Inactivation of Steroid Sulfatase by an Active Site-Directed Inhibitor, Estrone-3-*O*-Sulfamate†

Atul Purohit,‡ Gary J. Williams,§ Nicola M. Howarth,§ Barry V. L. Potter,§ and Michael J. Reed*,‡

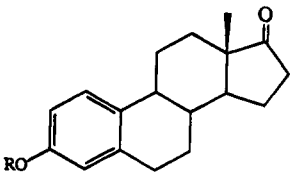
Unit of Metabolic Medicine, St. Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, London, W2 1PG, U.K., and Department of Medicinal Chemistry, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, U.K.

Received November 29, 1994; Revised Manuscript Received March 13, 1995⊗

ABSTRACT: Steroid sulfatases are responsible for the hydrolysis of 3β-hydroxy steroid sulfates, such as cholesterol and pregnenolone sulfate, and have an important role in regulating the synthesis of estrogenic steroids, from estrone sulfate and dehydroepiandrosterone sulfate, in endocrine-dependent tumors. Although little is known about the mechanism by which the sulfate group is removed from a steroid nucleus, an active site-directed sulfatase inhibitor has been developed. This inhibitor, estrone-3-*O*-sulfamate (EMATE), was synthesized by treating the sodium salt of estrone with sulfamoyl chloride. This compound inhibited not only estrone sulfatase but also dehydroepiandrosterone sulfatase activity in placental microsomes and in intact MCF-7 breast cancer cells. Pretreatment of MCF-7 cells or placental microsomes with EMATE, followed by extensive washing or dialysis indicated irreversible inhibition. This was confirmed by showing that EMATE inhibited estrone sulfatase activity in placental microsomes in a time-, concentration-, and pH-dependent manner. The enzyme is protected from inactivation by estrone sulfate, which is also consistent with active site-directed inhibition. EMATE is proposed to inactivate estrone sulfatase by irreversible sulfamoylation of the enzyme. Maximum enzyme activity was detected at pH 8.6, and the maximum rate of enzyme inactivation by EMATE also occurred at this pH. The pK_a values of the enzymatic reaction and pK_a of inactivation were 7.2 and 9.8, providing evidence that two active site residues are being modified by EMATE. As the phenolic pK_a of tyrosine (9.7) and the pK_a of histidine (6.8) are similar to the pK_a values of inactivation, these amino acid residues may play a role in the catalytic mechanism. The development of an active site-directed irreversible inhibitor of steroid sulfatase activity will allow the roles that these enzymes have in a number of physiological and pathological processes to be evaluated and also help to identify the active site of this enzyme.

Steroid sulfatases have a pivotal role in regulating the availability of biologically active steroids to several tissues in the body (Hobkirk, 1993). In the brain, pregnenolone sulfate is hydrolyzed to form several neurosteroids (Baulieu & Robel, 1991). In the female reproductive tract, the cleavage of cholesterol sulfate may be a crucial event in the ability of spermatozoa to penetrate the oocyte (Langlais et al., 1981). In normal mice, the balance of TH1 to TH2 cytokines released from lymphoid tissue in response to anti-CD3 is related to dehydroepiandrosterone sulfatase activity, and a similar mechanism may also be involved in regulating the immune response in humans (Daynes et al., 1990; Rook et al., 1994).

Currently there is considerable interest in the role that steroid sulfatases may have in regulating the synthesis of estrogenic steroids that are capable of supporting the growth of endocrine-dependent tumors of the breast and endometrium. This interest arises from the fact that in postmenopausal women, in whom breast and endometrial cancer most frequently occur, estrogens are formed almost exclusively in peripheral tissues. There is convincing evidence that the sulfatase pathway [i.e., formation of estrone (**1**) from estrone sulfate (**2**), Figure 1] as opposed to the aromatase pathway is the major source of estrogen in breast and endometrial tumors (Santner et al., 1984; Yamamoto et al., 1993). Furthermore, treatment of postmenopausal women with breast cancer by aromatase inhibitors, such as aminoglutethimide or 4-hydroxyandrostenedione, results in only a modest reduction in plasma estrogen concentrations (Santen et al., 1978; Reed et al., 1990). In aromatase inhibitor-treated patients, plasma estrone sulfate concentrations remain relatively high and hydrolysis of estrone sulfate, by estrone sulfatase, is considered to be the most likely source of plasma estrogens. The long half-life of steroid sulfates in blood (10 h) compared with their unconjugated derivatives (30 min), and high levels of steroid sulfatase activity in liver and normal and malignant breast tissues, lend support to this theory (James et al., 1987).



(1) R=H
(2) R=SO_3H
(3) R=PS(Me)OH
(4) R=SO_2NH_2
(5) R=SO_2NHMe
(6) R=SO_2NMe_2

FIGURE 1: Structures of estrone (**1**), estrone sulfate (**2**), estrone-3-*O*-methylthiophosphonate (**3**), estrone-3-*O*-sulfamate (**4**), estrone-3-*O*-(*N*-methyl)sulfamate (**5**), and estrone-3-*O*-(*N*,*N*-dimethyl)-sulfamate (**6**).

† This work was supported by grants from the Cancer Research Campaign and Imperial Exploitation Ltd. B.V.L.P. is a Lister Institute Research Professor.

‡ St Mary's Hospital Medical School.
§ University of Bath.
* Author to whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1995.

0006-2960/95/0434-11508$09.00/0

Although estrone and estrone sulfate have generally been considered to be the major sources of estrogenic steroids, which support breast tumor growth, there is now a considerable body of evidence to suggest that another steroid, androstenediol, may be of even greater importance as a promoter of tumor growth (Dauvois & Labrie, 1989). Androstenediol binds to the estrogen receptor with high affinity and can stimulate the growth of breast cancer cells *in vitro*, an effect that is blocked by the anti-estrogen, tamoxifen (Poulin & Labrie, 1986; Pizzini et al., 1992). In the rat, androstenediol stimulates the growth of nitrosomethylurea-induced mammary tumors and this ability is not inhibited by the coadministration of an aromatase inhibitor (Dauvois & Labrie, 1989).

In order to improve the response rate of breast cancer patients to some forms of endocrine therapy, such as aromatase inhibitors, it is necessary therefore to inhibit not only the formation of estrone from the large circulating reservoir of estrone sulfate (E1S)[1] but also the synthesis of the estrogenic steroid, androstenediol. In postmenopausal women, isotopic infusion studies have revealed that almost 90% of androstenediol originates from dehydroepiandrosterone sulfate (DHA-S), which is initially converted to either DHA or androstenediol sulfate (Poortman et al., 1980).

While it is still not clear whether the enzyme(s) that hydrolyze DHA-S and E1S are the same or different, the ability to inhibit the hydrolysis of these two steroid sulfates should represent a major advance in the therapies that are available to treat women with endocrine-dependent neoplasia. Additionally, although an impressive body of evidence has accumulated implicating steroid sulfates in several important physiological processes, a full examination of their role has been hampered by the lack of specific steroid sulfatase inhibitors. Using as model enzymes estrone sulfatase and dehydroepiandrosterone sulfatase, which are present in MCF-7 breast cancer cells and placental microsomal preparations, we have developed a series of potent inhibitors. One of the inhibitors, estrone-3-*O*-sulfamate (EMATE, **4**) inhibits steroid sulfatase via active site-directed inactivation.

MATERIALS AND METHODS

Synthesis of Sulfatase Inhibitors. Estrone-3-*O*-sulfamate (EMATE), -3-*O*-*N*-(methyl)sulfamate (MEMATE), and -3-*O*-(*N*,*N*-dimethyl)sulfamates (DEMATE) were synthesised by reaction of the anion of estrone with the appropriate sulfamoyl chloride, as previously described (Howarth et al., 1994). All compounds showed satisfactory spectroscopic and analytical data.

Inhibition of Sulfatase Activity. The ability of the compounds synthesised to inhibit steroid sulfatase activity was examined using placental microsomal preparations or intact MCF-7 breast cancer cells. Placental microsomes (100000*g* fraction) were prepared from a sulfatase-positive human placenta from a normal-term pregnancy (Duncan et al., 1993). To determine the IC_{50}s for the inhibition of estrone sulfatase and DHA-sulfatase, activity was measured in the presence of the inhibitor (0.05−1.0 μM) using either [^{3}H]E1S or [^{3}H]-DHA-S (4×10^5 dpm, NEN-Dupont, Boston, MA) adjusted to 20 μM with unlabeled substrate (Sigma). After incubation of the substrate ± inhibitor with placental microsomes (125 μg of protein/mL) for 30 min, the product formed was isolated from the mixture by extraction with toluene (4 mL), using [4-^{14}C]E1 or [4-^{14}C]DHA (7×10^3 dpm, Amersham International, Amersham, U.K.) to monitor procedural losses (Duncan et al., 1993).

MCF-7 breast cancer cells were maintained in minimal essential medium with 5% fetal calf serum and other essential nutrients (Duncan et al., 1993). For experiments, cells were seeded at 1×10^5 cells/flask and cultured in the above medium until 80% confluent. Steroid sulfatase activity was measured in intact monolayers after removal of the culture medium and washing with Earle's balanced salt solution (Purohit & Reed, 1992). Cells were incubated for 20 h at 37 °C with either [^{3}H]E1S (4×10^5 dpm, 2 nM) or [^{3}H]-DHA-S (1×10^5 dpm, 1 μM) in serum-free medium (2−5 mL) with or without inhibitor (1 fmol to 1 μmol). After incubation, medium (2.5 mL) was removed from each flask and sulfatase activity was determined using the procedure as described for the microsomal assay. Assays involving MCF-7 cells were usually carried out in triplicate, with the results being expressed as femtomoles of product formed per 20 h per million cells. Cell numbers were measured using a Coulter blood cell counter. Cell viability after exposure to inhibitors was assessed using the Trypan blue exclusion method.

Nature of EMATE Inhibition of Sulfatase Activity. To investigate the nature of EMATE inhibition of estrone sulfatase activity, intact monolayers of MCF-7 breast cancer cells were pretreated with medium ± EMATE, DEMATE, or E1-MTP (all tested at a 1 μM concentration) for 2 h at 37 °C. After removal of the medium and washing the cells five times with phosphate-buffered saline (5 mL), the remaining sulfatase activity was assayed using [^{3}H]E1S as previously described.

In addition, placental microsomes were incubated with EMATE (1 μM), DEMATE (2 μM), or E1-MTP (100 μM) for 5 min at 4 °C before incubating them at 37 °C for 30 min. An aliquot was taken from each sample for sulfatase assay at 37 °C with the remaining microsomes being subjected to dialysis at 4 °C for 16 h, prior to assaying for remaining sulfatase activity.

Placental microsomes were also used to examine the time- and concentration-dependent inactivation of estrone sulfatase by EMATE (**4**). Microsomes (200 μg of protein) were preincubated with EMATE (0−1.0 μM) for 0−60 min at 37 °C, followed by incubation with dextran-coated charcoal (0.5% and 5.0%) for 10 min at 4 °C.

Dextran-coated charcoal was sedimented by centrifugation, and portions of the supernatants were incubated with [^{3}H]-E1S (4×10^5 dpm, 20 μM) for 1 h at 37 °C to assess the remaining sulfatase activity, using duplicate assays for each concentration tested.

In addition, placental microsomes were used to examine the effect of pH on time-dependent inactivation of enzyme activity by EMATE (0.4 μM). For this, microsomes were incubated, in a total volume of 50 μL, at 37 °C for 0−15 min using 0.05 M citrate−phosphate and 0.1 M sodium carbonate−bicarbonate buffers over the range pH 2−11. The remaining enzyme activity was measured over 30 min by the addition of [6,7-^{3}H]E1S (20 μM) in 0.05 phosphate buffer (pH 7.5, 1 mL).

[1] Abbreviations: E1S, Estrone sulfate; E1-MTP, estrone-3-*O*-methylthiophosphonate; EMATE, estrone-3-*O*-sulfamate; MEMATE, estrone-3-*O*-(*N*-methyl)sulfamate; DEMATE, estrone-3-*O*-(*N*,*N*-dimethyl)sulfamate.

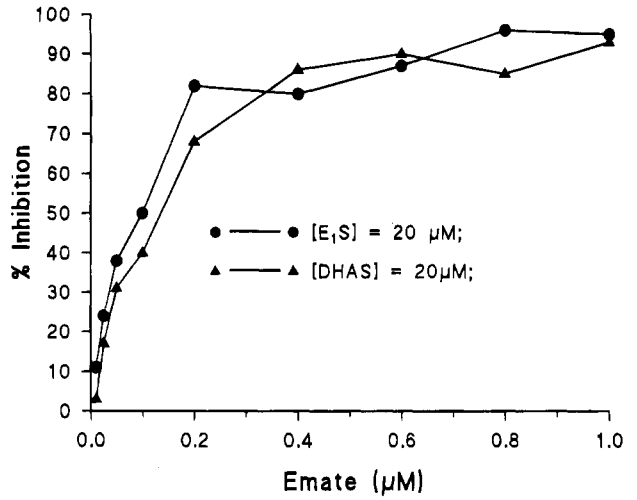


FIGURE 2: Inactivation of estrone sulfatase and dehydroepiandrosterone sulfatase activities by EMATE in placental microsome (100000*g*) preparations. [^{3}H]E1S or [^{3}H]DHA-S (4×10^5 dpm) adjusted to 20 μM with unlabeled substrate with or without EMATE at concentrations of 0.05−1.0 μM was incubated with placental microsomes (125 μg of protein/mL) for 30 min. The product formed was isolated by toluene extraction with [4-^{14}C]estrone or [4-^{14}C]DHA (7×10^3 dpm) being used to monitor procedural losses. Each point represents the mean of duplicate determinations. The IC_{50} values for estrone sulfatase and dehydroepiandrosterone sulfatase were 93 and 110 nM, respectively.

Substrate Protection Assay. To examine whether high substrate concentrations of estrone sulfate protected estrone sulfatase from inactivation by EMATE, placental microsomes (125 μg of protein) were preincubated with EMATE (1 μM) in the presence or absence of estrone sulfate (50 μM) for 0−30 min at 37 °C. Dextran-coated charcoal was then added to remove unbound substrate and inhibitor. Remaining sulfatase activity was then measured as previously described.

Effect of Amino Acid Modifying Reagents on Estrone Sulfatase Activity. To examine in a preliminary manner which amino acids at the active site of the enzyme may be involved in the removal of the sulfate moiety from the steroid nucleus, placental microsomes were treated with a number of amino acid modifying reagents at 10 and 100 μM concentrations, pH 7.5, at 37 °C and a reaction time of 2 h. Residual enzyme activity was measured at 37 °C at pH 7.5 using a saturating (20 μM) substrate concentration. Modifying reagents used included rose bengal, phenylglyoxal, *N*-bromosuccinimide, *N*-acetylimidazole, and phenylmethanesulfonyl fluoride, which are specific for histidine, arginine, tryptophan, tyrosine, and serine, respectively.

RESULTS

Inhibition of Sulfatase Activity. The IC_{50}s for the inhibition of estrone sulfatase and DHA sulfatase by EMATE, measured using a placental microsomal preparation and saturating substrate concentrations, were 93 and 110 nM, respectively (Figure 2). Using intact MCF-7 breast cancer cells and physiological concentrations of estrone sulfate (2 nM) or DHA-sulfate (1 μM), 1 nM EMATE inhibited estrone sulfatase activity by 95% and DHA-sulfatase activity by 82% (data not shown). EMATE appears to be a specific inhibitor of steroid sulfatase activity and did not inhibit placental microsome aromatase activity or estradiol dehydrogenase activity in MCF-7 cells (data not shown).

The IC_{50} for the inhibition of estrone sulfatase activity in MCF-7 cells by EMATE was about 65 pM. Inhibition of estrone sulfatase by 1 and 0.1 μM EMATE was 99.5% and 99.2%, respectively. For the other sulfamates, which were also tested for their inhibitory activity in intact MCF-7 breast cancer cells, inhibition of estrone sulfatase activity at 1 and 0.1 μM was 87% and 79% for MEMATE and 79% and 52% for DEMATE (Howarth et al., 1994).

Nature of EMATE Inhibition of Sulfatase Activity. The ability of 1 nM EMATE to inhibit estrone sulfatase activity by 95% in intact MCF-7 breast cancer cells suggested that this compound might be a mechanism-based or active site-directed inhibitor (Silverman, 1988). To examine this possibility, monolayers of MCF-7 cells were pretreated with inhibitors for 2 h at 37 °C with subsequent removal of the medium, washing, and assaying of remaining estrone sulfatase activity. Whereas no estrone sulfatase was detectable for EMATE-treated cells, after exposure of the cells to DEMATE or E1-MTP, estrone sulfatase activity had recovered by 81% and 88%, respectively.

In another experiment, placental microsomes were preincubated with the sulfamate analogues or E1-MTP for 30 min at 37 °C followed by dialysis for 16 h at 4 °C. Upon reassaying estrone sulfatase activity there was no recovery of activity with EMATE, whereas with the other inhibitors activity was restored by more than 80%. Thus, results from these studies suggested that EMATE acts as an irreversible inhibitor of estrone sulfatase activity.

Enzyme kinetic studies were then carried out to confirm the irreversible nature of the inhibition of sulfatase activity by EMATE. These investigations revealed that estrone sulfatase activity is inhibited by EMATE in a time- and concentration-dependent manner (Figure 3A). In contrast, neither MEMATE nor DEMATE, while potent, but some 100- and 1000-fold less potent than EMATE, inhibited estrone sulfatase in a time- and concentration-dependent manner (data not shown). Although the kinetics of inhibition were biphasic in nature (Figure 3A), initially the decrease in estrone sulfatase activity followed pseudo-first-order kinetics and the double-reciprocal plot of the first-order rates of inactivation versus inhibitor concentration revealed an apparent K_i for EMATE of 0.67 μM, and an overall rate constant for decrease in activity of 9×10^{-3} s^{-1}, as calculated by the method of Kitz and Wilson (1962) (Figure 3B).

pH Dependence of Inactivation. Maximum enzyme activity was detected at pH 8.6 (Figure 4), in the absence of EMATE (Segel, 1975). The maximum rate of inactivation of estrone sulfatase by EMATE was also detected at pH 8.6 (Figure 5A). Two ionizable groups appear to be required for activity and inhibition with pK_a values of about 7.2 and 9.8 (Figures 4 and 5B).

Substrate Protection Assay. Additional evidence for an active site-directed mode of inactivation of estrone sulfatase by EMATE was obtained by demonstrating that, at a high substrate concentration of estrone sulfate (50 μM), estrone sulfatase was protected from inactivation by EMATE. The rate constant of inactivation was reduced from $k = 2.03 \times 10^{-3}$ s^{-1} to $k = 0.63 \times 10^{-3}$ s^{-1}, a decrease of 69% (Figure 6).

Effect of Amino Acid Modifying Agents on Estrone Sulfatase Activity. Placental microsomes were treated with a number of amino acid modifying reagents to examine which amino acids at the active site may be involved in the

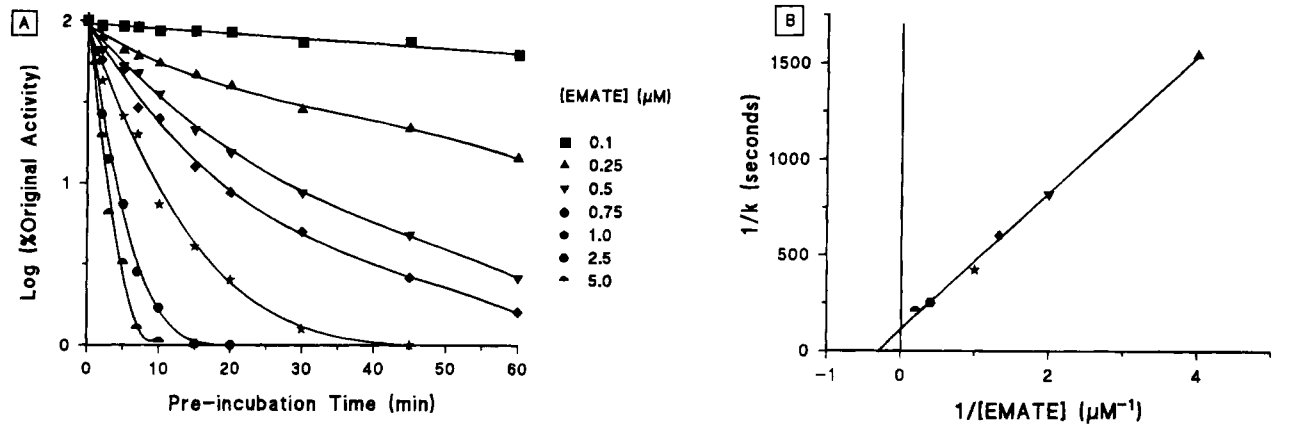


FIGURE 3: (A) Time- and concentration-dependent inhibition of estrone sulfatase activity in placental microsomes by EMATE. (B) Double-reciprocal plot of inactivation rate constant versus inhibitor concentration.

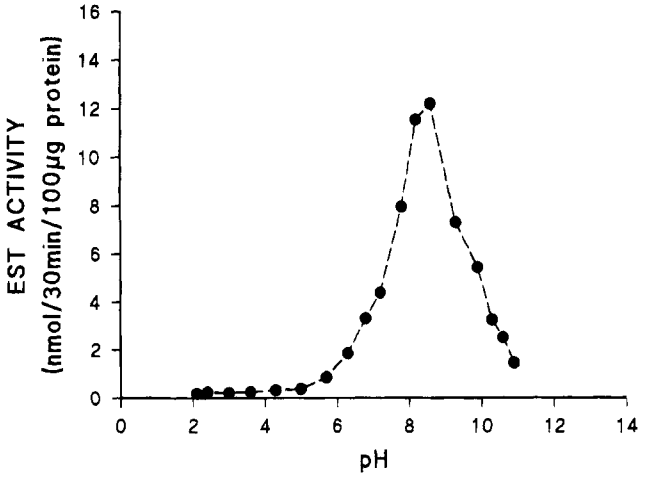


FIGURE 4: Effect of pH on estrone sulfatase (EST) activity in placental microsomes. From this, the pK_a values of the enzymatic reaction were estimated to be 7.2 and 9.8.

removal of the sulfate moiety. Of the compounds tested, rose bengal, which inactivates histidine (Wilson et al., 1961), reduced estrone sulfatase activity in a concentration-dependent manner (Figure 7).

DISCUSSION

After developing a lead compound, E1-3-MTP (Duncan et al., 1993) of moderate inhibitory potency, a series of steroidal and nonsteroidal analogues bearing sulfate surrogates was synthesized, of which the most potent inhibitor was EMATE. EMATE (1 nM) inhibited estrone sulfatase activity in intact MCF-7 breast cancer cells by 95% with an IC_{50} of 65 pM. This inhibitor is therefore about 1500 times more potent than the lead inhibitor (**3**) developed, which under the same conditions had an IC_{50} of 90 nM for inhibition of estrone sulfatase (Duncan et al., 1993). EMATE is about 100- and 1000-fold more potent than the N-methylated and N,N-dimethylated sulfamate analogues (Howarth et al., 1994). These estrone sulfamates therefore represent a new class of steroid sulfatase inhibitors. They are considerably more potent than danazol, which was previously reported to be an inhibitor of steroid sulfatase activity (Carlstrom et al., 1984), or other steroid sulfate surrogate inhibitors so far synthesized (Li et al., 1993). In addition to being active *in vitro*, preliminary experiments have confirmed the ability of EMATE and DEMATE to inhibit estrone sulfatase and

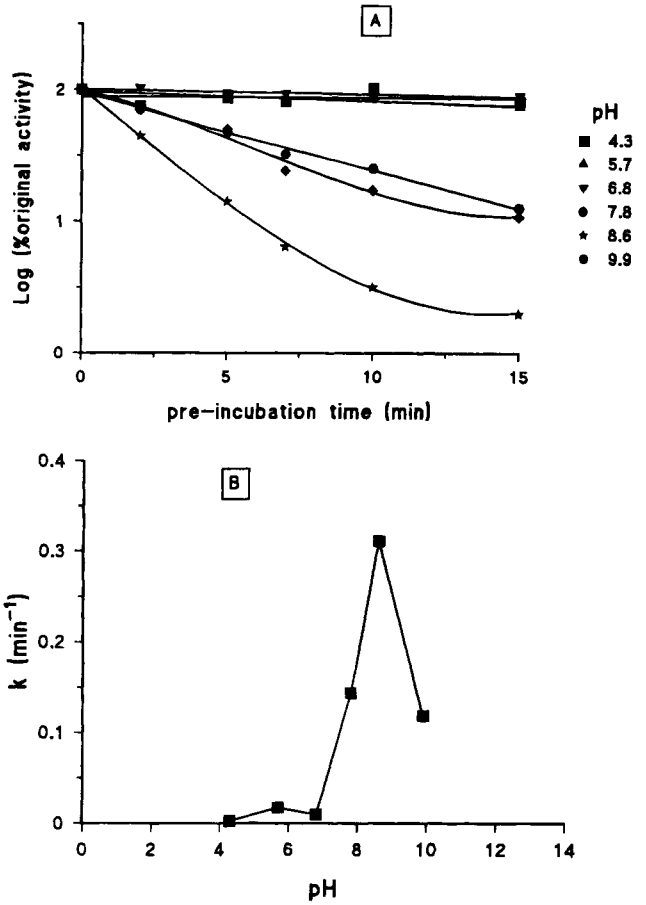


FIGURE 5: (A) Time- and pH-dependent inactivation of estrone sulfatase activity in placental microsomes. (B) Inactivation rate constant plotted versus pH. From this, the pK_a values of inactivation were found to be 7.2 and 9.8.

DHA-sulfatase activity *in vivo* in rat liver and NMU-induced mammary tumors (Purohit et al., 1995).

The ability of EMATE to inhibit estrone sulfatase activity by 95% in intact MCF-7 breast cancer cells initially suggested that this compound might be a mechanism-based or active site-directed inhibitor. Evidence to support such a possibility was obtained from a series of investigations. Pretreatment of MCF-7 cells with EMATE, but not DEMATE or E1-MTP, revealed that, after removal of the inhibitors by extensive washing, estrone sulfatase activity remained inactivated. Exposure of placental microsomes to EMATE, but not to the other inhibitors tested, with subse-

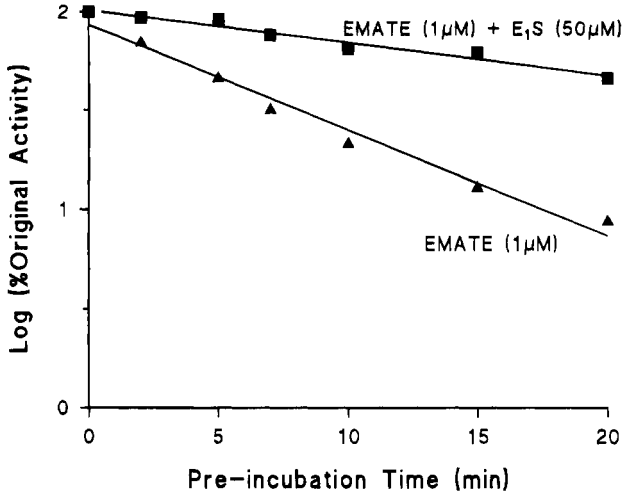


FIGURE 6: Substrate protection of estrone sulfatase from inactivation by EMATE. At high substrate concentrations of estrone sulfate (50 μM), estrone sulfatase activity was protected from inactivation by EMATE (1 μM).

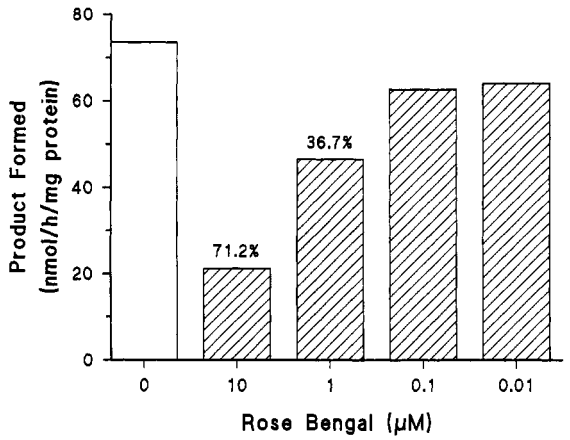


FIGURE 7: Effect of rose bengal, which inactivates histidine, on estrone sulfatase activity. Placental microsomes were treated with rose bengal (10 nM to 10 μM) and sulfatase activity was measured. Activity was inhibited in a concentration-dependent manner, suggesting that histidine residues may be involved in the catalysis of steroid sulfate hydrolysis.

quent dialysis and reassay of estrone sulfatase activity, also indicated that EMATE appeared to be an irreversible inhibitor. Kinetic studies were then carried out which confirmed that EMATE inhibited estrone sulfatase activity in placental microsomes in a time- and concentration-dependent manner. Modification of two amino acid residues may account for the biphasic nature of inactivation. High substrate concentrations of estrone sulfate protected estrone sulfatase from inactivation by EMATE, providing yet further evidence that this compound acts as an active site-directed inhibitor.

The analysis of pH dependence of enzyme activity and of enzyme inactivation by EMATE provides evidence that two ionizable groups with pK_a values of 7.2 and 9.8 are involved. Although further studies with a pure enzyme are required to confirm which amino acids are at the active site of the enzyme, the initial results obtained with a placental microsome preparation using rose bengal, which inactivates histidine (Wilson et al., 1961), suggests that this amino acid may play a role in the catalytic mechanism. However, the mechanism of aryl steroid sulfate hydrolysis is likely to be analogous to the proposed mechanism of sulfuryl transfer by aryl steroid sulfotransferase (Chai et al., 1992). For this it has been proposed that a ping-pong kinetic mechanism may be involved in the transfer between a tyrosine–enzyme substrate and a phenolic acceptor substrate. Studies on the stereochemical mechanism of sulfuryl transfer for steroid sulfotransferases demonstrate, through retention of configuration of a chiral sulfate group, the unambiguous involvement of a sulfuryl–enzyme intermediate (Chai et al., 1992; Chai & Lowe, 1992). The finding in the present study of a group with a pK_a of 9.8 and that a group with the same pK_a (9.8) is also required for inactivation by EMATE suggests that tyrosine (pK_a of phenolic hydroxy group 9.7) may also be involved in aryl steroid sulfate hydrolysis. It has also been proposed that histidine residues may act as general-acid–general-base catalysts in the mechanism of steroid sulfotransferases (Chai & Lowe, 1992). The fact that rose bengal inhibited estrone sulfatase activity in a dose-dependent manner also implicates a histidine residue as a potential amino acid at the active site of aryl steroid sulfatase.

The irreversible time- and concentration-dependent inhibition of estrone sulfatase by EMATE would seem to present a *prima facie* case for sulfamoylation of the active site of the enzyme. There is currently, however, little information available concerning the nature of the crucial amino acid residues in the active site of steroid sulfatase or aryl sulfatases in general (Roy et al., 1971; Nicholls & Roy, 1971), but possible mechanisms involve direct nucleophilic displacement of estrone from the sulfate group of E1S either by water in a one-step mechanism or by a two-step mechanism (Figure 8A) involving a sulfated enzyme intermediate, which is subsequently hydrolyzed by water to regenerate active enzyme. We propose that, in the inactivation of estrone sulfatase by EMATE, either the essential amino acid residue which is normally sulfated during catalysis or a neighboring residue becomes irreversibly sulfamoylated (Figure 8B). Two possible mechanisms are feasible: either direct nucleophilic attack at the sulfur atom of EMATE by the enzyme or the formation of a reactive aminosulfene intermediate from the anion of EMATE via an $E1_{cb}$ process, possibly by enzyme-catalyzed proton abstraction (Thea et al., 1986), followed by rapid sulfamoylation of the enzyme (Figure 8B). At present, however, it is difficult to distinguish between either mechanism, but the potential intermediacy of an aminosulfene, formed via a dissociative process, analogous to metaphosphate and sulfur trioxide, putative intermediates in phosphoryl and sulfuryl group transfers, is an attractive possibility.

Active site sulfamoylation bears some relationship to the inactivation of acetylcholinesterase by carbamate derivatives where carbamoylation of the enzyme is observed (Shinoda et al., 1992), although in this case the N-methylated carbamates are also potent inactivators. It seems likely for sulfatase that the less potent N-methylated sulfamates ME-MATE (**5**) and DEMATE (**6**) act by means of competitive inhibition in a fashion reminiscent of E1-MTP (**3**) (Duncan et al., 1993).

The development of an active site-directed irreversible inhibitor of steroid sulfatase activity will, for the first time, allow the roles that such enzymes have in a number of physiological and pathological processes to be evaluated. In contrast to other enzymes involved in estrogen synthesis,

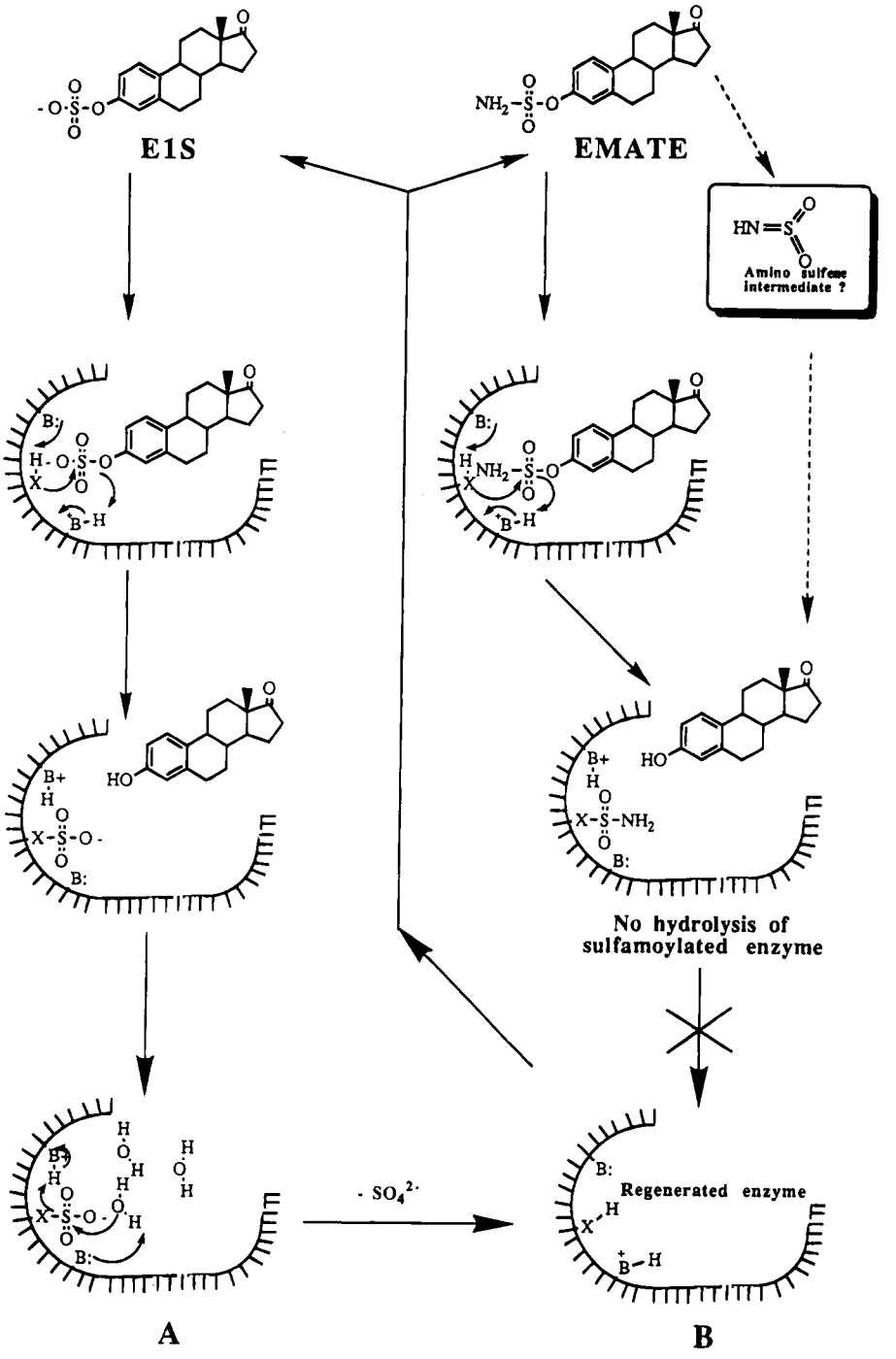


FIGURE 8: Proposed mechanisms of estrone sulfate hydrolysis by steroid sulfatase and enzyme inactivation by EMATE. (A) A possible mechanism for the hydrolysis of estrone sulfate involves direct nucleophilic displacement of estrone from the sulfate group of estrone sulfate (E1S). (B) It is proposed that the inactivation of estrone sulfatase by EMATE involves either the essential amino acid residue which is normally sulfated during catalysis or a neighboring residue becomes irreversibly sulfamoylated: either by direct nucleophilic attack at the sulfur atom of EMATE by the enzyme or by the formation of a reactive aminosulfene intermediate by proton abstraction at nitrogen and subsequent loss of estrone, followed by rapid sulfamoylation of the enzyme.

such as the aromatase complex, little is known about the structure of the active site of steroid sulfatase enzymes. The synthesis of a radioactively labeled irreversible inhibitor, e.g., of EMATE, should greatly facilitate the elucidation of key catalytic residues in the active site. Finally, if the hydrolysis of estrone sulfate and DHA-S to yield estrone and androstenediol is an important source of hormones to support the growth of endocrine-dependent tumors, then the development of a potent inhibitor should have considerable therapeutic potential for the treatment of conditions such as breast and endometrial cancer.

REFERENCES

Baulieu, E. E., & Robel, P. (1991) in *The New Biology of Steroids* (Hochberg, R. B., & Naftolin, F., Eds.) pp 251–263, Raven, New York.

Carlström, K., Doberl, A., Pousette, A., Rannevik, G., & Wilking, N. (1984) *Acta Obstet. Gynecol. Scand., Suppl. 123*, 107–111.

Chai, C. L. L., & Lowe, G. (1992) *Bioorg. Chem. 20*, 181–188.

Chai, C. L. L., Loughlin, W. A., & Lowe, G. (1992) *Biochem. J. 287*, 805–812.

Dauvois, S., & Labrie, F. (1989) *Breast Cancer Res. Treat. 13*, 61–69.

Daynes, R. A., Araneo, B. A., Dowell, T. A., Huang, K., & Dudley, D. (1990) *J. Exp. Med. 171*, 979−996.
Duncan, L. J., Purohit, A., Howarth, N. M., Potter, B. V. L., & Reed, M. J. (1993) *Cancer Res. 53*, 298−303.
Hobkirk, R. (1993) *Trends Endocrinol. Metab. 4*, 69−74.
Howarth, N. M., Purohit, A., Reed, M. J., & Potter, B. V. L. (1994) *J. Med. Chem. 37*, 219−221.
James, V. H. T., McNeill, J. M., Lai, L. C., Newton, C. J., Ghilchik, M. W., & Reed, M. J. (1987) *Steroids 50*, 269−279.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245−3249.
Langlais, J., Zollinger, M., Plante, L., Chapdelaine, A., Bleau, R., & Roberts, K. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7266−7270.
Li, P.-K., Pillai, R., Young, B. L., Bender, W. H., Martino, D. M., & Lin, F.-T. (1993) *Steroids 58*, 106−111.
Nicholls, R. G., & Roy, A. B. (1971) in *The Enzymes* (Boyer, V., Ed.) Vol. V, pp 21−41, Academic Press, New York.
Pizzini, A., Brignardello, E., Leonardi, L., Manaco, M. D., & Boccuzzi, G. (1992) *Int. J. Oncol. 1*, 709−712.
Poortman, J., et al. (1980) in *Adrenal Androgens* (Genazzani, A. R., Thijssen, J. H. H., & Siiteri, P. K., Eds.) pp 219−240, Raven, New York.
Poulin, R., & Labrie, F. (1986) *Cancer Res. 46*, 4933−4937.
Purohit, A., & Reed, M. J. (1992) *Int. J. Cancer 50*, 901−905.
Purohit, A., Williams, G. J., Roberts, C. J., Potter, B. V. L., & Reed, M. J. (1995) *Int. J. Cancer* (in press).
Reed, M. J., Lai, L. C., Owen, A. M., Singh, A., Coldham, N. G., Purohit, A., Ghilchik, M. W., Shaikh, N. A., & James, V. H. T. (1990) *Cancer Res. 50*, 193−196.
Rook, G. A. W., Hernandez-Pando, R., & Lightman, S. (1994) *Immunol. Today 15*, 301−303.
Roy, A. B. (1971) in *The Enzymes* (Boyer, V., Ed.) Vol. V, pp 1−19, Academic Press, New York.
Santen, R. J., Santner, S., Davis, B., Veldhuis, J., Samojlik, E., & Ruby, E. (1978) *J. Clin. Endocrinol. Metab. 47*, 1257−1265.
Santner, S. J., Feil, P. D., & Santen, R. J. (1984) *J. Clin. Endocrinol. Metab. 59*, 29−33.
Segel, I. H. (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, John Wiley & Sons, Inc. New York.
Shinoda, M., Hara, A., Nakayama, T., Deyashiki, Y., & Sawada, H. (1992) *J. Biochem. 112*, 834−839.
Silverman, R. (1988) *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, CRC Press, Boca Raton, FL.
Thea, S., Cerasco, G., Guanti, G., & Williams, A. (1986) *J. Chem. Soc., Chem. Commun.* 1582−1583.
Wilson, I. B., Harrison, M. A., & Ginsburg, S. (1961) *J. Biol. Chem. 236*, 1498−1500.
Yamamoto, T., Kitawaki, J., Urabe, M., et al. (1993) *J. Steroid Biochem. Mol. Biol. 44*, 463−468.

BI942744T