PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 351–358

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Inhibition of MCF-7 breast cancer cell proliferation and in vivo steroid sulphatase activity by 2-methoxyoestradiol-bis-sulphamate☆

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Abstract

The endogenous oestrogen metabolite, 2-methoxyoestradiol (2-MeOE2) inhibits the growth of breast cancer cells and is also a potent anti-angiogenic agent. We have previously shown that the 3-sulphamoylated derivatives of 2-methoxyoestrogens are more potent than the non-sulphamoylated compounds. In this study, we have compared the abilities of 2-methoxyoestradiol-bis-sulphamate (2-MeOE2bisMATE) and 2-MeOE2 to inhibit the growth of MCF-7 breast cancer cells. Both compounds inhibited cell growth with the IC_{50} for 2-MeOE2bisMATE (0.4 μM) being six-fold lower than that for 2-MeOE2 (2.5 μM). Oestrogen sulphamates are potent inhibitors of steroid sulphatase (STS) activity. 2-MeOE2bisMATE was found to retain its STS inhibitory activity and in a placental microsome assay system it was equipotent with oestrone-3-*O*-sulphamate (EMATE). An in vivo study was also carried out to compare the potency of 2-MeOE2bisMATE with that of EMATE and the non-steroidal STS inhibitor, 667 coumarin sulphamate (667 COUMATE). After a single oral dose (10 mg/kg) some recovery of STS activity was detected by day 3 (10%) with activity partially restored (55%) by day 7 after administration of 667 COUMATE. For the other two steroidal compounds, STS activity remained almost completely inactivated for up to 5 days with complete restoration of activity occurring by day 15. The anti-proliferative and STS inhibitory properties of 2-MeOE2bisMATE suggest that it has considerable potential for development as a novel anti-cancer drug.

Keywords: Oestrogens; Breast cancer; Steroid sulphatase; 2-Methoxyoestradiol; 2-Methoxyoestradiol-bis-sulphamate

1. Introduction

Breast cancer remains the major cause of death among women in Western countries. It has long been established that oestrogens act as important mitogens in the promotion of breast cancer and that breast tumours are able to produce oestrogens from circulating inactive precursors [1]. In post-menopausal women, in whom breast cancer most frequently occurs, the majority of the breast tumours are initially hormone-dependent. Although much emphasis has been placed on the prevention and early detection of the disease, there is a need for novel strategies to combat hormone-dependent breast cancer in postmenopausal women. The development of enzyme inhibitors to block the synthesis of oestrogens appears to be a promising approach for the treatment of hormone-dependent breast cancer [2,3]. So far, the research has been directed mainly towards the identification of aromatase inhibitors which block the conversion of androstenedione to oestrone (E1) [4,5]. In addition, there is another pathway which also contributes to the production of oestradiol and other oestrogenic steroids involving the enzyme steroid sulphatase (STS) [2]. A large proportion of oestrone (E1) synthesized by the aromatase is converted to oestrone sulphate (E1S). Hydrolysis of E1S to E1 by STS is thought to make a major contribution to the production of oestradiol (E2) within breast tumour tissues [2,3,6–8]. The STS enzyme is also responsible for the formation of dehydroepiandrosterone (DHEA) from DHEA-sulphate [9,10]. Reduction of DHEA gives rise to androstenediol (Adiol). Adiol, although an androgen can bind to the oestrogen receptor and can stimulate the growth of breast cancer cells in vivo and in vitro [11,12]. The production of Adiol is independent of the aromatase pathway but dependent on the sulphatase pathway. Therefore, in addition to aromatase enzyme inhibition, inhibition of STS, which prevents the formation of oestrogenic steroids such as E2 and Adiol, holds considerable potential for therapeutic development.

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology", Munich, Germany, 17–20 May 2002.

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0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00049-9

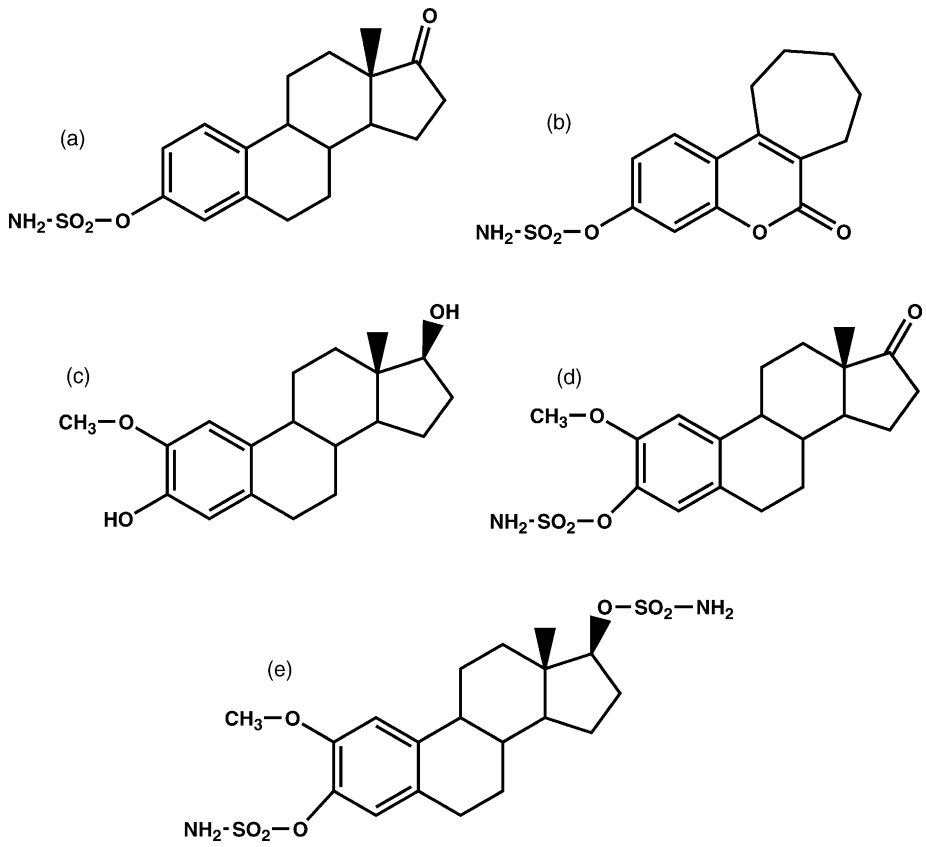

Fig. 1. Structures of (a) oestrone-3-*O*-sulphamate (EMATE), (b) 6-oxo-8,9,10,11-tetrahydro-7*H*-cyclohepta-[c][1]benzopyran-3-*O*-sulphamate (667 COUMATE), (c) 2-methoxyoestradiol (2-MeOE2), (d) 2-methoxyoestrone-3-*O*-sulphamate (2-MeOEMATE), (e) 2-methoxyoestradiol-3,17-*O*-*O*-bis-sulphamate (2-MeOE2bisMATE).

Since the identification of the active pharmacophore required for the inhibition of STS, i.e. an -*O*-sulphamate attached to an aromatic ring [13], many steroidal and non-steroidal derivatives have been developed and tested [14–16]. The E1S surrogate EMATE (Fig. 1a) was identified as the most potent, active site-directed, irreversible inhibitor of STS [17]. EMATE was active in vivo and had a prolonged duration of action in rodents [18] but, unexpectedly, it was highly oestrogenic, being five times more active than ethinyloestradiol on oral application [19]. Due to the sensitivity of endocrine-dependent tumours to oestrogens, EMATE would be unsuitable for use in such conditions. A number of different approaches were then used to identify non-oestrogenic STS inhibitors. This research led to the identification of a series of tricyclic coumarin sulphamates of which 667 coumarin sulphamate (667 COUMATE) (Fig. 1b) was amongst the most potent compound reported [20,21]. 667 COUMATE was non-oestrogenic and was active both in vitro and in vivo [22,23]. The ability of 667 COUMATE to inhibit STS activity was equipotent with EMATE in vivo. Like EMATE and other sulphamate based inhibitors developed so far, 667 COUMATE also acts to inhibit STS activity in an irreversible manner [20]. However, in contrast to the long term in vivo inhibition achieved with EMATE, the activity recovered fully within 1 week after dosing with 667 COUMATE [24].

As part of an attempt to develop non-oestrogenic, steroidal STS inhibitors, a number of A-ring modified analogues of EMATE were also synthesized and tested [15]. A lead compound that emerged from this study was 2-methoxyoestrone-3-*O*-sulphamate (2-MeOEMATE) (Fig. 1d). The rationale behind synthesizing 2-MeOEMATE was (a) the anti-proliferative activity associated with the methoxy-substituted E2 derivative, 2-methoxyoestradiol (2-MeOE2) (Fig. 1c), an endogenous metabolite of E2 known to suppress tumour growth and inhibit angiogenesis [25]; (b) substitution at the C2-position of the steroid nucleus reduces the oestrogenicity associated with the parent compound [26]. In keeping with this, 2-MeOEMATE, was found to possess anti-proliferative effects [24,27] yet retained its STS inhibitory potency but was devoid of any oestrogenic effects on uterine growth in ovariectomised rats [15]. Furthermore, 2-MeOEMATE was more potent than 2-MeOE2 as an anti-proliferative agent when tested on a wide range of human breast cancer cell lines [27]. In vivo there is evidence that sulphamoylated oestrogens are sequestered into red blood cells (RBCs) and it is possible that this class of drug also accumulates within other cells in the

body [28]. Little is known about possible side-effects that might arise from the inhibition of STS activity. Complete STS deficiency is associated with a condition characterised by ichthyosis, a pathological scaling of the skin due to prolonged retention of the stratum corneum. As ichthyosis can be readily treated with a cholesterol-containing ointment, no serious side-effects of STS inhibition are envisaged [29]. The exact mechanisms by which 2-MeOE2 or 2-MeOEMATE exert their effects are poorly understood. However, it has been shown that 2-MeOE2 binds to the colchicine site of tubulin and also inhibits the rate of tubulin polymerisation in vitro [30]. Based on these results, it was proposed that abnormal microtubule assembly, caused by 2-MeOE2, may be responsible for its anti-mitotic activity. In vitro, the sulphamoylated derivative, 2-MeOEMATE, inhibited taxol induced polymerisation of tubulin [27]. The mechanisms involved in the binding of these compounds to tubulin are currently under investigation.

The potent growth inhibitory effects, anti-microtubule activity and STS inhibitory property of 2-MeOEMATE, prompted us to further synthesize and test a wide range of 2-methoxy substituted and sulphamoylated E2 derivatives. In this paper, we have evaluated the anti-proliferative effects and STS inhibitory properties of a novel compound, 2-methoxyoestradiol-3,17-bis-sulphamate (2-MeOE2bisMATE) (Fig. 1e) and compared its potency with those of 2-MeOE2, EMATE and 667 COUMATE.

2. Materials and methods

2.1. Synthesis

All the chemicals, unless stated otherwise, were purchased from Sigma or Aldrich. All drugs used (Fig. 1) were synthesized at the Department of Pharmacy and Pharmacology, University of Bath. All compounds exhibited spectroscopic and analytical data in accordance with their structure and were pure, as shown by high performance liquid chromatography.

2.2. Cell proliferation assay

MCF-7 cells were cultured in growth medium (minimum essential medium (MEM) containing, phenol red, 10% foetal calf serum (FCS) and essential nutrients). When the cells reached 60% confluency, they were treated with the drugs (0.001–10 μM) in growth medium. After 72 h of incubation, photographs were taken under normal conditions of light and the number of attached cells in each flask was determined using a Coulter cell counter.

2.3. Steroid sulphatase enzyme assay

2.3.1. In placental microsomes

Placental microsomes were purified as described by Duncan et al. [31]. The assay was carried out using [6,7-^{3}H]E1S (4×10^5 dpm, NEN-DuPont, Boston, MA) adjusted to 20 μM with unlabelled E1S [31]. The enzyme (125 μg of protein) was incubated with the substrate ± inhibitor at different concentrations and the product formed was determined by partitioning into toluene [31].

2.3.2. In MCF-7 cells

MCF-7 breast cancer cells were maintained in MEM medium with 10% FCS and other essential nutrients. For experiments, the cells were seeded at 1×10^5 cells per flask and cultured in the above medium until 80% confluent. The STS assay was carried out using [^{3}H]E1S (2 nM) in serum free medium, with or without the inhibitor (1 pM–10 μM). After 20 h of incubation, STS activity was assayed in the medium by measuring the product formed [17]. The number of cells in each flask was determined using a Coulter cell counter.

2.3.3. In liver tissue

The assay methodology was essentially the same as that used for placental microsomes except that supernatants from the homogenized rat liver samples were used as a source of STS to test the extent of enzyme inhibition.

2.4. In vivo studies

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 the drug (10 mg/kg), with animals receiving a single dose. The animals receiving 667 COUMATE were sacrificed by an approved method on days 1, 3 and 7 and on days 1, 5, 10 and 15 for those receiving EMATE or 2-MeOE2bisMATE, to assess the duration of STS inhibition. Samples of liver tissues were removed and immediately frozen on solid carbon dioxide and stored at −20 °C until assayed. Tissues were homogenized in ice cold PBS-sucrose and, after centrifugation to remove the cell debris, aliquots of supernatants were used for STS assay [31]. Protein concentrations were determined by the method of Bradford [32].

3. Results

3.1. Effects on MCF-7 cell morphology and proliferation

Exposure of MCF-7 cells to 2-MeOE2 or 2-MeOE2bisMATE had a marked effect on their morphology (Fig. 2b and c), when compared to the control untreated cells (Fig. 2a). There was a significant increase in the number of rounded, shrunk, detached cells after treating the cells for 24 h with 2-MeOE2bisMATE at 1 μM. At the same concentration, 2-MeOE2 had less of an effect on cell morphology. However, both compounds induced a dose-dependent inhibition of MCF-7 cell proliferation (Fig. 3). The half-maximal inhibitory concentrations (IC_{50}) for 2-MeOE2

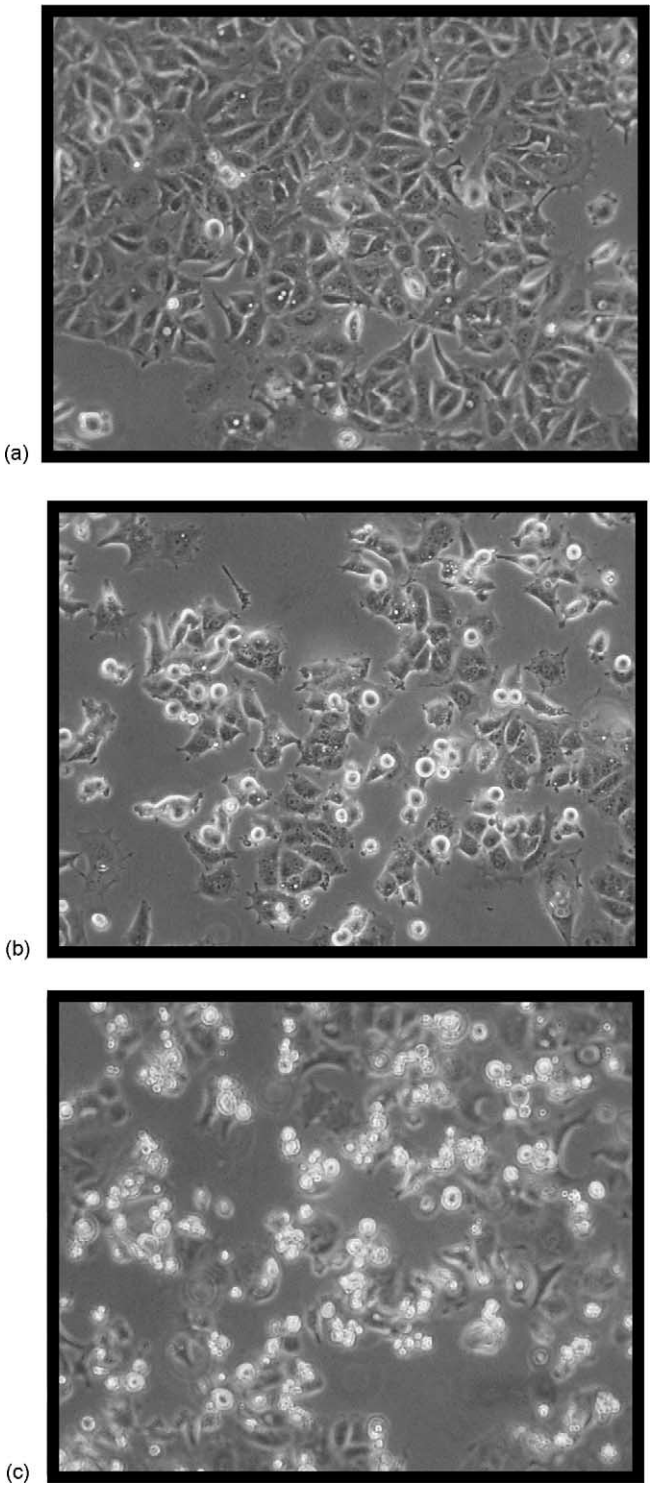

Fig. 2. Effect of (a) vehicle (THF), (b) 2-methoxyoestradiol (1 μM), (c) 2-methoxyoestradiol-3,17-bis-sulphamate (1 μM) on MCF-7 breast cancer cell morphology after 72 h of treatment.

and 2-MeOE2bisMATE were 2.5 and 0.4 μM, respectively. EMATE and 667 COUMATE when tested at 10 μM, did not have any effect on cell morphology or cell proliferation (data not shown).

Table 1
IC_{50}s for EMATE, 667 COUMATE, 2-MeOE2 and 2-MeOE2bisMATE determined using placental microsomes and MCF-7 cells

	IC_{50} values of STS inhibition	
	Placental microsomes	MCF-7 cells
EMATE (nM)	20	0.6
667 COUMATE (nM)	8	0.2
2-MeOE2 (μM)	>10	>10
2-MeOE2bisMATE (nM)	39	0.5

3.2. Inhibition of STS activity in vitro

The ability of all the compounds to inhibit STS activity was compared by determining their IC_{50} values using placental microsomes and MCF-7 cells (Table 1). In placental microsomes, with a 20 μM substrate concentration, 667 COUMATE was the most potent inhibitor with an IC_{50} value of 8 nM followed by EMATE and 2-MeOE2bisMATE with IC_{50}s of 20 and 39 nM, respectively. In MCF-7 cells, where the assays were carried out using a physiological concentration of E1S (2–3 nM), the overall IC_{50} values were lower than those obtained using placental microsomes. However, 667 COUMATE still remained the most potent inhibitor with an IC_{50} of 0.2 nM. EMATE and 2-MeOE2bisMATE had similar inhibitory potencies with IC_{50}s of 0.6 and 0.5 nM, respectively. 2-MeOE2, which lacks a sulphamate group, did not have any inhibitory effect on STS activity in both the assays when tested at 10 μM.

3.3. In vivo inhibition of STS activity

Having shown that 2-MeOE2bisMATE, a modified derivative of EMATE, was a potent inhibitor of STS activity in vitro, it was tested in vivo and compared with EMATE and 667 COUMATE. Animals received a single oral dose of the inhibitor at 10 mg/kg, with liver samples being collected 24 h later for the assay of STS activity. In animals receiving vehicle only, liver STS activity was 32.56 ± 8.1 nmol/h/mg protein. All the compounds tested were very effective and produced more than 95% inhibition of liver STS activity (Table 2).

Table 2
In vivo effects of selected compounds on rat liver STS activity

	Percent inhibition of STS activity
667 COUMATE	97.9 ± 0.06
EMATE	99.2 ± 0.05
2-MeOE2bisMATE	99.7 ± 0.08

Rats were treated with vehicle (propylene glycol), 667 COUMATE, EMATE or 2-MeOE2bisMATE (10 mg/kg, p.o., single dose). Samples of liver tissues were collected for assay of STS activity 24 h after the administration of the drug. The results are expressed as the percent inhibition of liver STS activity, compared with that of control animals (means ± S.D., $n = 3$).

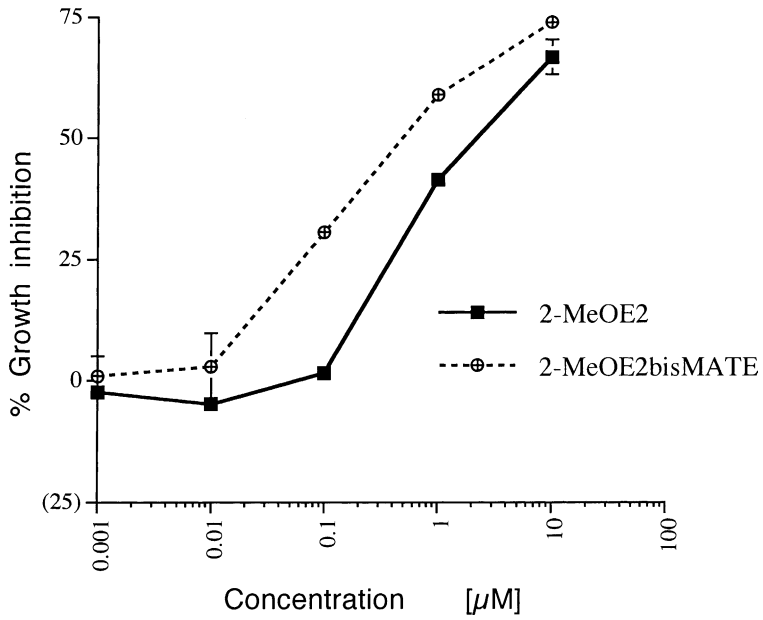

Fig. 3. Dose response of 2-methoxyoestradiol or 2-methoxyoestradiol-3,17-bis-sulphamate on the proliferation of MCF-7 breast cancer cells. The cells were treated for 3 days with or without drugs. The attached cells in each flask were counted using a Coulter cell counter. Values shown represent the mean ± S.D. ($n = 3$) of measurements at each drug concentration tested. Where no error bars are shown, the S.D. was <10%.

3.4. Recovery of STS activity in vivo

It was previously observed that, after administration of a single oral dose of 667 COUMATE, STS activity was completely restored within a week in contrast to EMATE when little recovery had occurred by this time. In the present study, we have compared the duration of inhibition of STS activity in vivo by EMATE, 667 COUMATE and 2-MeOE2bisMATE. The animals received a single oral dose at 10 mg/kg and the liver samples were collected on days 1, 3 and 7 for animals receiving 667 COUMATE and on days 1, 5, 10 and 15 for those receiving EMATE or 2-MeOE2bisMATE. As shown in Fig. 4, 10% of inhibited STS activity was recovered by day 3 in animals receiving

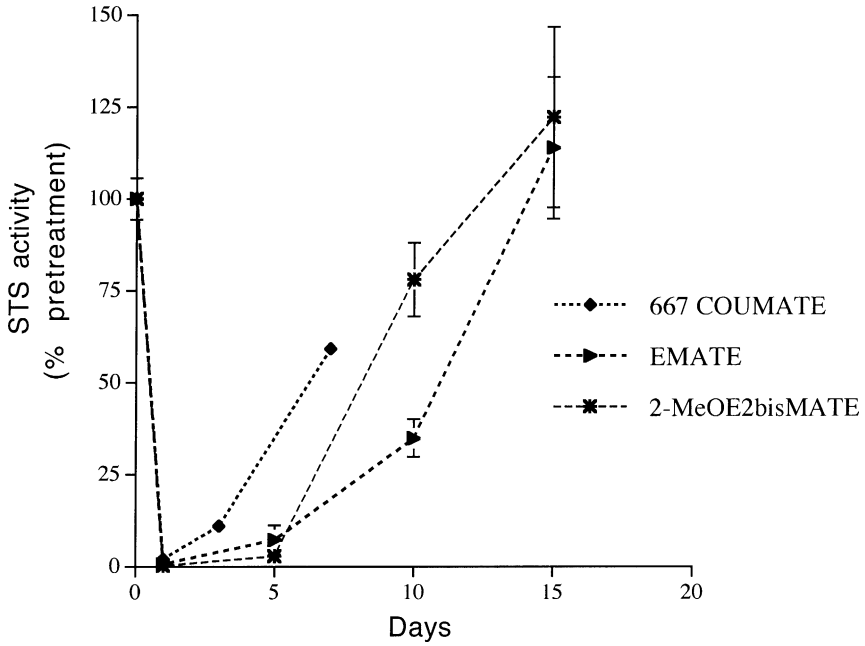

Fig. 4. Recovery of in vivo STS activity in rats. Rats were treated with the drugs at 10 mg/kg, with animals receiving a single dose p.o. Samples of liver tissues were collected after days 1, 3 and 7 for 667 COUMATE and on days 1, 5, 10 and 15 for EMATE and 2-MeOE2bisMATE. Results are expressed as percentage of pretreated STS activity. (means ± S.D., $n = 3$). Where no error bars are shown, the S.D. was <10%.

667 COUMATE, with activity partially restored to 55% of the controls by day 7. In animals receiving EMATE or 2-MeOE2bisMATE, STS activity remained inhibited up to 5 days. By day 10, 80% of STS activity was restored in 2-MeOE2bisMATE treated animals, while those with EMATE showed only 35% recovery by this time. However, complete recovery of the STS activity was observed by day 15 in both groups of animals.

4. Discussion

Steroid sulphatase inhibitors are due to enter Phase I clinical trials in the near future as a potential therapy for the treatment of postmenopausal women with hormone-dependent breast cancer. EMATE was identified as the first irreversible STS inhibitor [17], but unexpectedly proved to be oestrogenic [19]. In the pursuit of alternative non-oestrogenic mimics of EMATE, a large number of steroidal and non-steroidal sulphamates were then synthesized and tested as STS inhibitors. 667 COUMATE [20,21], a tricyclic coumarin sulphamate, resulted from such a study and this compound has been selected for clinical development. Using a different approach, an A-ring modified derivative of EMATE, 2-MeOEMATE was found to profoundly affect cell morphology [33]. 2-MeOEMATE was synthesized to exploit the biological advantages of EMATE and 2-MeOE2. 2-MeOEMATE induced a G2–M arrest and apoptosis in MCF-7 cells [27,33], but was more potent than 2-MeOE2. In addition, 2-MeOEMATE retained the STS inhibitory potency of EMATE making it a dual action drug. This initial result suggested that sulphamoylation may be an effective mechanism for enhancing the anti-proliferative activity, yet retaining the STS inhibitory potency, of 2-substituted oestrogens. We next synthesized the bis-sulphamoylated derivative of 2-MeOE2, 2-MeOE2bisMATE (Fig. 1e).

The results obtained from these investigations have provided further evidence of the fact that 2-methoxyoestrogens represent a new class of anti-cancer agents and are consistent with a previous study showing that sulphamoylation enhances the efficacy of the parent compounds. As an anti-proliferative agent, 2-MeOE2bisMATE was more potent than 2-MeOE2. EMATE or 667 COUMATE which lack a methoxy group, did not have any effect on cell proliferation, suggesting that the methoxy substitution at the C2-position of the steroid nucleus is an essential structural prerequisite for the compounds to possess anti-proliferative properties. 2-MeOE2bisMATE, being a derivative of EMATE, retained its STS inhibitory property. It was apparent that the 2-substitution did not markedly alter the inhibitory potency of these derivatives in spite of introducing a certain degree of steric bulk into the A-ring of the steroid nucleus. An interesting point to be noted here is that, 2-methoxy substitution of E1 or E2 greatly reduces the oestrogenicity of the parent compound. 2-MeOEMATE, a mono-sulphamoylated methoxy derivative was devoid of uterotropic activity in ovariectomised female rats [15]. It was previously reported that alkyl substitutions at the C2-position of oestrogens reduce the oestrogenicity associated with the parent compounds [26]. We have now identified 2-MeOE2bisMATE, which although a derivative of EMATE, is a non-oestrogenic [15] STS inhibitor and like 2-MeOE2, also has profound anti-proliferative properties. Substitutions at the C2 position of the steroid nucleus have attracted considerable interest in recent years. In order to define the structural parameters associated with the cytotoxicity of 2-methoxy derivatives, an array of analogues has been synthesized and evaluated [27,34,35]. Although the exact mechanisms by which these sulphamoylated oestrogen derivatives inhibit proliferation remain to be elucidated, it is likely that they induce cells to undergo apoptosis. The compounds induce cells to arrest at the G2–M stage of the cell cycle, induce phosphorylation of BCL-2 and cell death as confirmed by Tdt mediated dUTP nick end labelling (TUNEL) analysis [27]. In a previous study, a clear distinction was found between the duration of inhibition of in vivo STS activity by steroidal and non-steroidal STS inhibitors [24]. After oral administration of a single dose of 667 COUMATE, recovery of STS activity was almost complete within a week. In contrast, administration of EMATE resulted in a prolonged period of STS inhibition. In the present study, however, somewhat different results have been obtained to those previously reported. While the steroid based inhibitors still produced a sustained period of STS inhibition (greater than 90% at day 5 compared with a value of approximately 75% for 667 COUMATE) the difference between the two classes of inhibitors was less marked than previously observed. Also by day 15 recovery of STS activity after administration of the two steroidal inhibitors was complete. The reason for the discrepancy between the results obtained in the present study with those previously observed is not readily apparent but might have resulted from a different strain of animal being used for the current study. However, it is conclusive that the 2-methoxy substitution does not markedly alter the in vivo STS inhibitory potency of this class of compound. In addition to its potent inhibition of STS activity in vivo, other in vivo studies with 2-MeOE2bisMATE have produced encouraging results. In nude mice, with MDA-MB-435 xenografts, 2-MeOE2bisMATE inhibited tumour growth by about 50% at the end of a 4 weeks drug administration period and tumour volumes remained significantly lower for up to 4 weeks after the cessation of the treatment [36].

In this study, a novel compound 2-MeOE2bisMATE has been evaluated as potential anti-cancer agent. While this compound possesses potent STS inhibitory properties, it also inhibits cell proliferation and induces cells to undergo apoptosis. Since most cancers will eventually become resistant to either hormonal or chemotherapeutic agents, there is still a need for the development of novel therapeutic interventions. In view of the anti-proliferative and anti-angiogenic properties associated with 2-MeOE2 [25], its sulphamoylated

derivatives which possess enhanced efficacy, bio-availability, longer duration of action and most importantly also possess a dual mechanism of action, i.e. STS enzyme inhibition and anti-neoplasticity, have considerable therapeutic potential for the treatment of both hormone-dependent and hormone-independent cancers.

Acknowledgements

The authors would like to thank Mr. D. Bennetto for his technical assistance. This work was supported by Sterix Ltd.

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