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In vitro and in vivo models for the evaluation of new inhibitors of human steroid sulfatase, devoid of residual estrogenic activity☆

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Abstract

The goal of our research project is to develop a new class of orally active drugs, estrone sulfatase inhibitors, for the treatment of estrogen-dependent (receptor positive) breast cancer. Several compounds were synthesized and their pharmacological potencies explored. Based on encouraging preliminary results, three of them, TX 1299, TX 1492 and TX 1506 were further studied in vitro as well as in vivo. They proved to be strong inhibitors of estrone sulfatase when measured on the whole human JEG-3 choriocarcinoma and MCF-7 breast cancer cells and their IC_{50}s found to be in the range of known standard inhibitors. Their residual estrogenic activity was checked as negative in the test of induction of alkaline phosphatase (APase) activity in whole human endometrial adenocarcinoma Ishikawa cells. In addition, their effect on aromatase activity in JEG-3 cells was also examined, since the goal of inhibiting both sulfatase and aromatase activities appears very attractive. However, it has been unsuccessful so far. Then, in vivo potencies of TX 1299, the lead compound in our chemical series, were evaluated in comparison with 6,6,7-COUMATE, a non-steroidal standard, in two different rat models and by oral route. First, the absence of any residual estrogenic activity for these compounds was checked in the uterotrophic model in prepubescent female rats. Second, antiuterotrophic activity in adult ovariectomized rat supplemented with estrone sulfate (E_1S), showed that both compounds were potent inhibitors, the power of TX 1299 relative to 6,6,7-COUMATE being around 80%. This assay was combined with uterine sulfatase level determination and confirmed the complete inhibition of this enzyme within the target organ.

Preliminary studies indicated that other non-steroid compounds in the Théramex series were potent in vitro and in vivo inhibitors of estrone sulfatase in rats and further studies are in progress.

Keywords: Estrone sulfatase; JEG-3; MCF-7; Rat; Antisulfatase; 6,6,7-COUMATE

1. Introduction

The steroid sulfatase pathway has been the focus of recent interest in the context of breast cancer, with regard to the local intra-tissue formation of estrogens from the abundant circulating pool of estrone sulfate (E_1S) [1–4]. Inhibition of this enzyme would prevent E_1S to yield free estrone (E_1), which in turn can be transformed into estradiol (E_2) by enzymatic reduction. EMATE [1], estrone-3-sulfamate, is the historical standard steroidal sulfatase inhibitor but with the major drawback of being estrogenic because of its mechanism of inhibition: the sulfamate moiety is cleaved during the process of enzyme inactivation, which releases E_1, not from E_1S but from EMATE itself [5]. Other sulfamate derivatives have been designed with non-estrogenic precursors to give acceptable drug candidates such as 6,6,7-COUMATE, a standard non-estrogenic sulfatase inhibitor from the literature [6]. In our own search of sulfatase inhibitors, a strategy of in vitro and in vivo models has been deployed, described as follows.

First, the E_1-mimicking non-steroid moieties with the free OH groups are tested for potential estrogenic activity in vitro in the alkaline phosphatase (APase) induction assay on human Ishikawa endometrial cancer cells. Then, in a second round of chemical synthesis, the adequate precursors are converted into sulfamates and screened for estrone sulfatase inhibition in two in vitro models: whole human MCF-7 breast cancer cells and whole human JEG-3 choriocarcinoma cells, and again for estrogenic activity as

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sulfamates in the estrogenic test on Ishikawa cells. Next, selected compounds found active in vitro are checked for residual estrogenic activity in vivo in the classical uterotrophic assay after 3-day administration by oral route in prepubescent female rats. Finally, the steroid sulfatase inhibition is evaluated in vivo in adult castrated female rats receiving exogenous E_1S with or without an oral dose of the test compound, with uterine weight and uterine sulfatase activity as endpoints, which are well correlated with potent inhibitors such as 6,6,7-COUMATE or TX 1299, our own lead compound.

2. Materials and methods

2.1. Materials

Radioactive compounds were obtained from NEN, Perkin-Elmer (Boston, USA). Abiraterone, danazol, letrozole, raloxifene, 4OH-tamoxifen, EMATE, COUMATE, methyl-COUMATE, 6,6,7-COUMATE, TX 1299, TX 1506 and TX 1492 were synthesized by Théramex (Monaco). ICI-182,780 was purchased from TOCRIS (Bristol, UK). All media, sera and antibiotics for cell culture were obtained from Life Technologies (Cergy-Pontoise, France).

Other not listed or not specified compounds and reagents were from Sigma (St. Quentin Fallavier, France).

2.2. Cell lines

MCF-7 cells, derived from human breast adenocarcinoma, were purchased from the American Type Culture Collection (ATCC). They were routinely grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red (PR), glutamax I (4 mM), glucose (4.5 g/l) supplemented with 5% of decomplemented fetal calf serum (dFCS, by heat inactivation for 1 h at 56 °C) and penicillin–streptomycin (50,000 IU–50,000 μg/l) at pH 7.4. JEG-3 cells, derived from human choriocarcinoma, were purchased from ATCC. They were routinely grown as monolayers in Modified Eagle's Medium + PR with 1 g/l glucose supplemented with 10% of dFCS, penicillin–streptomycin (100,000 IU–100,000 μg/l), glutamax I (2 mM) and 1% of non-essential amino acid. Ishikawa cells, derived from human endometrial adenocarcinoma were from the European Collection of Animal Cell Cultures (ECACC). They were routinely grown as MCF-7 cells except that the medium contained 10% dFCS. Seeding was performed in Nunc culture plastic flasks and cells maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 ± 0.1 °C. Stocks were passaged once a week to maintain continuous exponential growth. When needed, steroids or test substances were solubilized in dimethyl sulfoxide (DMSO) or ethanol and added to the culture medium to obtain the final concentrations described in the figure legends. Ethanol or DMSO (≤0.2% (v/v)) were also added to control cells.

2.3. APase assay on Ishikawa cells

Effects of compounds on APase induction were measured in estrogen free conditions as already described [7,8]. In brief, cells were inoculated into 96-well microplate 48 h before studies. The next day, the medium was replaced by fresh medium containing 5% dFCS without PR. After an additional 24 h period, test substances were added. At the end of a 4-day treatment APase was measured with the *p*-nitrophenyl-phosphate assay, and the activity was expressed as fold increase above control levels, then as percent of 10^{-8} M E_2 effect which was taken to be equal to 100%.

2.4. Estrone sulfatase assay on cells

Whole-cell assays were performed as originally described by Duncan et al. [9] on intact MCF-7 cell monolayers. Assays were carried out with cells in logarithmic growth phase, on 96-well (JEG-3) or 24-well (MCF-7) microplates. Twenty-four hours (JEG-3) or 72 h (MCF-7) before studies, cells were seeded in dFCS supplemented medium. Then, seeding medium was removed and cells were rinsed with PBS to eliminate any trace of dFCS. Then, ^{3}H-E_1S was added, followed by test compounds. After 4 h (JEG-3) or 20 h (MCF-7) of treatment, the medium was transferred into either 96-deep-well microplates (JEG-3) or glass tubes (MCF-7) and centrifuged at 200 × *g* for 10 min to pellet cells for toluene extraction. The radioactivity in the toluene phase was determined by liquid scintillation counting (LSC), and estrone sulfatase activity was expressed in pmol of ^{3}H-E_1 + ^{3}H-E_2 formed per 4 or 20 h and per mg DNA, then in percent of control activity without inhibitor which was taken to be equal to 100%. IC_{50}s were then determined by non-linear regression analysis.

2.5. Aromatase assay

Effects of compounds on aromatase activity were measured on confluent JEG-3 cells with the tritiated water radiometric method [10,11]. Twenty-four hours before studies, cells were seeded in dFCS supplemented medium. Then, they were rinsed with MEM without dFCS and treatments were distributed. Cells were then incubated at 37 °C for 2 h with the substrate, 10 nM of 1β-^{3}H(*N*)-Δ4A. After incubation, the medium was transferred to a U-bottom 96-well microplate. A 1% charcoal solution was added to each well to trap unconverted substrate and other steroids. Plates were then centrifuged and the supernatant containing the tritiated water (^3H_2O) formed during aromatization of 1β-^{3}H(*N*)-Δ4A to estrone was measured by LSC. Aromatase activity was expressed as fmol of ^3H_2O (=E_1) formed per 2 h and μg DNA, then in percent of control activity without inhibitor which was taken to be equal to 100%.

2.6. Animals

Prepubescent Sprague–Dawley derived OFA strain female rats, and adult Wistar female rats (200–220 g) were selected for the present study. They were purchased from Iffa Credo (Elevage des Oncins, L'Arbresle, France). Rats were maintained in a controlled air-conditioned room and provided with a standard pellet diet and filtered mains water ad libitum.

2.7. Uterotrophic activity in prepubescent female rat

Uterotrophic activity assay was adapted to prepubescent rat from the well known method described by Hilgar and Palmore [12]. In brief, 21-day-old Sprague–Dawley female rats were treated orally with E_1S at 10 μg per rat per day or with 6,6,7-COUMATE, TX 1299, TX 1492 or TX 1506 at 1 mg per rat per day for 3 days. Animals were sacrificed on the day following the last treatment and uteri were removed and weighed.

2.8. Antiuterotrophic and antisulfatase activities in adult ovariectomized rat

The antiuterotrophic assay was performed as originally described by Purohit et al. [13] with slight modifications. Wistar female rats (200–220 g) were ovariectomized and left to rest for 4 weeks. Prior to the treatment, vaginal smears were performed in order to check the absence of cyclicity. Animals were supplemented with 50 μg/kg per day E_1S s.c., alone or combined with oral administration of test compounds at 1 mg/kg per day for four consecutive days. On the fifth day, a last administration was performed 2 h before the necropsy, for E_1S and E_2 plasma level determination; blood samples were taken from the abdominal aorta and serum estrogen levels were analyzed according to the supplier's standard procedure (DSL, Inc., Webster, TX, USA). The uteri were removed, freed of adjacent tissue, weighed and immediately frozen deeply until the determination of sulfatase activity, described as follows.

2.9. Estrone sulfatase levels in uterus

Estrone sulfatase activity in uterus was measured according to the method described by Purohit et al. [13] with slight modifications. Uteri were thawed at room temperature, dried and weighed. Tissues were minced and homogenized with a Turrax grinder in a 50 mM KH_2PO_4, NaOH pH 7.4 buffer. Nuclei and cell debris were removed by centrifugation for 20 min at 4 °C and 2000 × *g*. Aliquots of the supernatant were treated with dextran-coated charcoal and used for the sulfatase assay as described for cells. Briefly, E_1S activity was assessed after 30 min of incubation with 5 nM of ^{3}H-E_1S and 20 μM of unlabeled E_1S as substrate. The extraction yield was monitored by adding 1.25 nCi of ^{14}C-E_1 during the extraction process. Radioactivity was measured by LSC. Estrone sulfatase activity was expressed as pmol of ^{3}H-E_1 + ^{3}H-E_2 per hour per milligram of protein.

2.10. DNA, protein determination and data analysis

Cell homogenate DNA content was measured according to the Labarca and Paigen method [14] adapted to 96-well microplates. Protein estimation was made by the method of Bradford [15] with the Bio-Rad protein assay. Bovine serum albumin was used for protein standards. Data were reported as mean±S.E.M. for *n* determinations as specified in the legends to the figures. Data were analyzed with SAS software (version 6.12, SAS Institute, Grégy-sur-Yerres, France). The different tests used are specified in Section 3.

3. Results

3.1. No estrogenic potency in vitro

As shown in Fig. 1, between 10^{-12} and 10^{-6} M, E_2 induced a concentration-related stimulation of APase activity in Ishikawa cells, characterized by an EC_{50} of 0.2 nM. This effect of estradiol was purely estrogenic since at 10^{-7} M it was totally abolished by the pure antiestrogen ICI-182,780 with an IC_{50} of 1.95 nM and by the specific estrogen receptor modulator raloxifene with an IC_{50} of 30.8 nM. As shown in Table 1, ICI-182,780 and raloxifene, together with 4OH-tamoxifen were also tested for their intrinsic properties. In contrast with ICI-182,780 which was totally devoid of estrogenic potency in vitro, we found that both

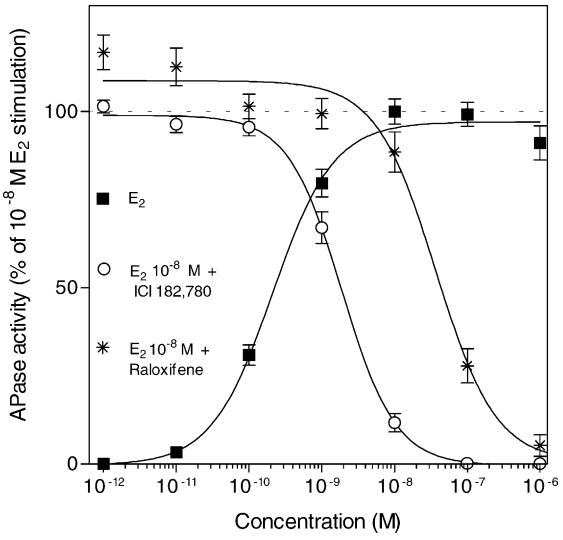

Fig. 1. Stimulation of the APase activity of Ishikawa cells by estradiol and inhibition of 10^{-8} M estradiol stimulation by ICI-182,780 and raloxifene. Exponentially growing cells were plated in 96-well microplates as described in Section 2. APase activity was measured after 4 days of incubation in the presence of the indicated concentrations of compounds. Each point represents the mean ± S.E.M. of at least five determinations, each made in quadruplicate wells in individual experiments.

Table 1
Lack of estrogenic potential of non-steroid antisulfatase compounds as revealed by APase assays in Ishikawa cells

Compounds (10^{-7} M)	APase activity in Ishikawa cells (percentage of E_2 effect)
EMATE	100
6,6,7-COUMATE	0
Methyl-COUMATE	0
COUMATE	0
TX 1299	2.5
TX 1506	3
TX 1492	5.4
4OH-tamoxifen	35.2
Raloxifene	14.5
Abiraterone	0
ICI-182,780	0
Danazol	0
Letrozole	0

Means for at least three determinations.

4OH-tamoxifen and raloxifene were partial agonists at 10^{-7} M. In the same model and at 10^{-7} M, among all the compounds tested here, including letrozole (antiaromatase), abiraterone (antilyase) and danazol, only EMATE, was able to induce APase activity, and to a similar extent than 10^{-8} M E_2.

3.2. Strong antisulfatase potencies in vitro

The ability of standard compounds to inhibit sulfatase activity was shown to follow the same decreasing order in both cell lines: EMATE > 6,6,7-COUMATE > methyl-COUMATE > COUMATE while danazol and letrozole were inactive (Figs. 2 and 3). The new Théramex compounds were all potent inhibitors but with a different pattern: TX 1506 and TX 1492 were more potent than TX 1299 in MCF-7 cells, while the contrary prevailed in JEG-3 cells. TX 1299, was as potent as the non-steroidal inhibitor 6,6,7-COUMATE in both models with IC_{50}s in the nmolar range as shown in Table 2. IC_{50}s were determined by non-linear regression analysis (GraphPad Prism Software, version 3.0), and analyzed by ANOVA followed by a non-parametric Kruskall–Wallis analysis and Wilcoxon tests.

Table 2
IC_{50} for the inhibition of sulfatase activity in whole-cell assays

Compounds	JEG-3 cells (nM)	MCF-7 cells (nM)
EMATE	3.2 ± 0.2	0.08 ± 0.03
6,6,7-COUMATE	5.1 ± 1.5	0.43 ± 0.1
Methyl-COUMATE	78.6 ± 18.6	25 ± 3
COUMATE	864.5 ± 105	Inactive
TX 1299	5.3 ± 2.2	0.76 ± 0.53
TX 1506	11.9 ± 1.6	0.06 ± 0.02
TX 1492	22.5 ± 0.9	0.07 ± 0.03
Danazol	Inactive	Inactive
Letrozole	Inactive	Inactive

Means ± S.E.M. for at least three determinations.

3.3. Specificity of sulfatase versus aromatase inhibition

Letrozole was shown to be a very potent inhibitor of JEG-3 cell aromatase activity with an IC_{50} of 0.6 ± 0.03 nM (Fig. 4). The only other compound found to display a little but negligible activity was 6,6,7-COUMATE with an IC_{50} of 1895.5 ± 73.4 nM.

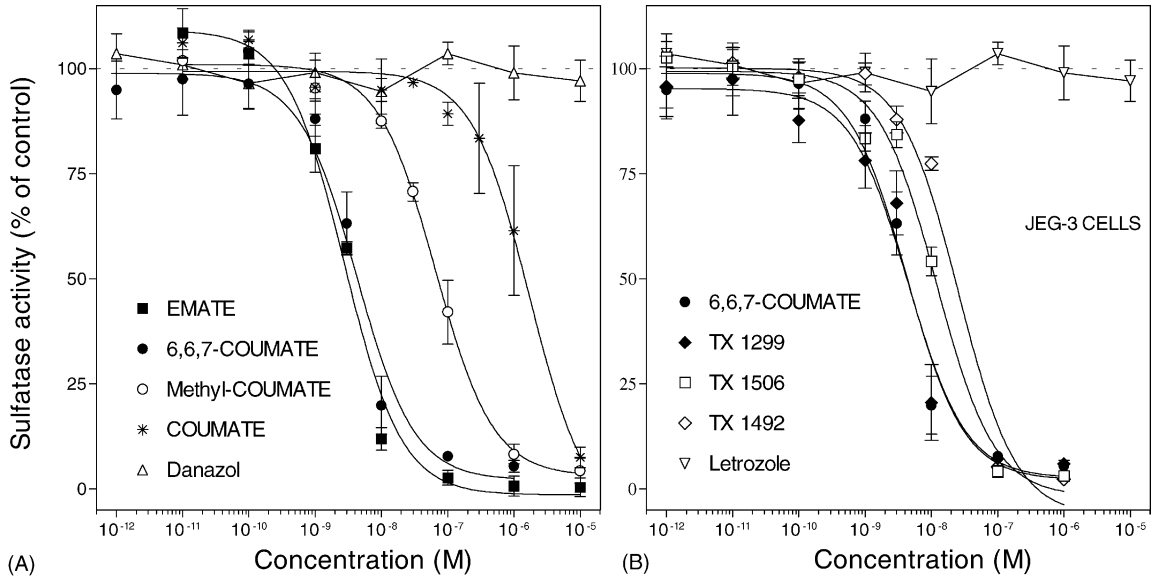

Fig. 2. Inhibition of estrone sulfatase activity in JEG-3 cells. Exponentially growing cells were plated in 96-well microplates as described in Section 2. Sulfatase activity was measured after 4 h of incubation in the presence of 5 nM ^{3}H-E_1S and the indicated concentrations of compounds. Each point represents the mean ± S.E.M. of at least three determinations each made in duplicate wells in individual experiments.

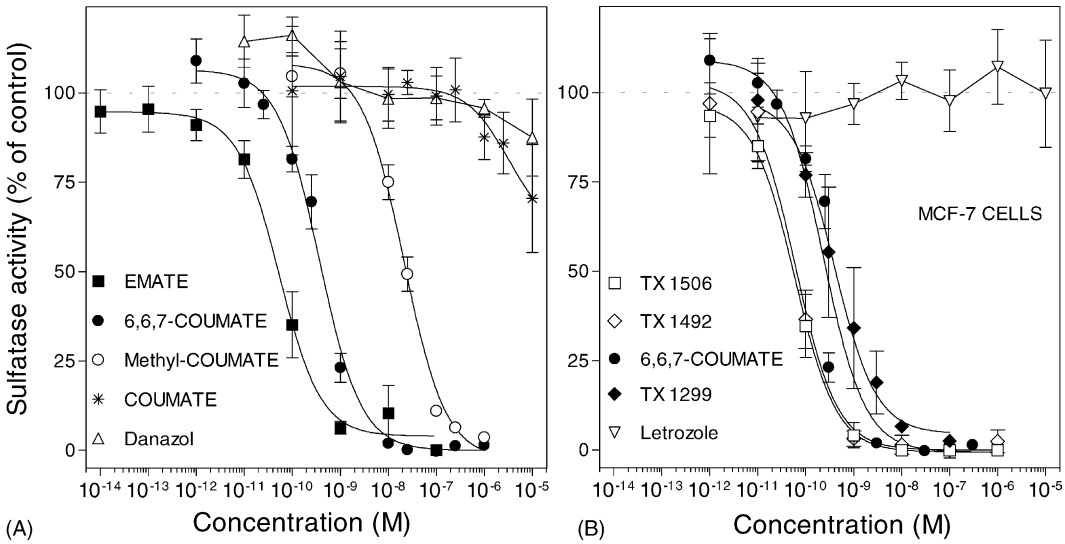

Fig. 3. Inhibition of estrone sulfatase activity in MCF-7 cells. Exponentially growing cells were plated in 24-well microplates as described in Section 2. Sulfatase activity was measured after 20 h of incubation in the presence of 5 nM ^{3}H-E_1S and the indicated concentrations of compounds. Each point represents the mean ± S.E.M. of at least three determinations each made in single wells in individual experiments.

3.4. Residual uterotrophic activity in prepubescent female rats

As shown in Fig. 5, E_1S at 10 μg per rat per day p.o. induced a four-fold increase in uterus weight. 6,6,7-COUMATE at 1 mg per rat per day p.o. was totally devoid of uterotrophic activity, as well as the non-steroidal compounds TX 1299 and TX 1506. TX 1492, however, was shown to display a weak (16%) but statistically significant effect on uterus weight ($P < 0.01$, ANOVA for unpaired values).

3.5. Positive correlation between antiuterotrophic and antisulfatase activity in adult ovariectomized rat

Fig. 6 shows that 50 μg/kg per day for 4 days of E_1S s.c. induced a 2.9-fold increase in uterus weight (Table 3). This stimulation was inhibited by the oral administration of 6,6,7-COUMATE, beginning at 0.2 mg/kg per day ($P < 0.05$) and was completely abolished at 1 mg/kg per day. The sulfatase levels measured on the same tissue were also inhibited, but from 0.03 mg/kg per day ($P < 0.05$) and the inhibition was complete at 0.3 mg/kg per day ($P < 0.001$).

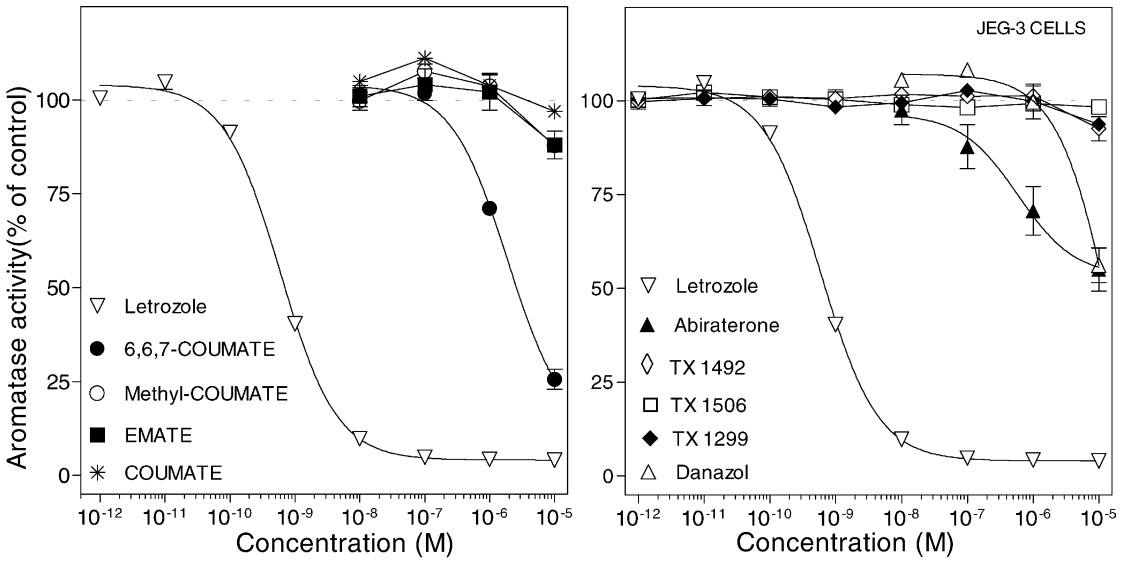

Fig. 4. Inhibition of aromatase activity in JEG-3 cells. Exponentially growing cells were plated in 96-well microplates as described in Section 2. Aromatase activity was measured after 2 h of incubation in the presence of 10 nM of 1β-^{3}H(*N*)Δ4A and the indicated concentrations of compounds. Each point represents the mean ± S.E.M. of at least three determinations each made in duplicate wells in individual experiments.

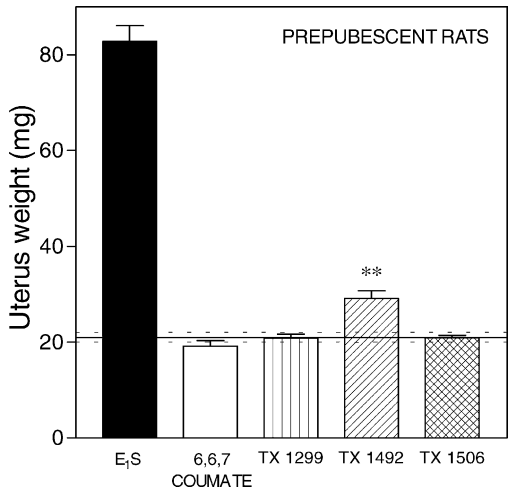

Fig. 5. Comparative residual uterotrophic activity of new Théramex compounds in the prepubescent rat. Immature Sprague–Dawley female rats were treated orally with E_1S at 10 μg per rat per day or with 6,6,7-COUMATE, TX 1299, TX 1492 or TX 1506 at 1 mg per rat per day for 3 days. Animals were sacrificed on the day following the last treatment and uteri were removed and weighed. Each point represents the mean ± S.E.M. of eight animals per group. (**) $P < 0.01$ as compared with control group.

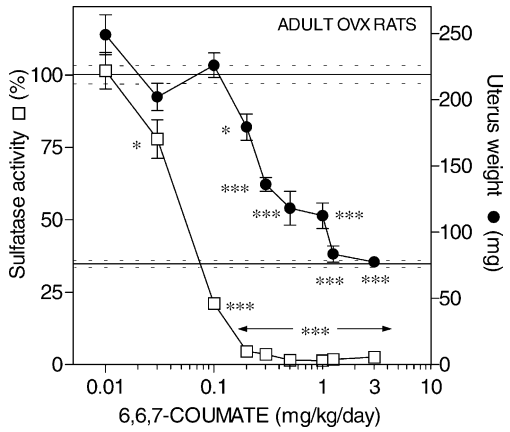

Fig. 6. Positive correlation between the antiuterotrophic activity of 6,6,7-COUMATE and its antisulfatase potency in the adult rat. Ovariectomized rats were treated as described in Section 2. They were supplemented with 50 μg/kg per day estrone sulfate (E_1S) s.c., alone or combined with oral administration of 6,6,7-COUMATE for four consecutive days. Uteri were removed and weighed and immediately frozen until the determination of sulfatase activity. Each point represents the mean ± S.E.M. of eight determinations. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ as compared with E_1S treated group.

There was a good correlation between sulfatase activity inhibition and antiuterotrophic activity (Pearson correlation coefficent = 0.7, $P < 0.05$, $n = 47$). So, sulfatase activity in uterus needed to be totally inhibited in order to trigger the reduction of this organ weight. Circulating estrogen levels when measured 2 h after the last E_1S treatment (Table 3) showed an increase in circulating E_1S and E_2 levels. For the three doses of 6,6,7-COUMATE used, all producing maximal effect on sulfatase activity (0.3, 1 and 3 mg/kg per day), there was an accumulation of E_1S, of about three times ($P < 0.001$), combined with a two-fold reduction of E_2 levels as compared with animals receiving E_1S alone ($P < 0.001$).

The effects of Théramex compounds at 1 mg/kg per day p.o. were compared in the same model with the effect of 6,6,7-COUMATE. Fig. 7 shows that the three new compounds were equivalent in reducing both uterus weight and uterine sulfatase levels and that they were as potent inhibitors as 6,6,7-COUMATE. ($P < 0.001$, Student's *t*-test between E_1S and negative control group (vehicle), Dunett's test for uterus weight between each group and E_1S group; ANOVA followed by a non-parametric Kruskall–Wallis analysis and Wilcoxon's test were used for sulfatase activity.)

The result obtained for TX 1299 was also compared with 6,6,7-COUMATE in a dose–response assay between 0.2 and 1.25 mg/kg per day (Fig. 8). Linear regressions were obtained ($r = -0.81$ ($P < 0.001$) for TX 1299, and $r = -0.82$ ($P < 0.001$) for 6,6,7-COUMATE) and it was shown that the power of TX 1299 relative to 6,6,7-COUMATE was of 80% (house-made software determination). Student's *t*-test was performed between E_1S and negative control group (vehicle), and each group was compared to E_1S group by Dunett's test.

Table 3
Uterus weight, sulfatase activity, and serum estrogen levels in adult ovariectomized rats after a 4-day treatment with E_1S alone or in combination with 6,6,7-COUMATE

Treatment	Uterus weight (mg)	Sulfatase activity (%)	E_1S levels (ng/ml)	E_2 levels (pg/ml)
Control	76.0 ± 2.62	0	7.5 ± 0.8	5.1 ± 0.1
E_1S	218.9 ± 6.79**	100	18.7 ± 2.5**	17.7 ± 1.8**
6,6,7-COUMATE (mg/kg per day)				
0.3	136.0 ± 5.15***	3.6 ± 0.4***	53.2 ± 5.9***	8.9 ± 0.8***
1.0	112.4 ± 9.55***	1.4 ± 0.14***	75.9 ± 4.6***	7.8 ± 0.3***
3.0	77.4 ± 3.25***	2.6 ± 0.33***	69.5 ± 8.1***	9.0 ± 0.7***

Means ± S.E.M. for eight determinations per group.
** $P \leq 0.01$ vs. control.
*** $P < 0.001$ vs. E_1S.

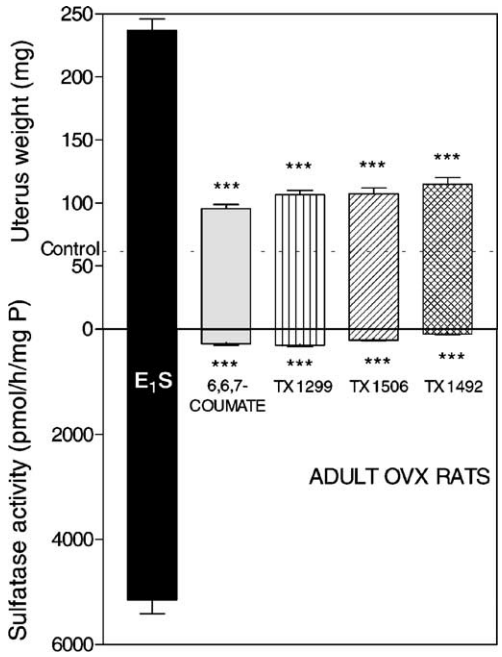

Fig. 7. Comparative antiuterotrophic activities and antisulfatase potencies of new Théramex compounds in the adult rat. For animal treatment see legend of Fig. 6. Each point represents the mean ± S.E.M. of eight animals per group. (***) $P < 0.005$ as compared with E_1S treated group.

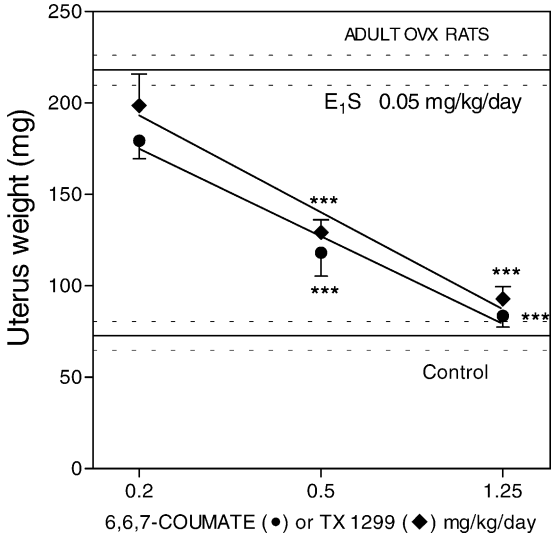

Fig. 8. Antiuterotrophic activity of TX 1299 as compared with 6,6,7-COUMATE in adult rat with E_1S. For animal treatment see legend of Fig. 6. Each point represents the mean ± S.E.M. of animals per group. (***) $P < 0.001$ as compared with E_1S treated group. Regression analysis was performed in the tested dose range: $r = 0.81$ for TX 1299 and $r = 0.82$ for 6,6,7-COUMATE. The relative power of TX 1299 vs. 6,6,7-COUMATE was 80% (house-made software determination).

4. Discussion

Estrone sulfatase activity was more than 80 times higher in the human choriocarcinoma JEG-3 cells than in the human breast cancer MCF-7 cells, with respectively 102 ± 5.8 pmol per 4 h per mg DNA and 1.2 ± 0.04 pmol per 4 h per mg DNA (results not showed). This characteristic allowed the simultaneous screening of (1) a larger number of molecules and/or concentrations with 96-well versus 24-well microplates; (2) a shorter duration of incubation, 4 h versus 20 h; and (3) duplicate versus single determinations.

In both cell lines, the four reference compounds were shown to possess the same pattern of inhibition (EMATE > 6,6,7-COUMATE > methyl-COUMATE and COUMATE), but with IC_{50}s in a totally different range of concentrations: from 3.2 to 864.5 nM in JEG-3 cells, and from 0.08 nM up to >10 μM in MCF-7. Then, if the JEG-3 cells proved to be more convenient, the MCF-7 cells proved to be more discriminative. In breast cancer cells, the Théramex compounds which were chosen from the results obtained in JEG-3, proved to be as potent inhibitors as 6,6,7-COUMATE ($P > 0.05$). It was therefore decided that molecules with an IC_{50} of ≤20 nM in JEG-3 cells were to be further tested in human breast cancer MCF-7 cells. The other interest of the JEG-3 cells is their high level of aromatase activity, which allowed for the parallel determination of inhibiting potential on that alternative estrogen supplier to tumor cells. Unfortunately, none of the new compounds were shown to possess such a potential.

Then, considering that antisulfatase molecules are intend to be used in the treatment of estrogen-dependent breast cancer, i.e. in estrogen receptor positive tumors, they are bound to be devoid of estrogenic potency. For that issue, the Ishikawa cell model which has been proved to be a very simple, useful and rapid tool to check out that potency [7,8] was used. TX 1299, TX 1506 and TX 1492 were found to be devoid of in vitro estrogenic potency, therefore they were identified as leads and underwent further in vivo investigations.

The lack of potential estrogenic side-effect was also checked in vivo, in immature rats. This model proved to be useful since TX 1492 was eliminated as a potential lead when a residual estrogenicity was demonstrated. This result showed that this in vivo assay is not redundant with the alkaline phosphatase model.

Then, the short-term model of antiuterotrophicity in adult ovariectomized rat supplemented with E_1S was assessed with 6,6,7-COUMATE, based on the one described by Purohit et al. [13] where animals were pretreated with antisulfatase compounds for 2 days before the administration of E_1S plus compounds for an additional 5-day treatment. Our shorter model displayed a positive correlation between the decrease in uterus sulfatase activity and the decrease in uterus weight. It showed that a complete inhibition of both parameters was achieved and that when plasma levels of estrogen where measured 2 h after a final treatment, there was an accumulation of circulating E_1S with a concomitant decrease of E_2. However, it showed that there was a gap between the dose triggering the complete decrease in uterus weight and the dose inducing full inhibition of sulfatase activity. As shown in Fig. 7, the three Théramex compounds

which were as active as 6,6,7-COUMATE also reduced partially uterus weight in adult OVX rats at a dose producing maximal inhibition of sulfatase activity.

This only partial effect of sulfatase inhibition on the decrease of uterus weight and the decrease of E_2, as the end-product of the estrogen biosynthesis, can be explained in part by the activity of other enzymes as sources of E_2. Besides 17β-hydroxysteroid-dehydrogenase (17βHSD) type 1 in peripheral tissue (conversion of E_1 to E_2), there is aromatase which converts Δ4A to E_1 and testosterone (T) to E_2 from adipose tissue and skin fibroblasts, the main source of Δ4A in OVX rats being the adrenal glands. Moreover, there are other substrates that need to be considered as well: dehydroepiandrosterone sulfate (DHEAS) which is converted in peripheral tissues to DHEA through a sulfatase, then to Δ4A and T and eventually to estrogens. Finally, DHEA can be converted through 17βHSD to androstenediol, which possesses intrinsic estrogenic properties [16–19]. Then, without the 2-day pretreatment used in the Purohit model, only high amounts of antisulfatase compounds were able to totally inhibit the production of estrogen from all estrogen sulfate precursors and from all steroidogenic tissues. Preliminary experiments (results not shown) revealed that our new compounds were also good inhibitors of the conversion of DHEAS to DHEA.

During the last three decades, research has been directed mainly toward the identification of aromatase inhibitors and potent, well-tolerated and highly selective aromatase inhibitors are now available for clinical use [20–23]. However, there is a growing awareness of the role that steroid sulfatase may have in regulating the formation of estrogenic steroids since the estrone sulfatase pathway as opposed to the aromatase pathway, is the major route of estrogen formation in breast tumors [24–26]. Evidence to support this hypothesis include: (i) an over thousand-fold higher steroid sulfatase activity than aromatase activity in liver and normal and malignant breast tissues [24], and (ii) the origin of E_1 from E_1S in breast cancer tissue is about 10 times more than that from Δ4A [25]. Androstenediol may be of even greater importance in the support of growth and development of hormone-dependent breast tumors since nearly 90% of androstenediol originates from DHEAS once it has been hydrolyzed to DHEA [27]. Hence, steroid sulfatase inhibitors, when used alone or in conjunction with an aromatase inhibitor, may enhance the response of hormone-dependent tumors, such as of the breast, to this type of endocrine therapy by reducing not only the formation of estrone but also the synthesis of androstenediol.

In conclusion it was shown that our in vitro–in vivo screening system allowed for the detection of promising new non-steroidal antisulfatase compounds which are devoid of estrogenic potency. Among them, TX 1299 and TX 1506 are currently under further investigations for testing in long-term models such as inhibition of induced mammary tumor in mice or rats, since those models (as well as human biological data) will remain invaluable to put the sulfatase paradigm into perspective with the other current approaches to hormone-dependent breast cancer management: estrogen antagonism at the receptor level and aromatase inhibition.

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