In Vivo Activity of 4-Methylcoumarin-7-O-Sulfamate, a Nonsteroidal, Nonestrogenic Steroid Sulfatase Inhibitor

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ABSTRACT

Steroid sulfatase regulates the formation of estrone from estrone sulfate (E1S) and dehydroepiandrosterone (DHA) from DHA sulfate. DHA can be converted to androstenediol, a steroid with potent estrogenic properties, and inhibition of steroid sulfatase activity is therefore an important therapeutic target. Because nonsteroidal steroid sulfatase inhibitors may offer some advantage for use in the treatment of breast cancer, 4-methylcoumarin-7-O-sulfamate (COUMATE) was synthesized and shown to be active in vitro. In this study, in vitro and in vivo techniques have been used to confirm that COUMATE, in contrast to the steroidal steroid sulfatase inhibitor estrone-3-O-sulfamate, is devoid of estrogenic activity. COUMATE did not stimulate the growth of MCF-7 breast cancer cells or uteri of ovariectomized rats, in contrast to estrone-3-O-sulfamate. COUMATE was orally active in vivo and after multiple dosing (10 mg/kg/day for 7 days) inhibited liver estrone sulfatase activity by 85%. Seven days after single or multiple dosing with COUMATE, liver estrone sulfatase activity was almost fully restored. Measurement of estrone sulfatase activity in WBCs revealed a degree of inhibition similar to that detected in liver samples. COUMATE was able to completely block the ability of E1S to stimulate uterine growth in ovariectomized rats. The development of a potent nonsteroidal, nonestrogenic steroid sulfatase inhibitor should allow the therapeutic potential of this type of therapy to be evaluated.

INTRODUCTION

Estrogens continue to support the growth of hormone-dependent tumors of the breast and endometrium after menopause (1, 2). The enzymes which are involved in estrogen synthesis are therefore important targets for therapeutic intervention. In postmenopausal women, with the cessation of ovarian activity, estrogens continue to be produced by the conversion of androstenedione to estrone by the aromatase enzyme complex which is present in peripheral tissues (3, 4). Several potent aromatase inhibitors have now been identified, some of which are currently undergoing clinical evaluation (5). However, in a recent definitive Phase III trial in which a new potent aromatase inhibitor was evaluated, the response rate of 11% (complete response + partial response) in patients with ER+ positive tumors was disappointing (6). The results from this study raised questions as to what other mechanisms might be responsible for a lack of tumor shrinkage when using a drug with higher aromatase inhibitory properties (7).

Research into the role that another enzyme may have in regulating estrogen synthesis in postmenopausal women could provide an explanation for the failure of potent aromatase inhibitors to achieve a response rate of >11%. For some time it has been apparent that much of the estrone (Fig. 1, compound 1) formed from androstenedione can be converted to E1S (Fig. 1, compound 2) by estrone sulfotransferase (8). Plasma and tissue concentrations of estrogen sulfates are much higher than for unconjugated estrogens (9, 10), and the half-life of E1S (10–12 h) is much longer than that for estrone or estradiol (20–30 min; Ref. 11). Thus, it is likely that E1S in plasma and tissues can act as a reservoir for the formation of unconjugated estrogens by the action of estrone sulfatase (12, 13). The activity of this enzyme is much higher than that for the aromatase in both normal and malignant breast tissues (14). Furthermore, it has been shown that formation of estrone in breast tumors via the sulfatase pathway provides at least 10 times as much estrone as that synthesized by the aromatase route (15). In three recent studies in which the effects of new aromatase inhibitors on plasma estrogen concentrations were examined, for women receiving CGS 16949A, fadrozole hydrochloride, or vorozole, while plasma E1S concentrations decreased by about 28–67%, they remained relatively high (400–1000 pg/ml) during therapy (16–18). If, as is now evident, E1S is an important source of estrogen in postmenopausal women, then the development of potent inhibitors of estrone sulfatase activity could be of considerable therapeutic value (19).

In addition to estrone and estradiol, there is a growing realization that if enzyme inhibitor therapy is to succeed in postmenopausal women, it will be important to block the formation of another steroid that also has potent estrogenic properties (12, 20). This steroid is androstenediol (Adiol; Fig. 1, compound 5), which can bind to the ER and can stimulate the growth of ER-positive breast cancer cells and dimethylbenzanthracene-induced mammary tumors in the rat (21, 22). Almost 90% of Adiol originates from DHA-S (Fig. 1, compound 4) after it has been hydrolyzed to DHA (Fig. 1, compound 3) by DHA sulfatase (23). Transient transfection of a placental steroid sulfatase into COS-1 cells revealed that the expressed protein was able to hydrolyze both E1S and DHA-S (24). Therefore, the ability to inhibit steroid sulfatase activity in vivo should reduce not only the formation of estrone from E1S but also the synthesis of Adiol from DHA-S.

Several steroid sulfatase inhibitors have now been developed and include estrone-3-O-methylthiophosphonate (25), estrone sultone chloride (26), and a series of estrone sulfamates (27, 28). Of these compounds, EMATE (Fig. 1, compound 6) is the most potent inhibitor of estrone sulfatase activity in vivo and after multiple dosing (29). However, this sulfatase inhibitor is an estrone analogue, and it is possible that estrone may be released from EMATE during sulfatase inactivation (28, 30). Also, unexpectedly, this compound was recently reported to possess potent estrogenic properties (31), making it unsuitable for use in the treatment of women with hormone-dependent tumors.

To avoid the problems associated with using analogues of steroids as inhibitors, a number of nonsteroidal inhibitors have been developed, including sulfate derivatives of 2-phenylindoles (32) and sulfamate derivatives of tetrahydrocanthph-2-ol (19, 27). More recently, a series of coumarin sulfamates was synthesized, one of which, COUMATE (Fig. 1, compound 8), inhibited estrone sulfatase activity in MCF-7 breast cancer by >90% at 10 μM (33). Furthermore, COUMATE, like EMATE, inhibited estrone sulfatase activity in a time-dependent and concentration-dependent manner, suggesting that it may have a mechanism of action similar to that of EMATE. In the present study, in vitro and in vivo methods have been used to examine
whether COUMATE has any estrogenic properties. The ability of COUMATE to inhibit estrone sulfatase activity in vivo and the duration of inhibition were also investigated.

MATERIALS AND METHODS

Synthesis of COUMATE. COUMATE (Fig. 1, compound 8) was synthesized by first treating a solution of 7-hydroxy-4-methylcoumarin (Fig. 1, compound 7) in anhydrous dimethylformamide with sodium hydride (1 equivalent) at 0°C under N₂. Sulfamoyl chloride (—1.5 equivalent) was then added, and after the mixture was warmed to room temperature overnight and the reaction quenched, the crude product was purified by flash chromatography and recrystallization (yield 61%). The compound was fully characterized by spectroscopic and combustion analysis (33).

In Vitro Cell Growth. The ER-positive MCF-7 breast cancer cell line was used to assess whether COUMATE possessed any estrogenic properties. Cells were routinely cultured in 25-cm² tissue culture flasks in Eagle’s modified MEM with Earle’s salts and HEPES buffer (20 mM), hereafter referred to as MEM (34). This medium was routinely supplemented with L-glutamine (2 mM), sodium hydrogen carbonate (10 mm), 1% nonessential amino acids, and 5% FCS. Cells were grown in this medium until approximately 40% confluent. Since phenol red, which is used as a pH indicator, can act as a weak estrogen, this medium was removed prior to the experiments to investigate the possible estrogenicity of COUMATE. Cells were washed twice with PBS and cultured in phenol red-free medium (MEM-AUTO POW; ICN Flow Biomedicals, Bicester, United Kingdom). Groups of rats, with three rats in each group for each experiment, were treated p.o. with vehicle (propylene glycol) or 0.1, 1.0, or 10.0 mg/kg COUMATE. Animals received either a single dose of COUMATE (single-dose study) or seven doses at daily intervals (multiple-dose study). After completing the dosing schedules, groups of animals were killed, using an approved procedure, at 24 h after the last dose to assess the extent of inhibition, but also 3, 7, and 14 days later to obtain information about the duration of estrone sulfatase inhibition.

Uterotrophic Studies. To examine in vivo possible estrogenic effects of COUMATE, rats were ovariectomized and 14 days later treated with vehicle (propylene glycol s.c.) or COUMATE (1 mg/kg/day s.c. for 5 days). For comparison, other ovariectomized animals received either a single dose of EMATE (10 mg/kg s.c.) or 0.1 mg/kg or 1.0 mg/kg, s.c. per day for 5 days.

Animals were killed 24 h after administration of the last dose of drug, with the exception of animals receiving a single dose of EMATE, which were killed 6 days after receiving the drug. Uteri were excised of fat and weighed. Total body weights of the animals were also recorded, and the results were expressed as uterine weight × 100/total body weight. Estrone sulfatase activity was measured in uteri obtained from the animals used for this study.

The ability of COUMATE to inhibit the E1S-stimulated growth of uteri in ovariectomized animals was also examined. For this, animals received either vehicle (propylene glycol s.c.) or COUMATE (10 mg/kg/day s.c.) initially for 2 days. Animals either continued to receive vehicle only or COUMATE (10 mg/kg) plus E1S (50 μg/day) or E1S only (50 μg/day s.c.) for another 5 days. Twenty-four h after administration of the last dose, animals were killed and uterine and total body weights were recorded.

Estrone Sulfatase Activity in Tissues. Liver and uterine tissues obtained from rats were immediately frozen on solid carbon dioxide and stored at −20°C until assayed. For this, tissues were minced and homogenized, and nuclei and cell debris were removed by centrifugation (2000 × g at 4°C for 20 min). Aliquots of the supernatant, which contained the microsomal fraction, were used for the assay of sulfatase activity (25). The protein concentration in supernatants was measured using the Lowry method (35). Estrone sulfatase activity was measured using [6,7-3H]E1S (4 × 10⁴ dpm, 3 mCi; NEN-DuPont, Boston, MA) adjusted to a final concentration of 20 μM with unlabeled E1S (Sigma) in a final reaction mixture volume made up to 1 ml with PBS, [4,14C]Estrone (1 × 10⁴ dpm; Amersham, Aylesbury, United Kingdom) was included in the mixture to monitor procedural losses. Samples were processed as described previously (25). Using this assay method, the extent of E1S hydrolysis has been shown to be directly proportional to incubation time and protein concentration (25).

A similar assay procedure, using placental microsomes and [6,7-3H]DHA-S (NEN-DuPont), was used to examine the ability of COUMATE to inhibit DHA sulfatase activity as described previously (29).

Estrone Sulfatase Activity in WBCs. WBCs possess significant estrone sulfatase activity (36), and the effect of COUMATE on sulfatase activity in these cells was monitored. For this, blood from each group of three animals was pooled and an equal volume of Histopaque 1077 (Sigma) added. The blood was centrifuged (500 × g, 30 min) and the WBCs removed and washed with PBS containing sucrose (250 mm) and 0.9% NaCl. WBCs were again collected by centrifugation (250 × g, 15 min), and the cell pellet was resuspended in the wash buffer (0.2 ml/1 ml whole blood). Aliquots were used for the measurement of estrone sulfatase activity as previously described for other tissues and for determining the number of cells using a Coulter Counter. For WBCs, estrone sulfatase activity was shown to be linear with respect to the number of cells used and the incubation period (data not shown).

Statistics. The significance of differences for cell growth, uterine weights, and estrone sulfatase activity in tissues for control and treated animals were assessed using Student’s t test.

RESULTS

Effect of COUMATE on Cell Growth

The MCF-7 cells used for this investigation were responsive to estrogens with both estradiol (100 pm) and E1S (1 μM) significantly stimulating cell numbers (Fig. 2). The steroidal sulfatase inhibitor EMATE (0.1 μM) also stimulated cell proliferation. In contrast, COUMATE (0.1 μM) and its parent compound 7-hydroxy-4-methylcoumarin (0.1 μM) had no effect on cell growth. Examination of the effect of EMATE on cell growth over the concentration range of 0.01 μM-10 μM revealed that whereas the lower concentrations were stimulatory, the highest concentration (10 μM) inhibited cell growth (Fig. 3).
Recovery of Estrone Sulfatase Activity. To examine the duration of inhibition of estrone sulfatase activity by COUMATE, in addition to the blood and tissue samples obtained at 24 h after a single or last multiple dose, additional samples were obtained after 3, 7, and 14 days. Recovery of estrone sulfatase activity after the cessation of COUMATE administration was rapid, as monitored using liver or WBCs (Fig. 6), with activity being almost fully restored by day 7. The time taken for enzyme activity to recover to 50% of its original activity was about 3.5 days.

Uterotrophic Studies

After demonstrating that COUMATE did not stimulate growth of MCF-7 cells in vitro, it was tested for estrogenicity in vivo using the rat uterotrophic model and compared with the effects of EMATE on uterine growth. In ovariectomized control animals and animals receiving COUMATE (1 mg/kg/day for 5 days), no significant difference in uterine weights was detected (Fig. 7a). In contrast, EMATE, given as either a single (10 mg/kg) dose, with evaluation of its effect on uterine growth made 6 days later, or daily doses (0.1 and 1.0 mg/kg/day for 5 days) resulted in significant increases in uterine weights (Fig. 7a). Uterine weights in EMATE-treated animals were similar to those of intact untreated controls. Estrone sulfatase activity was also assessed in uteri obtained from these animals. Single or multiple doses of EMATE inhibited uterine sulfatase activity by >99% with COUMATE at 1 mg/kg/day for 5 days, inhibiting activity by almost 90% (Fig. 7b).

Because COUMATE is being developed as a potential therapeutic agent to inhibit the hormone-dependent growth of tumors, the uterotrophic assay was also used to test whether it could block the E1S-stimulated uterine growth in ovariectomized animals. For this experiment, animals were pretreated with COUMATE for 2 days to suppress estrone sulfatase activity. Animals then received either COUMATE plus E1S or E1S only daily for 5 days. Measurements of uterine weights 24 h after administration of the last dose of drug or hormone revealed that E1S significantly stimulated uterine growth (Fig. 8). However, the ability of E1S to stimulate uterine growth was completely blocked by the coadministration of COUMATE.

**Effect of COUMATE on DHA Sulfatase Activity**

Using a placental microsomal preparation, COUMATE (10 μM) effectively inhibited DHA sulfatase activity by 94% (Fig. 4).

**In Vivo Studies**

**Dose Response.** Administration of single doses of COUMATE inhibited liver estrone sulfatase activity in a dose-dependent manner, with maximum inhibition (67%) being achieved with the 1.0 mg/kg dose with no further increase in the extent of inhibition occurring at the higher dose (Fig. 5). Daily dosing with COUMATE for 7 days resulted in a greater degree of inhibition of liver estrone sulfatase activity with 85% inhibition at the 10 mg/kg dose.

After multiple dosing, COUMATE inhibited liver and WBC estrone sulfatase activity to a similar extent. However, a single dose of COUMATE resulted in a greater degree of inhibition in WBCs than in liver tissue.

**Recovery of Estrone Sulfatase Activity.** To examine the duration of inhibition of estrone sulfatase activity by COUMATE, in addition to the blood and tissue samples obtained at 24 h after a single or last multiple dose, additional samples were obtained after 3, 7, and 14 days. Recovery of estrone sulfatase activity after the cessation of COUMATE administration was rapid, as monitored using liver or WBCs (Fig. 6), with activity being almost fully restored by day 7. The time taken for enzyme activity to recover to 50% of its original activity was about 3.5 days.

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SULFATASE AND BREAST CANCER

ulated a search for potent steroid sulfatase inhibitors (12, 13, 19). Such an inhibitor, EMATE, was previously identified (27–29) but unexpectedly was subsequently discovered to have potent agonist properties (31), making it unsuitable for use in the treatment of women with breast cancer. The reason why EMATE is such a potent estrogen is not yet known. As such it does not appear to bind to the ER (31), but it is possible that estrone may be released during sulfamoylation of an amino acid at the active site during irreversible inhibition of sulfatase activity (28, 30).

Results obtained from both the *in vitro* and *in vivo* studies in the present investigation have confirmed that EMATE is a potent estrogen. Like other estrogens, however, which have previously been used at high doses for the treatment of breast cancer (37), the highest concentration of EMATE tested in vitro significantly inhibited the growth of MCF-7 breast cancer cells. The enhanced potency of EMATE as an estrogen in vivo was confirmed by the finding that a

**Fig. 5.** Dose response for the effect of COUMATE on rat liver or WBC estrone sulfatase (E1-STS) activity. Animals received either a single (sd) or multiple doses (md) of COUMATE p.o. in propylene glycol, with liver (sd or md) and WBC (sd wbc or md wbc) estrone sulfatase activity being measured in samples obtained 24 h after the last dose. Estrone sulfatase activity was measured in liver tissue supernatants obtained after centrifugation of homogenized tissues, or WBCs, using [6,7-3H]E1S (4 x 10^5 dpm, 3 nm) adjusted to a final concentration of 20 μM with unlabeled E1S to monitor procedural losses. For liver tissue samples, means (n = 3) are shown. **P < 0.01 and ***P < 0.001. In control animals, liver estrone sulfatase activity was 53.2 ± 5.4 pmol/30 min/mg protein. WBCs from three animals were pooled to provide sufficient cells for the measurement of estrone sulfatase activity. Bars, SD.

**Fig. 6.** Recovery of estrone sulfatase (E1-STS) activity in rat livers (sd or md) and WBCs (sd wbc, or md wbc) obtained 3, 7, and 14 days after administration of a single (sd) or last multiple (md) dose of COUMATE (10 mg/kg s.c. in propylene glycol). Details of the assay procedure are as indicated in the legend to Fig. 5. For liver tissue samples, means (n = 3) are shown: *P < 0.05, **P < 0.01, and ***P < 0.001. Bars, SD.

**DISCUSSION**

The realization that E1S and DHA-S are important sources for the formation of estrogenic steroids in postmenopausal women has stimulated a search for potent steroid sulfatase inhibitors (12, 13, 19). Such an inhibitor, EMATE, was previously identified (27–29) but unexpectedly was subsequently discovered to have potent agonist properties (31), making it unsuitable for use in the treatment of women with breast cancer. The reason why EMATE is such a potent estrogen is not yet known. As such it does not appear to bind to the ER (31), but it is possible that estrone may be released during sulfamoylation of an amino acid at the active site during irreversible inhibition of sulfatase activity (28, 30).

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**Fig. 7.** a, effect of COUMATE or EMATE on uterine growth in ovariectomized rats. Animals were ovariectomized 14 days before the start of the study and received COUMATE s.c. in propylene glycol at the doses indicated daily for 5 days. EMATE was also administered using the same dosing schedule but in addition was given in a single 10 mg/kg dose. Uterine weights were measured 24 h after administration of the last dose for animals receiving multiple doses or after 6 days for animals given a single injection of EMATE. Results are expressed as uterine weight x 100/total body weight (means; n = 3). Bars, SD.

b, estrone sulfatase activity in uteri of ovariectomized or intact control animals and in animals receiving COUMATE or EMATE. Estrone sulfatase (E1-STS) activity was measured as described in the legend to Fig. 5. EMATE at all doses and schedules tested and COUMATE significantly (P < 0.001) inhibited uterine estrone sulfatase activity. Activity in uteri of intact animals was significantly lower (P < 0.05) compared with that in uteri of ovariectomized animals (means; n = 3). Bars, SD.
single dose resulted in an increase in uterine weight when measured 6 days later.

In contrast to the estrogenic properties associated with EMATE, the results from the in vitro and in vivo studies confirm that COUMATE is devoid of any estrogenic properties. COUMATE did not stimulate the growth of MCF-7 cells in vitro and had no uterotrophic effect in vivo. Furthermore, COUMATE completely blocked the ability of E1S to stimulate uterine growth in ovariectomized animals. Because COUMATE was also shown to be active by the p.o. route, this compound represents a potential lead inhibitor for further development.

Although potent, it is apparent that COUMATE is less active than EMATE as an inhibitor of estrone sulfatase activity. Whereas EMATE at a dose of 0.1 mg/kg inhibited rat liver estrone sulfatase activity by 87% (29), a dose of 10 mg/kg COUMATE is required to achieve a similar degree of inhibition. However, in view of the result obtained showing that COUMATE at this dose can block E1S-stimulated uterine growth, it may be of sufficient potency for therapeutic investigations.

Using a placental microsomal preparation, COUMATE was also shown to effectively inhibit the hydrolysis of DHA-S. Thus, COUMATE should block the synthesis of Adiol from DHA-S in addition to preventing the formation of estrone from E1S.

Single or multiple doses of EMATE, when given to rats, were previously found to produce a prolonged period of steroid sulfatase inhibition (29). Seven days after a single 10 mg/kg dose of EMATE, rat liver and ovarian estrone sulfatase activity remained almost completely inhibited. Fifteen days after the cessation of multiple dosing with EMATE, only a small (13%) recovery of liver estrone sulfatase activity had occurred. Since the half-time for the turnover of estrone sulfatase is about 3–4 days (38), it is not yet clear why the inhibitory effects of EMATE are so prolonged.

In contrast, for COUMATE, recovery of estrone sulfatase activity, after either single or multiple doses, is rapid with almost complete recovery of activity occurring within 7 days. Like EMATE, COUMATE acts as an active site-directed irreversible inhibitor and it is likely that restoration of enzyme activity results from the synthesis of new enzyme.

Monitoring the extent and duration of inhibition of estrogen synthesis in humans can be difficult. For aromatase inhibition, measurement of plasma estrogen concentrations does not appear to accurately reflect the extent of inhibition of estrogen synthesis because the problems associated with measurements of low estrogen levels in plasma. Isotopic infusion techniques can be used to obtain information about the effectiveness of these inhibitors (39) but the use of isotopes precludes their widespread use.

In an attempt to overcome such problems, advantage has been taken of the fact that a readily accessible tissue source, i.e., WBCs, possesses significant estrone sulfatase activity. A simple, robust method was therefore established to rapidly measure estrone sulfatase activity in these cells. Using this method, inhibition of estrone sulfatase activity can be readily monitored. Although there was some discrepancy between the extent of inhibition detected from assaying liver and WBC estrone sulfatase activity after a single dose of COUMATE, multiple dosing resulted in a similar degree of inhibition for both liver and WBC enzyme activity. This assay should be of considerable value in monitoring the extent and duration of steroid sulfatase inhibition in humans during clinical trials with steroid sulfatase inhibitors.

The development of a nonsteroidal, nonestrogenic steroid sulfatase inhibitor should allow the contribution that the sulfatase pathway makes to estrogen synthesis in breast tumors to be determined. The development of COUMATE and related analogues, which can block the E1S-stimulated growth of hormone-sensitive target organs, should also allow their potential as therapeutic agents for use in the treatment of postmenopausal women with breast cancer to be established.

REFERENCES

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