

In Vivo Inhibition of Estrone Sulfatase Activity and Growth of Nitrosomethylurea-induced Mammary Tumors by 667 COUMATE¹

Atul Purohit, Lawrence W. L. Woo, Barry V. L. Potter, and Michael J. Reed²

Endocrinology and Metabolic Medicine and Sterix Ltd., Imperial College School of Medicine, St. Mary's Hospital, London W2 1NY, United Kingdom [A. P., M. J. R.], and Wolfson Laboratory of Medicinal Chemistry and Sterix Ltd., Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom [L. W. L. W., B. V. L. P.]

Abstract

The development of potent steroid sulfatase inhibitors is an important new therapeutic strategy for the treatment of postmenopausal women with breast cancer. A series of tricyclic coumarin sulfamates were synthesized, and their inhibitory properties were examined *in vitro* and *in vivo*. In a placental microsomal assay system, 667 COUMATE emerged as the most potent inhibitor with an IC_{50} of 8 nM. Administration of a single dose (10 mg/kg, p.o.) of 667 COUMATE inhibited rat liver estrone sulfatase activity by 93%. 667 COUMATE was devoid of estrogenicity, as indicated by its failure to stimulate the growth of uteri in ovariectomized rats. *In vivo*, estrone sulfate-stimulated growth of uteri in ovariectomized rats was inhibited by 667 COUMATE. Using the nitrosomethylurea-induced mammary tumor model, we found that 667 COUMATE caused regression of estrone sulfate-stimulated tumor growth in a dose-dependent manner. The identification of 667 COUMATE as a potent steroid sulfatase inhibitor will enable the therapeutic potential of this type of therapy to be evaluated.

Introduction

The development of enzyme inhibitors to block estrogen synthesis offers a new approach for the treatment of postmenopausal women with hormone-dependent breast tumors. Thus far, research has been directed mainly toward the identification of aromatase inhibitors, which block the conversion of androstenedione to estrone (1, 2). There is, however, a growing awareness of the role that steroid sulfatase, which hydrolyses E1S³ to estrone, may have in regulating the formation of estrogenic steroids (3–5). The steroid sulfatase that hydrolyses estrone sulfate is also responsible for the formation of DHEA from DHEA sulfate (6). Reduction of DHEA gives rise to Adiol which, although an androgen, can bind to the estrogen receptor. Adiol can stimulate the growth of breast cancer cells *in vitro* and carcinogen-induced mammary tumors *in vivo* in ovariectomized rats (7, 8). The E1S surrogate, EMATE, was identified as a potent, active site-directed irreversible inhibitor (9, 10). EMATE was active *in vivo* and had a prolonged duration of action (11) but, unexpectedly, EMATE proved to be a potent estrogen, being five times more active than ethinylestradiol on oral application to rats (12). A series of structure-activity relationship studies revealed that the active pharmacophore required for potent steroid sulfatase inhibition is a sulfamate

group attached to an aromatic ring (13). This finding led to the design and synthesis of a number of sulfamated 1–4 ring compounds that are potent non-estrogenic steroid sulfatase inhibitors (14–16). Such inhibitors included COUMATE which, although less potent than EMATE, was active *in vivo* (15). In this paper, we report on the *in vitro* and *in vivo* activity of a number of tricyclic coumarin sulfamates, one of which was selected for testing in an induced mammary tumor model in rats.

Materials and Methods

Synthesis of Tricyclic Coumarin and Oxepin Sulfamates. The series of 665–668 tricyclic coumarins were prepared by a Pechmann synthesis of the starting coumarin by reacting resorcinol with the corresponding 5–8-membered cyclic β -ketoester (*e.g.*, methyl 2-oxocycloheptane carboxylate for 667 COUMARIN) in the presence of concentrated sulfuric and trifluoroacetic acids. The tricyclic oxepin was prepared in a similar manner from resorcinol and ethyl (2-oxocyclohexyl) acetate.

The resulting phenolic compounds were sulfamoylated to give: 665 COUMATE (Fig. 1, 1), 666 COUMATE (Fig. 1, 2), 667 COUMATE (Fig. 1, 3), 668 COUMATE (Fig. 1, 4), and 676 OXEPIN (Fig. 1, 5). All compounds exhibited spectroscopic and analytical properties consistent with their structure. Full details of the synthesis and characterization of the tricyclic coumarins and oxepin will be reported elsewhere.

In Vitro and in Vivo Inhibition of Estrone Sulfatase Activity. The ability of the tricyclic coumarin and oxepin sulfamates to inhibit E1-STS activity and determinations of IC_{50} s were carried out using placental microsomes (100,000 \times g fraction; Ref. 17). For *in vivo* studies, female Wistar rats (Harlan Olac, Bicester, Oxon, United Kingdom) were treated p.o. with vehicle (propylene glycol) or drug (0.1–1.0 mg/kg) with either a single dose or five multiple doses at daily intervals.

Uterotrophic Study. To examine *in vivo* for possible estrogenic effects of 667 COUMATE, rats were ovariectomized and 14 days later received vehicle (propylene glycol, 200 μ l, p.o.) or 667 COUMATE (2 mg/kg/day, p.o.) for 5 days. The ability of 667 COUMATE to inhibit E1S-stimulated uterine growth in ovariectomized animals was also investigated. For this, animals received either vehicle or 667 COUMATE (2 mg/kg/day, p.o.) initially for 2 days to suppress E1-STS activity. Animals either continued to receive vehicle p.o. plus E1S (50 μ g/day, s.c.) or 667 COUMATE (2 mg/kg/day, p.o.) plus E1S (50 μ g/day, s.c.) for another 5 days.

Animals were killed 24 h after administration of the last dose of drug, and uteri were excised of fat and weighed. Total body weights of the animals were also recorded, and the results were expressed as uterine weight \times 100/total body weight.

Inhibition of Mammary Tumor Growth. Ludwig rats were obtained from Harlan Olac after induction of mammary tumors with NMU (16). Tumor development was monitored, and animals were ovariectomized when tumors reached 0.8–1.5 cm in diameter. Tumors were allowed to regress over a 12- to 13-day period to confirm their hormone-dependent status. Regrowth of tumors was stimulated with E1S (50 μ g/day, s.c.). When tumors had regrown, animals continued to receive either E1S alone or E1S plus 667 COUMATE at 10 mg/kg/day or 2 mg/kg/day, p.o., until tumor regression had occurred. Tumor volumes were calculated from two measured diameters (11).

Estrone Sulfatase Activity in Tissues. Liver and tumor tissues obtained from rats were immediately frozen on solid carbon dioxide and stored at

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² To whom requests for reprints should be addressed, at Endocrinology and Metabolic Medicine, Imperial College School of Medicine, St. Mary's Hospital, London W2 1NY, United Kingdom. Telephone: 44-207-886-1738; Fax: 44-207-886-1790; E-mail: m.reed@ic.ac.uk.

³ The abbreviations used are: E1S, estrone sulfate; E1-STS, estrone sulfatase; DHEA, dehydroepiandrosterone; Adiol, 5-androstenediol; EMATE, estrone-3-*O*-sulfamate; COUMATE, 4-methylcoumarin-7-*O*-sulfamate; NMU, nitrosomethylurea; 667 COUMATE, 6-oxo-8,9,10,11-tetrahydro-7H-cyclohepta-[c][1]benzopyran-3-*O*-sulfamate.

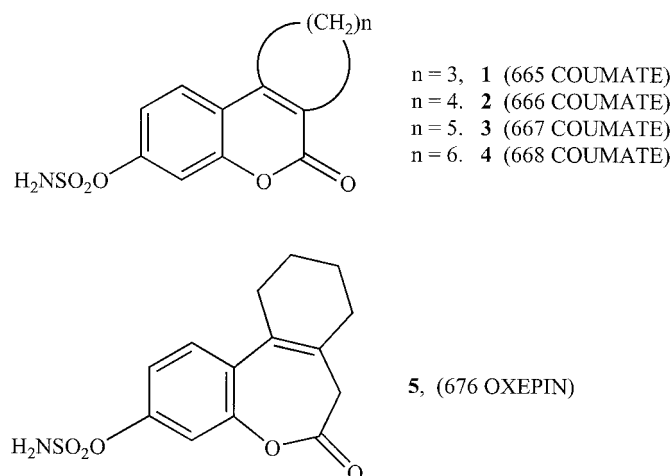


Fig. 1. Structures: compound 1, 4-oxo-2,3-dihydro-1*H*-cyclopenta-*c*[1]benzopyran-7-*O*-sulfamate (665 COUMATE); compound 2, 6-oxo-7,8,9,10-tetrahydro-dibenzo[*b*,*d*]pyran-3-*O*-sulfamate (666 COUMATE); compound 3, 6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta-*c*[1]benzopyran-3-*O*-sulfamate (667 COUMATE); compound 4, 6-oxo-7,8,9,10,11,12-hexahydro-cycloocta-*c*[1]benzopyran-3-*O*-sulfamate (668 COUMATE); compound 5, 6-oxo-6,7,8,9,10,11-hexahydrodibenz[*b*,*d*]oxepin-3-*O*-sulfamate (676 OXEPIN).

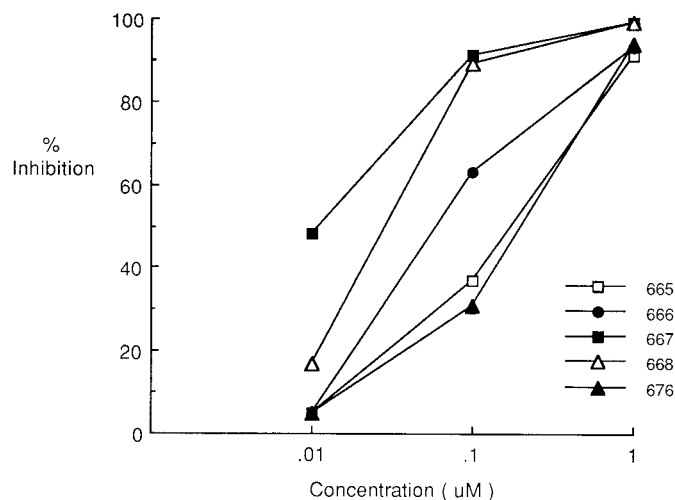


Fig. 2. Inactivation of E1-STS activity in placental microsomes by 665 COUMATE, 666 COUMATE, 667 COUMATE, 668 COUMATE, and 676 OXEPIN. [³H]E1S adjusted to 20 μM with unlabeled substrate with or without inhibitors at concentrations of 0.01–1.0 μM was incubated with placental microsomes (125 μg of protein/ml) for 30 min. The product formed was isolated by toluene extraction with [4-¹⁴C]estrone being used to monitor procedural losses. Each point represents the mean of triplicate measurements for which the coefficients of variation were <10%.

–20°C until assayed. Tissues were homogenized and, after centrifugation to remove cell debris, aliquots of the supernatant were used for the sulfatase assay (17).

Statistics. Student's *t* test was used to assess the significance of the effect of 667 COUMATE on uterine growth. The paired Student's *t* test was used to assess the significance of changes in tumor volumes after ovariectomy, E1S-stimulated regrowth, and after inhibitor treatment.

Results and Discussion

Inhibition of E1-STS Activity *in Vitro* and *in Vivo*. The series of tricyclic coumarin and oxepin sulfamates were all potent inhibitors of *in vitro* E1-STS activity (Fig. 2), with almost complete (91–99%) inhibition being achieved at 1 μM. Determination of IC₅₀s for this series revealed that 667 COUMATE was the most potent, with an IC₅₀ of 8 nM. IC₅₀s for the other derivatives were 200, 70, and 30 nM for the 665, 666, and 668

COUMATES and 250 nM for the 676 OXEPIN sulfamate. The relative potency of 667 COUMATE to that of COUMATE and EMATE was assessed using the placental microsomes assay. The IC₅₀ for EMATE was 25 nM and for COUMATE, 800 nM. Thus, 667 COUMATE (IC₅₀, 8 nM) is three times more potent than EMATE and 100 times more potent than COUMATE in inhibiting E1-STS activity.

On the basis of its highest *in vitro* inhibitory potency, 667 COUMATE was selected for testing *in vivo*. Single or multiple doses of 667 COUMATE (1 mg/kg) inhibited rat liver E1-STS activity by >90% (data not shown). 667 COUMATE, therefore, has a similar potency *in vivo* to that of EMATE (11). The duration of inhibition of E1-STS activity by 667 COUMATE was assessed

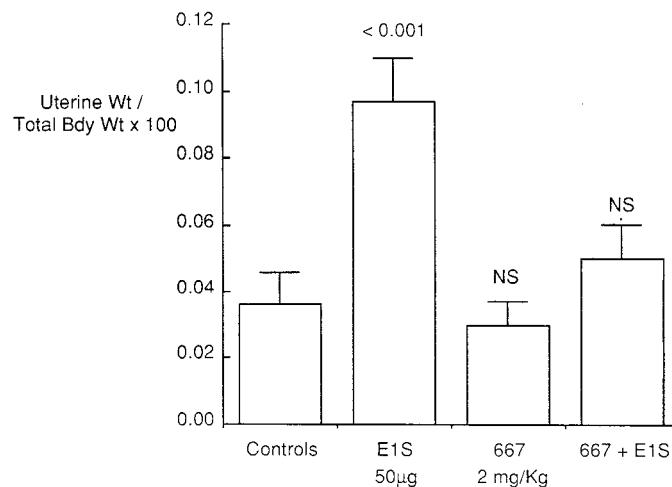


Fig. 3. Effect of E1S, 667 COUMATE, or E1S plus 667 COUMATE on uterine growth. Animals were ovariectomized and 14 days later received vehicle (propylene glycol, p.o.), E1S (50 μg/day, s.c.), or 667 COUMATE (2 mg/kg/day, p.o.) for 5 days. Animals receiving E1S plus 667 COUMATE were initially treated for 2 days with 667 COUMATE (2 mg/kg, p.o.) to suppress E1-STS activity and then received a combination of E1S (50 μg/day, s.c.) plus 667 COUMATE (2 mg/kg/day, p.o.) for an additional 5-day period. Twenty-four h after the last dose was administered, uteri were obtained and weighed. Results are expressed as uterine weight × 100/total body weight (means; *n* = 3; bars, SD). NS, not significant.

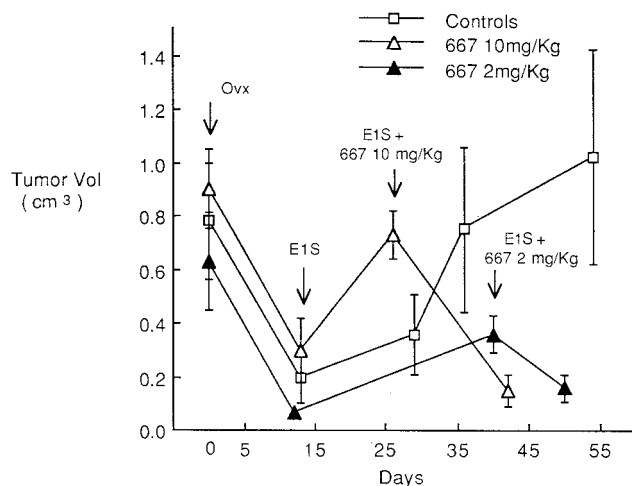


Fig. 4. Effect of E1S or E1S plus 667 COUMATE on the growth of NMU-induced mammary tumors in ovariectomized rats. After ovariectomy and tumor regression, tumor regrowth was restimulated with E1S (50 μg/kg/day, s.c.). Animals continued to receive either E1S alone or E1S (50 μg/kg/day, s.c.) plus 667 COUMATE (10 mg/kg/day or 2 mg/kg/day, p.o.) as indicated. Tumors were measured in two diameters, and tumor volumes were calculated. A significant decrease ($P < 0.02$) in tumor volumes occurred after ovariectomy and an increase ($P < 0.02$) after E1S administration and decrease ($P < 0.02$) after inhibitor co-administration with E1S ($n = 4$ for E1S only; $n = 3$ for E1S + 10 mg/kg 667 COUMATE; and $n = 3$ for E1S + 2 mg/kg 667 COUMATE; means; bars, SE).

using samples of liver tissue obtained 1, 3, and 7 days after a single dose (10 mg/kg). Complete recovery of E1-STS activity had occurred by 7 days after drug administration (data not shown). The *in vitro* and *in vivo* hydrolysis of DHEA-sulfate was also efficiently inhibited by 667 COUMATE (data not shown). 667 COUMATE has been identified as a potent nonsteroidal inhibitor of steroid sulfatase and is considerably more potent than the two-ringed COUMATE. The two rings of COUMATE were thought to act as mimics for the A and B rings of the steroid nucleus. The introduction of a third ring in the tricyclic coumarin sulfamates has increased the potency of 667 COUMATE 100-fold compared with that of COUMATE. EMATE and COUMATE have been shown previously to act in a time- and concentration-dependent manner (10, 13). 667 COUMATE also inhibits the enzyme in a similar manner, but the inactivation of E1-STS by this inhibitor was more rapid than by EMATE (data not shown), confirming its enhanced potency. Although the mechanism by which 667 COUMATE inactivates E1-STS is not yet fully elucidated, it has been postulated that, like EMATE, it acts via irreversible sulfamoylation of one or more residues in the enzyme active site.

Although 667 COUMATE has a similar potency to that of EMATE *in vivo*, there is a marked difference in the time taken for E1-STS activity to be restored after a single dose of these drugs. For 667 COUMATE, similar to COUMATE (15), E1-STS activity had recovered by 7 days. This is much shorter than the recovery period seen after EMATE administration, which only increased by 10–15% 15 days after cessation of the drug (11).

Inhibition of E1S-stimulated Uterine and Tumor Growth. Although previous studies revealed that 667 COUMATE did not stimulate the growth of estrogen-sensitive MCF-7 breast cells *in vitro* (18), it was tested for estrogenicity *in vivo* using the rat uterotrophic model. In ovariectomized rats receiving E1S (50 µg/day, s.c.) for 5 days, uterine growth was stimulated by 170% compared with animals receiving vehicle (Fig. 3). In contrast, for animals receiving 667 COUMATE (2 mg/kg, p.o.) for 5 days, uterine weights did not differ significantly from those of control animals. This model was also used to test the ability of 667 COUMATE to inhibit E1S-stimulated uterine growth. Measurements of uterine weights 24 h after administration of the last dose of drug and E1S revealed that the ability of E1S to stimulate uterine growth was blocked by the co-administration of 667 COUMATE (Fig. 3). Thus, although 667 COUMATE is a potent inhibitor of E1-STS activity, unlike EMATE it is devoid of estrogenicity. It was evident, however, from this model system that 667 COUMATE was effective in blocking the ability of E1S to stimulate uterine growth.

Further evidence of the ability of 667 COUMATE to inhibit E1S-stimulated tissue growth was obtained from a NMU-induced mammary tumor study (Fig. 4). In this model, tumor growth is maintained after ovariectomy by the s.c. administration of E1S. Ovariectomy resulted in a significant decrease ($P < 0.02$) in tumor volumes, and administration of E1S led to a significant increase ($P < 0.02$) in tumor volumes. For animals receiving a combination of E1S plus 667 COUMATE, a significant reduction ($P < 0.02$) in tumor volumes occurred. At a dose of 10 mg/kg, the decrease was $81 \pm 5\%$ (mean \pm SE; $n = 3$), whereas at 2 mg/kg the decrease was $56 \pm 13\%$ (mean \pm SE; $n = 3$). Measurements of E1-STS activity in representative samples of tumors confirmed that activity was effectively sup-

pressed (data not shown). Using this model, EMATE and its *N*-methylated derivatives were shown previously to inhibit E1S-stimulated mammary tumor growth (11).

Since the identification of EMATE as a steroid sulfatase inhibitor, there has been considerable progress in developing potent, non-estrogenic inhibitors. Such compounds include modifications at the C17 position of EMATE to give a series of alkylamido and *N*-alkylcarbamoyl sulfamates (19) or 17 α -*p*-*tert*-butylbenzyl sulfamates (20). The development of 667 COUMATE represents an important advance in identifying a nonsteroidal inhibitor that, although three times more potent than EMATE *in vitro*, is not estrogenic. 667 COUMATE has been identified as a potent inhibitor with therapeutic potential and is scheduled to enter a Phase I clinical trial for use in postmenopausal women with breast cancer in the near future.

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