 665 COUMATE proved to be 4-times more potent than the 2-ringed COUMATE, with an IC_{50} of 200 nM. This suggested that such a strategy could lead to the development of more potent STS inhibitors than the original lead compound EMATE. Testing a series of tricyclic COUMATEs has proved

the validity of this approach, indicating, at least in vitro, that the optimal size of the third ring was achieved with 6610 COUMATE. This compound is 800-times more potent than COUMATE in vitro and 8-times more potent than 667 COUMATE. One possibility for the optimal degree of STS inhibition achieved with 6610 COUMATE might be that the third, 10-membered carbon ring, folds to mimic the C and D rings of the steroid nucleus. In vitro 6610 COUMATE is 25-times more potent than the steroid-based inhibitor EMATE. In vitro further increases in the size of the third ring was associated with a marked reduction in inhibitor potency with 6615 COUMATE being 370-times less potent than 6610 COUMATE.

On the basis of their in vitro inhibitory potencies a range of compounds were selected for further testing in vivo. For the series of 665–6610 COUMATEs, inhibition of STS activity in vivo at 1 mg/kg closely reflected the IC_{50}s for the compounds obtained from in vitro studies. 665 COUMATE (IC_{50} 200 nM) inhibited liver STS by 75% whereas 6610 COUMATE (IC_{50} 1 nM, the most potent compound in vitro) inhibited activity by 89%. Unexpectedly, 6615 COUMATE with an IC_{50} value 370-times higher than that for 6610 COUMATE proved to be the most potent inhibitor in vivo. 6615 COUMATE at 1 mg/kg inhibited STS activity by 94%, a significantly higher degree than that achieved with 6610 COUMATE. While further studies are required to account for this unexpected finding it is possible that the larger third ring of 6615 COUMATE increases its lipophilicity thus facilitating its entry into tissues.

Overall, the tricyclic COUMATEs are more potent in vivo inhibitors than the original 2-ringed COUMATE which, at a single dose of 1 mg/kg, p.o., inhibited liver STS activity by only 68% [12]. However, in other studies with EMATE and a number of its 2-substituted derivatives, a single 0.1 mg/kg dose resulted in almost 50% inhibition of liver STS activity (unpublished observations). In the present investigations only 6615 COUMATE at 0.1 mg/kg produced a small (23%) decrease in liver STS activity. Thus, in vivo steroid-based inhibitors are more potent than those based upon other ring structures. It has been previously observed that the duration of STS inhibition in vivo by steroid-based inhibitors is much longer than that achieved with non-steroidal inhibitors [12,23]. Whether this is due to differences in the stability, rates of metabolism of the different types of inhibitors or to steroid-based inhibitors being sequestrated in tissues and slowly released remains to be determined.

As it is proposed to use STS inhibitors in the treatment of hormone-dependent breast cancer, a method using WBCs was previously developed to monitor the extent and duration of STS inhibition [24]. Using this assay both 6610- and 6615-COUMATE were found to inhibit WBCs STS activity almost completely at the higher dose tested. This finding confirms that WBCs STS activity will provide a means of monitoring the extent and duration of inhibition of STS activity if these compounds are selected for clinical trials.

There is evidence, that in addition to their potential therapeutic roles in oncology and immunology, STS inhibitors may be of value in the treatment of some disorders of cognitive function [25]. The ability of 6610 COUMATE to inhibit brain STS activity was therefore tested. As previously found for EMATE [23], 6610 COUMATE effectively inhibited STS activity in brain tissue.

In conclusion, the results from these investigations have identified additional potent STS inhibitors that are active in vivo. Unexpectedly 6615 COUMATE proved to be the most potent inhibitor in vivo. It is possible that further enlargement of the third ring size (e.g. 6616–6620) might further enhance in vivo activity.

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