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INHIBITION OF ESTRONE SULFATASE ENZYME IN HUMAN PLACENTA AND HUMAN BREAST CARCINOMA

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Summary—Estrone sulfatase is an important mechanism of local synthesis of biologically active estrogens in human breast cancer. The human placental microsome and breast carcinoma mitochondrial/microsomal estrone sulfatase activity were characterized and inhibition studies performed. The K_m of the placental tissue enzyme was 6.83 μM, V_{max} 0.015 nmol/min/mg, and for the breast carcinoma tissue K_m was 8.91 μM and V_{max} 0.022 nmol/min/mg. Danazol produced a significant inhibition of estrone sulfatase (20% with 50 μM danazol). No significant inhibition was seen in the presence of aminoglutethimide, rogletimide, tamoxifen, 4-hydroxyandrostenedione, stilboestrol, or any metabolites of danazol or tamoxifen. Studies with synthetic and naturally occuring steroids demonstrated that the presence of a sulfate group at the 3 position to be the most important factor in determining inhibition, and the most potent inhibitor was 5α-androstene-3β,17β-diol-3-sulfate (K_i of 2.0 μM). The naturally occuring 3-sulfated steroids all demonstrated competitive inhibition. These studies could form the basis for the design of a potent estrone sulfatase inhibitor which would have potential therapeutic activity in the management of breast cancer.

INTRODUCTION

Approximately one third of human breast carcinomas are hormone-dependent [1]. Animal studies and clinical trials of antiestrogens and inhibitors of estrogen biosynthesis have confirmed that estrogens are the most important hormones involved in supporting growth of hormone-dependent breast tumours [2, 3]. Indeed, estrogen deprivation by these means results in objective tumour regression.

Plasma levels of estrone and estradiol in postmenopausal women are very low, however the estrogen concentration in breast tumour tissues is of an order of magnitude higher than in the plasma [4], suggesting local intratumoral production of estrogens in breast tumour cells from precursor substrates. Although the predominant source of estrogen production in postmenopausal women is the extraglandular conversion of androstenedione to estrone by peripheral tissues catalyzed by the aromatase enzyme [5], estrone sulfate is the most abundant estrogen in peripheral blood [6]. The plasma estrone sulfate level is higher in postmenopausal women with breast cancer than in normal postmenopausal women [7]. Although the estrone sulfate itself is unable to bind to the estrogen receptor and stimulate a biological response, studies involving MCF-7 cell lines have shown that estrone sulfate can be taken up by these cells and hydrolyzed to the unconjugated estrone in sufficient amounts to stimulate a biological response [8].

In addition, a comparative study of intratumoural aromatase and sulfatase activities in breast cancer cells showed that under enzyme saturating conditions, the capacity of the sulfatase pathway in forming estrone is one million-fold greater than the aromatase pathway, and even at the physiological concentration of substrate, the sulfatase enzyme appears to be at least ten times more important than the aromatase enzyme. Therefore the sulfatase pathway is likely to be an important means of local production of biologically active estrogens in breast carcinoma tissue [9].

Previous studies have investigated the inhibition of steroid sulfatase using dehydroepiandrosterone sulfate (DHEAS) as substrate by naturally occurring and synthetic steroids in human placenta [10], chorion and decidua [11], prostate [12], testis [13] and rat testis [14]. More recently the inhibition of steroid sulfatase using

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estrone sulfate as the substrate by naturally occurring steroids in human chorion [15] and by danazol in human breast carcinoma tissue [16] has been reported. We therefore considered it pertinent to further characterize the estrone sulfatase enzyme in human placenta and human breast carcinoma and investigate its inhibition by both naturally occurring and synthetic steroids, and by antiendocrine agents currently in use for the management of human breast carcinoma.

EXPERIMENTAL

Reagents

[6,7-^{3}H]estrone sulfate (sp.act. 47.7 Ci/mmol) was purchased from New England Nuclear Division (Du Pont, U.K. Ltd.). Purity was checked by thin layer chromatography (Silica gel Merck 5415 Kieselgel F254) using the following solvent system: ethyl acetate–methanol–ammonium hydroxide (75:25:2, vol by vol). [14-^{14}C]estrone (sp.act. 60 Ci/mmol) was purchased from Amersham, Int. (Amersham, U.K.). Tamoxifen, and its metabolites, were kindly supplied by Dr R. McCague (Institute of Cancer Research, Sutton, U.K.), rogletimide (pyridoglutethimide) was synthesized at the Institute of Cancer Research, Sutton, U.K. Aminoglutethimide and 4-hydroxyandrostenedione were supplied by Ciba Geigy (Horsham, U.K.), danazol and its metabolites were supplied by Sterling-Winthrop (Guildford, U.K.). FCE 24304 (6-methylenandrosta-1,4 diene-3,17-dione) was supplied by Farmitalia Carlo Erba (Milan, Italy). Estra-1,3,5,(10)triene-3,16α,17β-triol-3-sulfate,5-β androsten-3αol-11,17-dione-3-sulfate, 17β estradiol disulfate, androst-5-en-3β ol-17-one-19al-3-sulfate, androst-5-ene-3β, 17β-diol-disulfate, 5β androsten-3-α-ol-17-one 3 sulfate and 5β pregnene-3α,20α-diol 3 sulfate were all supplied by the Steroid Reference Collection by kind permission of the curator, Professor D. Kirk (London, U.K.). All other steroids were purchased from Sigma Chemical Co. (Poole, U.K.).

Tissues and tissue preparation.

Full term human placenta were obtained immediately after delivery from the labour ward of St George's Hospital, London, U.K., and transferred to the laboratory on ice for preparation of tissue fractions. Human breast carcinoma samples were obtained from the tissue bank of the Oncology Unit, St George's Hospital Medical School.

All procedures were carried out at 0–4°C. Placental tissue was freed from connective tissue and large blood vessels, and washed twice with chilled 1.15% KCl, dried and weighed. The placenta was chopped with scissors and dissolved in 0.25 M sucrose in 50 mM Tris–HCl buffer pH 7.4 (1 g tissue to 1 ml buffer) then homogenized with a Polytron for two periods of 20 s. The homogenate was subjected to subcellular fractionation. The nuclear pellet was obtained by centrifugation at 1500*g* for 15 min, followed by 10,000*g* for 30 min to obtain the mitochondrial pellet and finally 100,000*g* for 70 min to separate the microsomes from the cytosol. Human breast carcinoma samples were pooled and processed as above except that the 10,000*g* centrifuge spin was omitted, so the mitochondrial and microsomal fractions were not separated. All pellets were resuspended in 50 mM Tris–HCl pH 7.4 and together with the cytosols were snap frozen at −80°C and stored at −20°C. All samples were assayed for estrone sulfatase activity and the protein content determined by the method of Hartree [17].

Estrone sulfatase assay

Before use in the assay, estrone sulfate was purified by solvent partition with diethyl ether (5:1 vol by vol) in order to remove any unconjugated steroids. Radiolabelled estrone sulfate was added to the unlabelled compound to achieve the required concentration. All assays were carried out in duplicate at 37°C in a shaking waterbath. Tubes were preincubated for 1 min before initiating the reaction by addition of the tissue samples. The assay tubes (vol 0.3 ml) contained 10 mM DTT (dithiothreitol), 1 mM EDTA, 20 μM [^{3}H]estrone sulfate (approx. 4×10^5 cpm), tissue sample and 50 mM Tris–HCl buffer at the relevant optimum pH. Control tubes contained boiled tissue samples.

Aliquots (0.1 ml) were removed from each assay tube after 10 and 20 min of incubation to ensure linearity of product formation. The reaction was terminated by addition of each aliquot to a chilled tube containing 0.1 ml of 0.1 M sodium carbonate and [4-^{14}C]estrone (approx. 5000 cpm) as internal standard. The unconjugated product was separated from the substrate by adding 3 ml of ether and left to stand at room temperature. After drying with anhydrous sodium sulfate, the ether layer was separated by centrifugation and added to a

scintillation vial. The sample was evaporated to dryness under nitrogen and reconstituted with 10 ml scintillation fluid and radioactivity determined by liquid scintillation counting. The recovery of the internal standard was used to correct the amount of tritiated product formed.

The method was modified in order to identify the products of the estrone sulfatase enzyme. The extracted ether phase was dried and centrifuged as before, an aliquot of 200 μl taken for counting and then the remainder taken to dryness by rotary evaporation and reconstituted with a small volume (25 μl) of ethyl acetate. This sample was run on a TLC plate using dichloromethane–ether (9:1 vol by vol) as solvent system. The plates were scanned using a Berthold LB 283 linear analyzer and the radioactive peaks compared to authentic steroids. The radioactive peaks were scraped off the TLC plate, the silica dissolved in methanol, an aliquot retained for counting and the remainder taken to dryness under nitrogen and reconstituted in 25 μl ethyl acetate as before. These samples were run on a TLC plate using a second solvent system, namely ethyl acetate–benzene (1:1 vol by vol). The plate was scanned using the Berthold LB 283 linear analyzer, the radioactive peaks identified and scraped off and dissolved in methanol before counting.

For enzyme inhibition studies, a stock solution of each compound dissolved in methanol was prepared and added to the assay tubes such that the solvent did not exceed 2% of the total volume. An equal volume of methanol was added to the control tubes. Each compound was initially assayed at a final concentration of 20 and 50 μM, using a saturating substrate concentration of 20 μM. The estrone sulfatase activity was determined from the linear plots of product released against time, comparison of this value with the control activity enabled the percentage inhibition to be determined. Inhibition of $<10\%$ was considered not significant. Kinetic analysis of inhibitors was carried out using two inhibitor concentrations and a range of substrate concentrations. The K_i values and type of inhibition were determined from the resulting Lineweaver–Burk plots.

RESULTS

Evaluation of optimal assay conditions

Under the standard assay conditions the control tubes containing boiled tissue gave values of approx. 1% for the conversion of estrone sulfate to estrone. Tissue samples were diluted to give conversion rates of between 5 and 20% at a substrate concentration of 20 μM. The intra-assay coefficient of variation was 7.08% ($n = 6$), the inter-assay coefficient of variation was 7.66% ($n = 8$).

Table 1. Specific activity of estrone sulfatase in subcellular fractions

Tissue fraction	Sp. act. (nmol/min/mg)	% Total activity
Human placenta		
Nuclear	2.47	27.6
Mitochondrial	2.26	25.3
Microsomal	4.20	47
Cytosolic	NA	
Human breast carcinoma		
Nuclear	0.19	18.1
Mitochondrial/ microsomal	0.89	81.9
Cytosolic	NA	
Homogenate	0.13	

NA, no activity.

Table 1 shows the specific activities of the sulfatase enzyme in the subcellular fractions of both human placenta and human breast carcinoma tissues, and the percentage that each of these fractions represents of the total activity seen in that particular tissue. In the human placenta the microsomal fraction is the predominant source of enzyme activity, whilst in human breast carcinoma the pooled mitochondrial/microsomal fraction is the predominant source of enzyme activity. In both tissues, there was a linear relationship of increasing enzyme activity with increasing protein concentration, demonstrated up to 121.5 μg of mitochondrial/microsomal fraction of breast carcinoma tissue, and up to at least 52 μg of placental microsomal fraction. In addition, all assays displayed linear product formation up to 20 min, demonstrating that the enzyme was assayed under saturating conditions.

The K_m and V_{max} were calculated by the Lineweaver–Burk method. In the human placental microsomal fraction the K_m was 6.83 μM, and the V_{max} 0.015 nmol/min/mg (Fig. 1). In the human breast carcinoma mitochondrial/microsomal fraction K_m was 8.91 μM, V_{max} 0.02 nmol/min/mg. The reciprocal plots for both enzymes were linear in nature with no evidence of substrate activation or product inhibition. The optimum pH was determined over a range of pH from 5.5 to 9.0, using Mes/NaOH [2(*N*-morpholino) ethane sulphonic acid], Tris–HCl and glycine–NaOH as buffering systems. The optimum pH for placental microsomal fraction was found to be Tris–HCl pH 7.4 and for breast carcinoma mitochondrial/microsomal fraction, Tris–HCl pH 7.2.

Table 2. Purity of [^{3}H]estrone product

Sample	$^3H/^{14}C$ ratio		
	Ether extraction	Dichloromethane–ether (9:1)	Ethylacetate–benzene (1:1)
Human placenta	16.0:1	15.8:1	15.58:1
Human breast carcinoma	5.64:1	5.52:1	5.53:1

With both solvent systems only one radioactive peak was seen for both the placental enzyme product and the pooled breast carcinoma enzyme product, in each case corresponding to the R_f value of cold estrone as visualized by u.v. light. With the dichloromethane–ether (9:1 vol by vol) system, the R_f value was 0.39. With the ethylacetate–benzene (1:1 vol by vol) the R_f value was 0.47. The $^3H/^{14}C$ ratio of the products remained constant after the ether extraction and after the sequential TLC analysis. This demonstrates that the only ^{3}H-labelled non-polar metabolite formed is estrone and this is essentially pure at the ether extraction stage (Table 2).

Inhibitors of placental estrone sulfatase

The inhibition of placental estrone sulfatase by antiendocrine drugs and their major metabolites is shown in Table 3. Danazol was the most potent inhibitor in this group, although only 20% inhibition was seen at the maximum concentration used (50 μM). None of its major metabolites had significant effect, and none of the other compounds in this group had any significant (< 10%) inhibition at 50 μM.

Twenty-four naturally occurring and synthetic steroids were similarly investigated for potential inhibition. The results are shown in Table 4. Of the estrogens, significant inhibition was seen in the presence of 50 μM estrone (41%) and 50 μM estradiol (42%), but 2-hydroxyestradiol and 2-methoxyestrone were inactive. Of the unconjugated androgens only

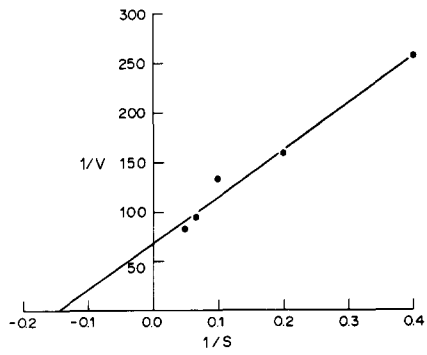

Fig. 1. Lineweaver–Burk plot to determine the K_m and V_{max} of placental estrone sulfatase. V = velocity of reaction in nmol/min/mg. S = substrate concentration in μM. Each point is the mean (±10%) of duplicate determinations at each of two time points.

Table 3. Inhibition of placental estrone sulfatase by antiendocrine drugs

Compound	% Inhibition	
	10 μM Inhibitor	50 μM Inhibitor
Aminoglutethimide	8	0
Pyridoglutethimide	3	0
Danazol	14	20
Ethisterone	7	0
17-Hydroxy-2-(hydroxyl)17α-pregna-1,4-dien-20-yn-3-one	0	0
17-Hydroxy-2α-(hydroxymethyl)-17α-pregna-4-en-20-yn-3-one	7	0
Tamoxifen	8	10
4-Hydroxytamoxifen	0	0
N-desmethyltamoxifen	0	0
Cis-tamoxifen	0	6
4-Hydroxyandrostenedione	7	5
Diethylstilboestrol	8	4
FCE 24304	6	0

Each value is the mean of duplicate determinations at each of two time points, and all values are within 10% of the mean value shown. Any result > 10% is regarded as significant.

Table 4. Inhibition of placental estrone sulfatase by steroid compounds

Compound	% Inhibition	
	10 μM Inhibitor	50 μM Inhibitor
Estrogens		
17β-Estradiol	13	42
Estrone	20	41
2-Hydroxyestradiol	0	0
2-Methoxyestrone	0	1
Androgens		
5-Androsten-3β-17β-diol-3-acetate	31	38
5α-Androstane-3α,17β-diol	20	37
Testosterone	6	5
Dihydroepiandrosterone	0	14
5α-Androstene-3β,17β-diol	13	6
Dihydrotestosterone	0	0
Sulfated steroids		
5-Androsten-3β,17β-diol-3-sulfate	58	87
Pregnenolone-3-sulfate	33	77
17β-Estradiol-3-sulfate	26	52
DHEAS	23	50
Androsterone-3-sulfate	11	47
Cholesterol-3-sulfate	15	43
5β-Pregnene-3α,20α,diol-3-sulfate	0	43
Estriol-3-sulfate	13	12
5β-Androstan-3α-ol-17one-3-sulfate	8	18
5β-Androstan-3α-ol-11,17dione-3-sulfate	0	5
Androst-5-en-3β-17one-19al-3-sulfate	13	0
5-Androstene-3β,17β-diol-17-sulfate	8	24
17β-Estradiol disulfate	0	0
Androst-5-ene-3β,17β-diol-disulfate	13	16

Each value is the mean of duplicate determinations at each of two time points, and each value is within 10% of the mean. Any result > 10% is regarded as significant.

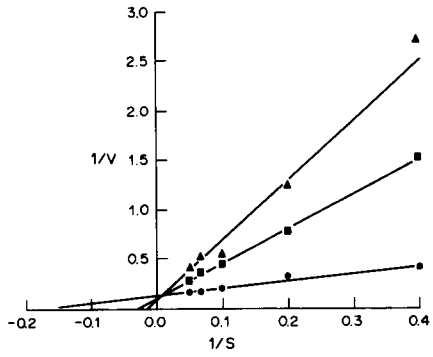

Fig. 2. Lineweaver–Burk plot for the inhibition of placental estrone sulfatase by pregnenolone sulfate. V = velocity of reaction in nmol/min/mg. S = substrate concentration in μM. Each point is the mean of duplicate determinations at each of two time points. Control = ●—●; 5 μM pregnenolone sulfate = ■—■; and 10 μM pregnenolone sulfate = ▲—▲.

5-α-androstene-3α,17β-diol had any significant activity (37% inhibition with 50 μM inhibitor).

The most potent inhibitors of estrone sulfatase examined all had a sulfate group in the 3 position of the steroid ring. The compound with the greatest activity (87% inhibition at 50 μM) was 5-α-androstene-3β,17β-diol-3-sulfate. In each case where both a 3-sulfated steroid and its unconjugated parent steroid were investigated, the sulfated compound was more potent. The results of the 3-sulfated steroid inhibition is shown in Table 4. In contrast, considerably less inhibition was seen with the 17-sulfated steroids and with the disulfated steroids. It is likely that the 3-sulfated steroid compounds act as alternative substrates as they demonstrate competitive inhibition on Lineweaver–Burk plots (Fig. 2), which would support this hypothesis. The K_i values of four 3-sulfated steroid compounds are shown in Table 5.

Inhibitors of human breast carcinoma estrone sulfatase

A sample of the compounds which had been screened on the placental enzyme were investigated on the human breast carcinoma enzyme. Results are shown in Table 6. Again the most potent inhibitor (75% at 50 μM) was 5-androstene-3β-17β-diol-3-sulfate. Significant inhibition was also seen with the other compounds screened.

Table 5. K_i constant of steroid 3-sulfates on placental estrone sulfatase

Compound	K_i (μM)
5-Androstene-3β,17β-diol-3-sulfate	2.0
Pregnenolone-3-sulfate	3.1
Cholesterol-3-sulfate	6.25
DHEAS	9.2

All displayed competitive inhibition.

Table 6. Inhibition of estrone sulfatase in human breast carcinoma tissue

Compound	% Inhibition 50 μM
5-Androstene-3β,17β-diol-3-sulfate	75
DHEAS	62
Estradiol	41
Estrone	30
Danazol 20 μm	24
Danazol 60 μm	26
Danazol 100 μm	32

Each value is the mean of duplicate determinations of each of two time points, and each value is within 10% of the mean value. Any result > 10% is regarded as significant

In view of the report demonstrating that danazol is a potent estrone sulfatase inhibitor in human breast carcinoma [16], we investigated its inhibition of the breast carcinoma enzyme, over a range of concentrations. Table 6 shows the results. Danazol appears to cause a concentration dependent decrease in estrone sulfatase activity in breast carcinoma, but high concentrations are required.

DISCUSSION

Estrone sulfatase has similar enzyme characteristics (K_m, V_{max} and optimum pH) in both human placenta and human breast carcinoma. The enzyme is predominantly microsomal in the human placenta and microsomal/mitochondrial in human breast carcinoma.

Danazol significantly inhibits estrone sulfatase in both human placenta and human breast carcinoma. However Carlstrom [16] reported greater inhibition with danazol. Although they continued their *in vitro* reaction for a longer time period than in our study, we added considerably higher concentrations of danazol.

In both studies the danazol levels were higher than those observed in plasma *in vivo*. The most likely explanation of this discrepancy is that Carlstrom investigated the formation of [^{3}H]estradiol from [^{3}H]estrone sulfate in the present of NADH and NADPH. This conversion is a two step reaction, involving the initial conversion of estrone sulfate to estrone by estrone sulfatase, followed by conversion of estrone to estradiol by 17β-hydroxysteroid dehydrogenase, for which NADH and NADPH are cofactors, although these are not required by the sulfatase enzyme. Estrone sulfatase inhibition by danazol contributes to the decreased conversion of [^{3}H]estrone sulfate to [^{3}H]estradiol, but the major site of this inhibition is likely to be on 17β-hydroxysteroid

dehydrogenase [18, 19]. Another possible reason for this discrepancy is that we used a saturating concentration of substrate in our assay, and under these conditions weak inhibitors would display less inhibition than at lower substrate concentrations. None of the three major danazol metabolites shows significant inhibition. Similarly a decreased production of estradiol from estrone sulfate in many cancer cell lines has been demonstrated in the presence of tamoxifen and its metabolites [8, 20, 21]. However, in our *in vitro* study there was no significant inhibition of estrone sulfatase by tamoxifen nor by any of its major metabolites. This suggests that inhibition of 17β-hydroxysteroid dehydrogenase, or the inhibition of estrone sulfate uptake by these cells may explain these findings. Aminoglutethimide does not inhibit estrone sulfatase. *In vivo* aromatization studies have demonstrated that despite almost complete inhibition of aromatase by aminoglutethimide, there is not complete suppression of estrone formation, and it has in addition a significant effect in increasing estrone sulfate metabolism and clearance [22], which may be an important mechanism in its inhibition of estrone formation. 4-Hydroxyandrostenedione, stilboestrol and rogletimide demonstrate no significant inhibition of estrone sulfatase.

Although it has been reported that estrone sulfate is hydrolyzed by an enzyme distinct from that which hydrolyses the other steroid sulfates on the basis of physicochemical and kinetic studies [23, 24], physical separation of these enzymes has never been achieved, and it is likely that it is the presence of detergent in the purification procedure that is responsible for these physicochemical and kinetic differences [25]. In addition, studies of placental sulfatase deficiency and X-linked icthyosis have demonstrated a defect of estrone sulfate metabolism as well as of DHEAS and cholesterol sulfate metabolism, and the use of cDNA probes has shown that this defect of metabolism is a consequence of a deletion of the steroid sulfatase gene [26, 27, 28]. Therefore, it is likely that steroid sulfatase is one enzyme and hydrolyses several sulfated steroids as substrates. However, a range of substrate specificities exit.

In comparison with previously published works, the inhibition of human placental and breast carcinoma estrone sulfatase by sulfated and unconjugated steroids differs from the inhibition observed on steroid sulfatase when DHEAS or pregnenolene sulfate is used as a substrate [10–14]. Agents which have been screened on estrone sulfatase, DHEAS sulfatase and pregnenolone sulfatase produce a consistently greater degree of inhibition of pregnenolone sulfatase than estrone sulfatase, although the difference is less than that observed between DHEAS and estrone sulfatases. This occurs both in the placenta and in other tissues. There is some difference observed in the inhibition studies of estrone sulfatase by steroids between human chorion [15], placenta and breast carcinoma. However, this is less than the variation seen when an alternative substrate for steroid substrate is investigated. One possible explanation of these discrepancies is that different authors have used different substrate concentrations. Comparison of K_i values overcomes this problem. Unfortunately, not all the previously published works include K_i values. The K_i values we report when estrone sulfate is used as substrate for the human placental enzyme, is similar to those values reported where the same substrate is used for human chorion [15], but not for rat liver [29]. However, in this latter study, the inhibition of the enzyme by other 3-sulfated steroids was non-competitive, probably because they used Miranol H2M detergent in the extraction and solubilization procedure, rather than any difference in the enzyme. The K_i values were different where DHEAS was used as substrate, both in human placenta [10] and in other tissue [11]. This evidence suggests that steroid sulfatase exhibits a range of substrate specificities. Inhibition studies of human breast carcinoma estrone sulfatase and human placenta estrone sulfatase are sufficiently comparable so that the placental tissue enzyme can be used as a screening investigation for substrates which may be of value as inhibitors of the human breast carcinoma tissue enzyme.

The most significant factor in inhibition of estrone sulfate is the presence of a sulfate group in the 3 position of the steroid ring. In each case, where both a 3-sulfated steroid and its unconjugated parent counterpart were investigated, the degree of inhibition was considerably and significantly greater in the presence of the 3-sulfated steroid. 17-Sulfated steroids, however are not hydrolyzed by the enzyme. The most potent inhibitor is 5-α androsten-3β,17β-diol-3-sulfate. Other bulky chemical groups in the 3 position, such as an acetate group, also result in greater inhibition than seen in the presence of the corresponding unconjugated parent steroid. However, even in the presence of a sulfate group

in the 3 position, modifications elsewhere in the steroid molecule lead to modification of inhibitory activity. The presence of a sulfate group at the 17 position, an aldehyde group at the 19 position, a keto group at the 11 position, a methoxy or hydroxy group at the 2 position, all considerably reduce sulfatase inhibition.

It is anticipated that these screening inhibition studies on human placental tissue, with selection of the most potent inhibitors for evaluation on human breast carcinoma tissue enzyme will lead to the rational design of a potent estrone sulfatase inhibitor, which may be a useful novel endocrine agent in the management of human breast cancer.

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