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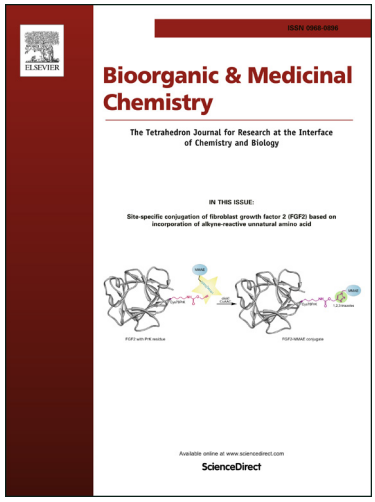

A prodrug design for improved oral absorption and reduced hepatic interaction

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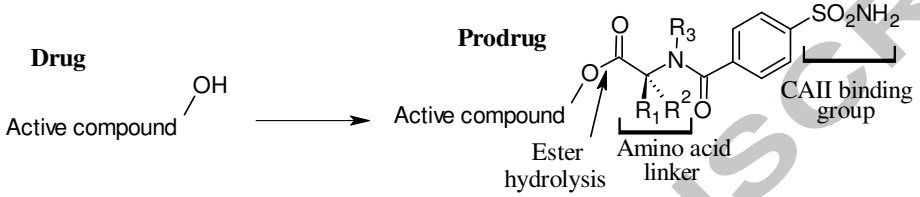

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A prodrug design for improved oral absorption and reduced hepatic interaction

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ABSTRACT

A series of estradiol-17-β esters of *N*-(*p*-sulfomylbenzamide)-amino acids were prepared and evaluated for systemic and hepatic estrogenic activity after oral administration in ovariectomized rats. The alkyl substitution at nitrogen of amino acids such as proline or *N*-methyl-alanine produced compounds that exhibit potent oral activity. The proline analog (EC508) was further evaluated along with 17β-estradiol (E2) and ethinyl-estradiol (EE) and compared their effects on the uterus, angiotensin and HDL-cholesterol after oral administration to ovariectomized female rats. Orally administered EC508 produced systemic estrogenic activity 10 times greater than EE and a 100 times higher activity than E2 with no influence on levels of angiotensin and HDL-cholesterol, whereas EE and E2 reduced the HDL-cholesterol and increased the angiotensine plasma levels. EC 508 might offer significant advantages in indications like fertility control and HRT based on its high oral bioavailability and lack of hepatic estrogenicity.

1. Introduction

There are drugs on the market with sub-optimal bioavailability or adverse effect, and there are many more compounds in clinical development that fail due to hepatic toxicity or low bioavailability[1a]. The pharmaceutical industry generally focuses on improving the characteristic of these molecules through chemical manipulation to convert them into compounds with desirable pharmaceutical properties. Some of these drugs or clinical compounds can be rescued by converting them into covalent derivatives (prodrugs) with little or no biological activity resulting in improved physicochemical properties. Upon administration, these prodrug compounds can undergo *in vivo* biochemical transformation to release the active drug before reaching their therapeutic target site[1]. All the small molecule drugs approved during 2000 to 2008 consisted of approximately 20% of prodrugs while prodrugs accounted for 33% of all drugs approved in 2010 alone[2b].

Prodrug concepts are utilized more and more in drug development in order to attain desired pharmaceutical outcomes such as improved permeability, absorption and distribution. Prodrugs can also alter solubility, metabolism, toxicity or elimination. Prodrugs can be produced by modifying diverse functional groups such as carboxylic acids, amines, sulfides and alcohols. The esters are among the common functional groups utilized in prodrug design (figure 1). These ester prodrugs usually pass through the gastrointestinal (GI) track unaffected. Once absorbed into the blood, the drugs can be released by the action of esterases[2].

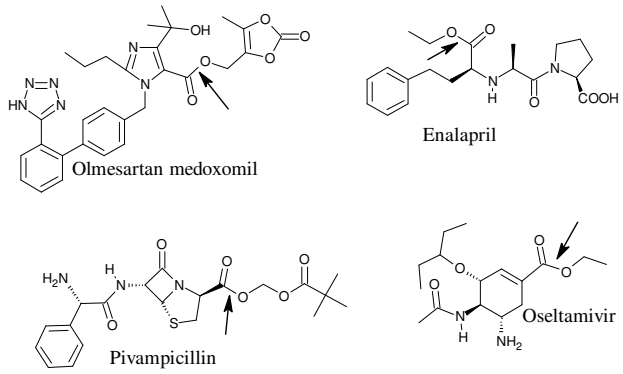

Figure 1. Examples of prodrug esters, and arrows mark the side where the ester function is hydrolyzed.

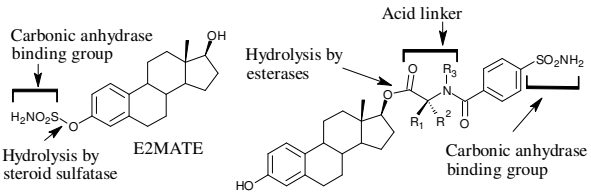

Figure 2. A sulfamate prodrug, E2MATE and a new sulfonamide-ester prodrug.

Regardless of many efforts, it can be stated that a general applicable prodrug concept which could be applied broadly in different therapeutic classes is still missing. In our search for a broadly applicable concept, we analyzed earlier work by Elger et.

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al. utilizing the sulfamate moiety[3]. The sulfamate prodrug of estradiol (E2MATE) exhibits high oral exposures which can be rationalized by their reversible binding to carbonic anhydrases (CA) located in high concentrations inside erythrocytes. The reversible binding to CA is advantageous because once the prodrug absorbs through the intestine and enters the portal vein, these molecules bind specifically to CAII inside the erythrocytes before entering the liver. This provides a shielding effect for these prodrug molecules to avoid the interaction with the hepatic enzymes or first pass effect[4]. A prodrug, E2MATE (also known as J995 or ZK190628) was developed utilizing this concept and was successful in preclinical studies (figure 2). However, it failed in the human trials due to an adverse side effect resulting from strong inhibition of human sulfatase[5], which was also required for the hydrolysis of the prodrug. It was therefore hypothesized that separating the CAII binding function from the parent molecule (modified as an ester) that could be cleaved *in vivo* to release the parent molecule might be a useful approach (figure 2 & 3)[6].

Herein, we are reporting a prodrug concept by linking an active drug estradiol (E2)[7,8] with sulfonyl-amino acids to improve bioavailability and bypass liver metabolism (figure 2).

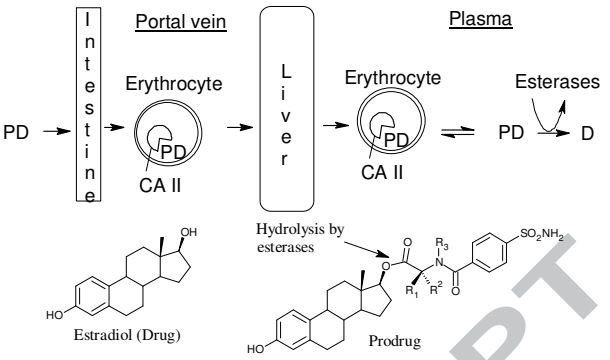
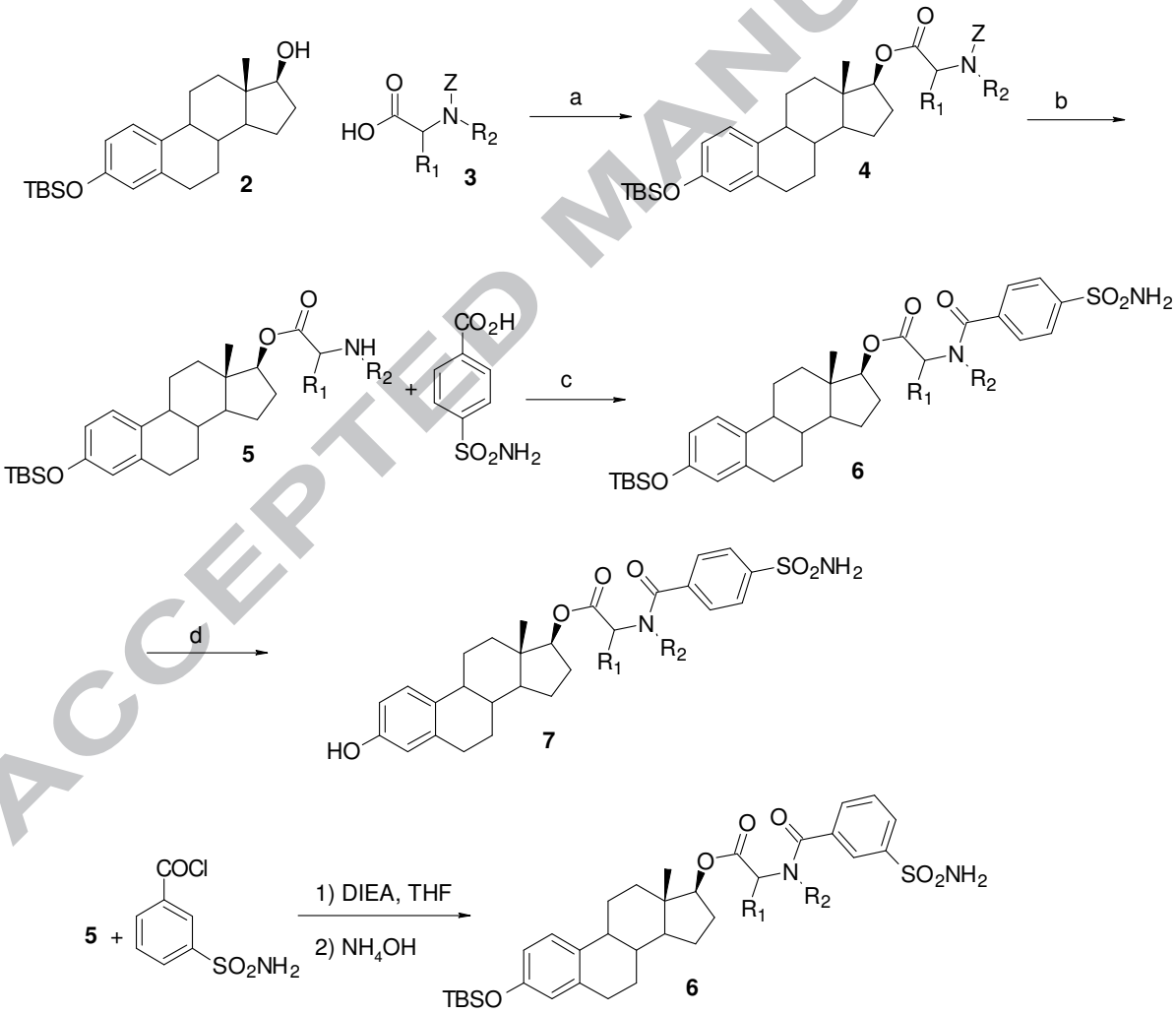


Figure 3: Schematic for sulfonamide prodrug bypassing hepatic interaction inside erythrocyte (PD= prodrug, D= drug).

2. Chemistry

Synthesis of our target molecules were carried out as detailed in Scheme 1. The appropriate *N*-protected amino acids **3a-p** were esterified via dicyclopropylcarbodiimide (DIC) or N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI) coupling with 3-*tert*-butyldimethylsilyl-estradiol **2** to afford esters **4a-p**. Subsequent deprotection of amines under

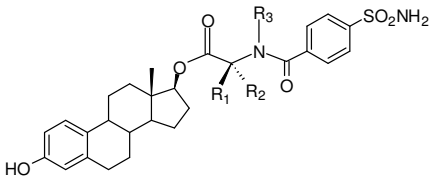


Scheme 1. Method A (Z=Cbz) reagents and conditions: (a) DIC, DMAP, DCM, rt; (b) 10% Pd/C, 30 psi H2, ethyl acetate; (c) DIC, DMAP, DCM, rt; (d) p-TsOH, DCM, acetone, MeOH, H_2O, rt or TBAF, THF, rt; or Method B (Z=BOC): (a) EDCI, DMAP, DCM, rt; (b) TFA, DCM.

usual palladium catalyzed hydrogenation for benzyloxycarbonyl or triflouroacetic acid for *tert*-butoxycarbonyl groups rendered amino-acid esters **5a-p**. 4-Sulfamoylbenzoic acid was first treated with DIC in DCM, followed by the amines **5** to afford the amides **6**. The poor solubility of the compounds containing sulfamoyl substituent in most organic solvents causes the coupling reaction to be sluggish. The removal of the silyl group **6** under standard TBAF/THF conditions worked, but we have observed cleaner reaction profiles when the deprotection was carried out with *p*-toluenesulfonic acid to afford phenols **7a-g** (Table 1).

Target molecule with m-sulfamoyl group was synthesized by treating amine **5** in THF with m-chlorosulphonylbenzoylchloride followed by ammonium hydroxide, and subsequent silyl group removal to afford the phenol **7h**.

Table 1: Compound described in Scheme 1.



Compound	R_1	R_2	R_3
7a	H	H	H
7b	Me	H	H
7c	Me	Me	H
7d	Isopropyl	H	H
7e	Benzyl	H	H
7f	Me	H	Me
7g	R_1-R_3 = propyl	H	-
7h*	R_1-R_3 = propyl	H	-

* 3-sulfamoylbenzamide

3. Results

Our hypothesis was that an ideal estrogenic prodrug molecule must bind to carbonic anhydrase (CA)[9] inside erythrocytes and consequently avoid exposure to metabolic effect of liver. Human carbonic anhydrase II (hCAII) binding affinity was evaluated for compounds **7a-t**. It was encouraging to observe hCAII inhibition in moderate ranges (IC_{50} > 50 nM to < 500 nM) as shown in Table 2 & 4. It was assumed that strong binding affinity (IC_{50} < 50 nM) towards CAII of these compounds might cause poor efficacy because of slow release of the prodrug into plasma and a weak binding compound (IC_{50} > 500 nM) might not protect these prodrugs properly inside the erythrocytes. All the tested compounds showed hCAII inhibition in the moderate range as desired with the exception of compounds **7o**, **7p** and **7s** (these compounds possessed no affinity towards CAII).

In order to generate the active drug from the prodrug once it is released into the plasma, compounds **7a-g** were subjected to *in vitro* human plasma incubation assay (ELISA) to monitor the release of drug estradiol by hydrolysis of prodrug ester molecules in the presence of plasma esterases. It is evident from the data in Table 2 that our synthesized compounds readily hydrolyze once exposed to esterases and over 80% of the parent compounds on average converted to estradiol in about 10 minutes which is primarily responsible for the efficacy. The high rate of saponification is advantageous in order to avoid any unforeseen clearance pathway of the prodrug.

Table 2: *In vitro* data for prodrug molecules

Compound	hCAII inhibition (IC_{50} nM)*	hPlasma/Esterase (% saponified, 10 min)
Acetazol-amide	309	-
7a	65	87
7b	250	70
7c	232	97
7d	202	89
7e	100	75
7f	325	84
7g	110	83
7h	300	-

* Standard deviation (SD) ranges 5 to 11

Ovariectomized female rats were treated orally with the test compounds and their uterine weight increase was reported as compared to 100% for vehicle control (Table 3). The increase in the uterine weight compared to vehicle indicates the efficacy of the test compound. The compound **7a** (glycine as amino acid linker) caused minimal increase in uterine weight while compound **7b** with alanine linker had a positive effect on the treated animals. The estrogenic effect noticeably dropped to baseline when *gem*-dimethyl-glycine was employed as linker compound **7c**. We had further explored the size of alpha substitution on glycine compound **7d** and **7e**. It is evident that isopropyl group compound **7d** is well tolerated but the benzyl **7e** has negative influence. The increase of systemic oral estrogenic activities were highest when *N*-substituted amino acids were employed. Compounds **7f** (*N*-methyl-alanine) and **7g** (proline) were the most active prodrug molecules in this series. Compound **7h** with 3-sulfamate-benzamide showed no improvement in estrogenic activity. The effect of the enantiomer of amino acids was also explored. The *S*-proline derived compound **7g** exhibited excellent estrogenic activity, while the *R*-proline derived diastereomer of the compound **7g** showed no improvement over the vehicle control (compound not shown). In our opinion, the differences in the estrogenic activities of these compounds depend on oral bio-availability, otherwise there is not much difference in their hydrolysis activity by esterases or carbonic anhydrase binding affinity.

Table 3: *In vivo* ovariectomized rat's uterine weight (po)

Compound	% uterine wt 1.0 µg dose	% uterine wt 10 µg dose
Vehicle	100	100
7a	-	152
7b	163*	271''
7c	107	97
7d	105	267
7e	95	117
7f	145	289
7g	244	340
7h	-	108

* 3 µg dose; '' 30 µg dose.

The activation of estrogen receptor by an agonist is responsible for uterine weight increases[6]. Whether the prodrug or estrogen (hydrolyzed parent drug) was causing this efficacy, estrogen receptor (ER) agonist affinity was determined for prodrug **7g** and estradiol (E2). The compound **7g** showed EC_{50} value equal to 432 nM for ER while estradiol had a value of 2.3 nM (determined by Life Technologies' SelectScreen® Profiling Service). It is evident that the prodrug has poor affinity for ER, so the *in vivo* activity is due to the hydrolyzed parent drug (E2).

The effects of sulfonamide bearing moieties have been further explored with fixing the linker as proline and the results are summarized in table 4. The N-arylsulfonamide **7i** resulted in no positive effect on uterine growth when administered at 10 mg dose, while N-benzylsulfonamide **7j** exhibited an increase of 272% uterine growth. 4-Sulfomoylphenyl-acetamide **7k**, the furyl **7l** and **7m**, the pyridyl **7n** and **7o** resulted in good to excellent uterine weight increases. The sulfonamide **7p** showed good estrogenic effect without having any hCAII binding affinity, which might not reduce hepatic toxicity. The methyl-**7q** (**7g**-methyl) retained all the activity compared with **7g**. The biaryl analogs **7s**, **7r** and **7t** resulted in a wide range of activity with the para-para **7r** as most active. This exercise shows, a wide variety of sulfonamide bearing moieties can be employed without losing efficacy which can be beneficial when working with other drug targets.

In order to show the general scope of this strategy, the compound **7g** also known as EC508 was chosen for further evaluation. A side by side study of **7g** with estradiol (E2) and ethinyl-estradiol (EE) (Figure 4) in ovariectomized rats has demonstrated that at 10 µg doses **7g** caused the uterine weight to more than double, whereas under E2 treatment no effect was seen at all and only a small weight increase could be observed at this dose level for EE. These data suggest that EC 508 might be around 100 times more potent than E2 and around 10 times more potent than EE after oral treatment. The plasma of these rats was also analyzed for HDL-cholesterol levels (Figure 5) and angiotensin concentration (Figure 6) at the end of the studies. Compound **7g** did not impact in anyway at all doses on the level of HDL studied while EE and E2 clearly reduced the level of HDL indicating hepatic interaction with EE and E2. Hepatic functions were also monitored by analyzing the angiotensin concentration. Again compound **7g** exerted no influence on angiotensin production while EE and E2 showed elevated angiotensin.

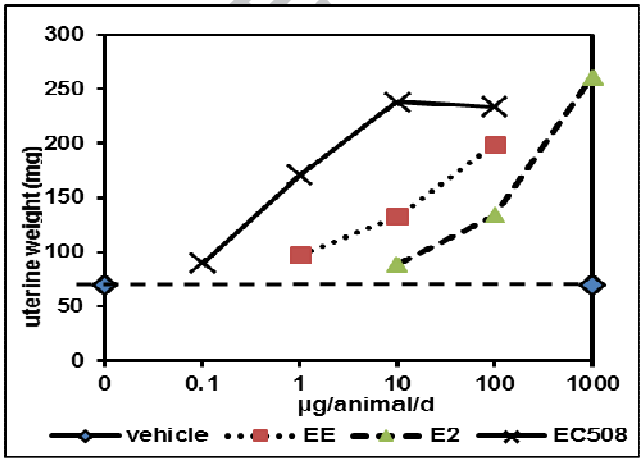
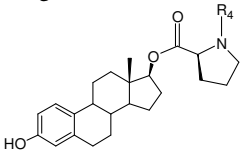
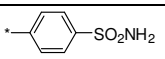
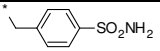
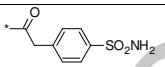
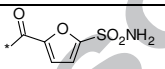
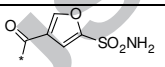
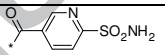
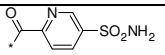
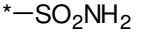
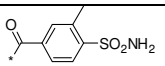
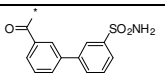
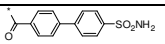
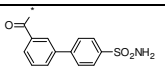


Figure 4: Comparison of estrogenic action of estradiol (E2), ethinyl estradiol (EE) and EC508.

Table 4: *In vivo* ovariectomized rat's uterine weight (po) increase of proline-linked prodrugs



Compound	R_4	% uterine wt 10 µg dose	hCAII inhibition (IC_{50} nM)
7i		92	198
7j		272	129
7k		267	500
7l		318	250
7m		313	7
7n		259	250
7o		268	5000
7p		268	10000
7q		258	210
7s		216	10000
7r		309	290
7t		153	120

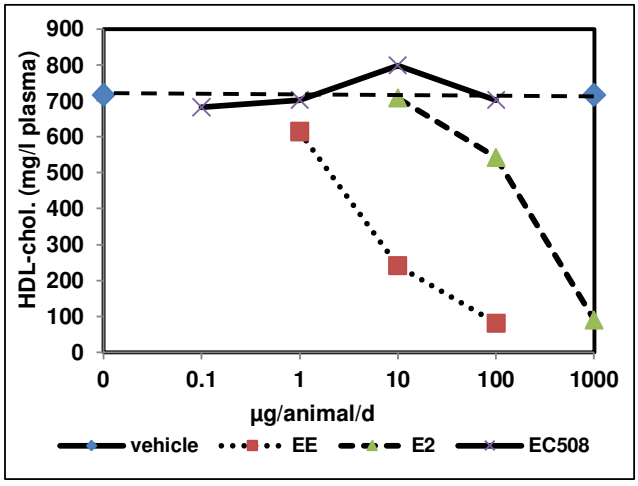


Figure 5: Effects on HDL-cholesterol levels in plasma of the studied rats.

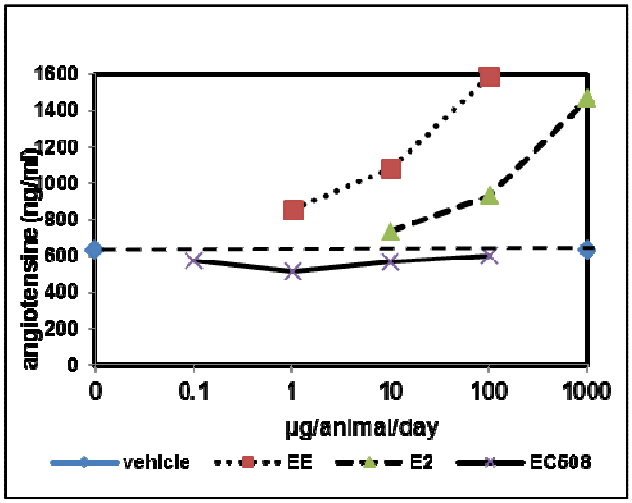


Figure 6: Angiotensin concentration in the treated rats.

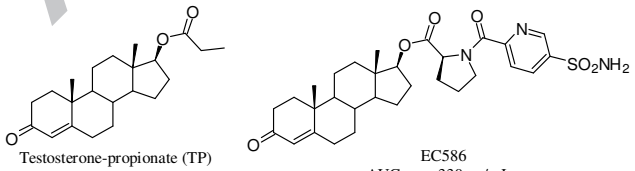
Pharmacokinetic (PK) properties of compound **7g** was evaluated in rat. We were please to find that this compound displayed excellent oral bioavailability with prolonged half-life and low in vivo clearance in blood (Table 5). We analyzed the plasma as well as blood and found 20 times more compound **7g** concentration in blood compared to plasma as was found for J995[10]. It is consistent with the argument that this compound bind to carbonic anhydrase inside the erythrocytes and avoid the metabolic action of hepatocytes. It is noteworthy to mention that the clearance of compound **7g** is high in plasma due to the fact of its saponification by the plasma esterases.

Table 5: Pharmacokinetics of EC508 dosed at 1.0 mg/kg iv and 5.0 mg/gk po Male SD rat in comparison to E2

Entry	Plasma	Blood
Cl[a] (mL/min/kg)	62.76	3.86
Vd[b] (L/kg)	5.68	0.24
$T_{1/2}$ (h)[c]	4.58	4.90
F (%)[d]	102.08	122.08

[a] In vivo clearance after iv dosing.
[b] Volume of distribution at steady state after iv dosing.
[c] Half-life after oral dosing.
[d] Bioavailability after oral dosing.

In order to test the applicability of the concept to other drug classes, a small pilot study was performed producing some prodrugs of testosterone following the same concept. One compound, EC586 (figure 7) showed a significantly enhanced serum exposure of testosterone after oral administration in the male rats compared to testosterone-propionate (TP, a marketed prodrug).



Testosterone-propionate (TP)
AUC_{0-3h} = 2.5 ng/mL
dose = 3.0 mg/rat, n = 5 rats

EC586
AUC_{0-3h} = 330 ng/mL
dose = 3.0 mg/rat, n = 5 rats

Figure 7: Examples of testosterone prodrugs with PK data.

4. Conclusion

A series of amino acid-linked benzenesulfonamide estradiol compounds was prepared. These prodrugs possess moderate affinity for carbonic anhydrase II (hCAII) which allows them to hide inside the erythrocytes and avoid interaction with the liver enzymes. Once released from erythrocytes into the plasma, they are readily hydrolyzed by esterases to produce estradiol. Optimization based on the amino acid substitutions led to EC508, an orally efficacious compound capable of producing excellent estrogenic activity, while producing no effects on plasma HDL-cholesterol and angiotensin levels. Side by side comparison studies of estradiol, ethynyl-estradiol and EC508 have shown that EC508 offered superior estrogen dependent uterine weight growth and no impact on the liver function as measured by the HDL- cholesterine and angiotensine levels.

The presented findings might have two distinct application. A new estrogenic principle that combines high oral bioavailability with reduced hepatic estrogenicity could set new safety standards in hormonal contraception and hormone replacement therapy.

In addition the concept of combining an ester linker with an aromatic sulfonamide moiety should be applicable to other drug classes that have problems with low or varying oral bioavailability and high hepatic toxicity or clearance. The testosterone prodrugs (e.g. EC586) study will be published in detail soon. The finding supports that the concept might be broadly applicable, but for each class of compounds a structural optimization has to be performed. The work is ongoing to apply this concept to other steroidal and non-steroidal drugs, and other drugs incorporating this prodrug concept will be disclosed in due time.

5. Experimental

5.1 Chemistry

Nuclear magnetic resonance spectra were recorded on a Bruker ARX (300 MHz) spectrometer as deuterochloroform ($CDCl_3$) solutions using tetramethylsilane (TMS) as an internal standard (δ = 0) unless noted otherwise. 'Flash column' chromatography was performed on 32-64 µM silica gel obtained from EM Science, Gibbstown, New Jersey. Thin-layer chromatography (TLC) analyses were carried out on silica gel GF (Analtech) glass plates (2.5 cm x 10 cm with 250 µM layer and pre-scored). Most chemicals and solvents were analytical grade and used without further purification. Commercial reagents were purchased from Aldrich Chemical Company (Milwaukee, WI).

General Method B: Boc-glycine-OH **3a** (2.0 g, 2 eq.) was treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI, 2.2 g, 2 eq.) in DCM (30 mL) for 1 hour at rt. Estradiol **2** (2.1 g, 1 eq.) and 4-dimethylaminopyridine (DMAP, 0.7 g, 1 eq) were then added and the resulting mixture was stirred at rt for 20 h. The reaction was concentrated and the residue was purified by silica gel chromatography using 60 – 100 % DCM in hexanes as eluent to afford the ester product as white solid **4a** (2.1 g, 68% yield). The ester **4a** (2.0 g) was treated with triflouroacetic acid (TFA, 6 mL) in DCM (30 mL) at rt for 24 h. After the completion of reaction, the reaction was diluted with toluene (30 mL) and triflouroacetic acid was removed under anhydrous conditions to yield the amine compound **5a** (2.1 g as TFA salt). The compound **5a** (1.0 g, 1 eq.) was then treated with p-aminosulfamoyl-benzoic acid (0.54 g, 1.5 eq.) in the presence of DIC (0.42 mL, 1.5 eq.), HOBt (0.41 g, 1.5 eq.), and Hunig's base (DIEA, 1.3 mL, 4 eq.) in DCM (20 mL) at rt for 72 h. The reaction was concentrated and the residue was purified by silica gel chromatography using 15% acetone in DCM as eluent to

afford the desired product **6a** as white solid (0.4 g, 36 % yield). The compound **6a** (0.4 g, 1 eq.) was reacted with tetrabutylammonium fluoride trihydrate (TBAF, 0.2 g, 1 eq.) in THF (20 mL) at rt for 60 min. The reaction was quenched with aq. ammonium chloride, extracted with ethyl acetate (3 x 25 mL), the combined organic was dried under sodium sulfate, filtered and concentrated. This residue was purified by silica gel chromatography using 5 % acetone in 1:1 hexanes:ethyl acetate to afford (13S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15-,16,17-decahydro-6H-cyclopenta[a]phenan-thren-17-yl 2-(4-sulfamoylbenzamido)acetate (0.15 g, 46% yield) as white solid **7a**: ^{1}H NMR (δ, DMSO-d_6 300 MHz): 9.15 (t, 1H, -NH, *J*= 5.82 Hz), 8.01 (d, 2H, ArH, *J*= 8.56 Hz), 7.91 (d, 2H, ArH, *J*= 8.54 Hz), 7.50 (bs, 1H, ArOH), 7.02 (d, 1H, ArH, *J*= 8.46 Hz), 6.49 (dd, 1H, ArH, *J*= 2.46, 8.37 Hz), 6.42 (d, 1H, ArH, *J*= 2.42 Hz), 4.67 (t, 1H, 17-CH, *J*=7.08 Hz), 4.02 (d, 2H, *J*= 5.75 Hz), 0.73 (s, 3H, CH_3). IR (cm^{-1}): 3369, 3278, 2933, 1736, 1656, 1529. $[\alpha]_D^{23}$ = +18^o (c=0.5, 1,4-dioxane).

(2S)-((13S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,-16,17-decahydro-6H-cyclopenta[a]phenan-thren-17-yl) 2-(4-sulfamoylbenzamido)propanoate (**7b**) was synthesized according to method B starting with Boc-alanine **3b**, as white solid (75 mg): ^{1}H NMR (δ, 5:1 $CDCl_3$:DMSO_d_6 300 MHz): 8.54 (s, 1H, ArOH), 8.18 (d, 1H, NH, *J*= 7.03 Hz), 7.99 (dd, 4H, ArH, *J*= 2.4, 8.88 Hz), 7.07 (d, 1H, ArH, *J*= 8.35 Hz), 6.97 (s, 2H, NH_2), 6.62 (dd, 1H, ArH, *J*= 2.60, 8.39 Hz), 6.54 (d, 1H, ArH, *J*= 2.46 Hz), 4.77 (t, 1H, 17-CH, *J*=7.99 Hz), 4.71 (t, 1H, 17-CH, *J*=7.30 Hz), 1.54 (d, 3H, CH_3, *J*= 7.29 Hz), 0.75 (s, 3H, CH_3). IR (cm^{-1})**:** 3496, 3381, 2930, 22911, 1743, 1706, 1650. $[\alpha]_D^{23}$ = +34^o (c=0.5, 1,4-dioxane).

General Method A: N-Cbz-methylalanine **3f** (2.45 g, 2 eq.) was treated with DIC (1.6 mL, 2 eq.) in DCM (33 mL) for 30 min under nitrogen at rt. TBS-Estradiol **2** (2.0 g, 1 eq.) and DMAP (0.063 g, 0.1 eq.) were then added and the resulting white mixture was stirred for 16 h rt. After filtration, the filtrate was concentrated and the residue was purified by silica gel chromatography using 5 – 40 % ethyl acetate in hexanes as eluent to afford the white solid product **4f** (2.85 g, 91% yield). The Cbz group of ester **4f** (2.85 g) was then removed using Pd/C (10% Pd, 0.51 g) and hydrogen (30 psi) on a Parr shaker in ethyl acetate (35 mL) as solvent for 16 h. After filtration through celite and concentration of the solvent, the white solid product **5f** (2.2 g, quantitative) was obtained. The amide formation of the amine **5** was carried out using p-sulfamoylbenzoic acid (2.22 g, 2.4 eq.) and DIC (1.7 mL, 2.4 eq.) in DCM (75 mL) followed by the addition of the amine **5f** (2.2 g, 1 eq.) and DMAP (56 mg, 0.1 eq.) . The reaction was not soluble initially so ethyl acetate (35 mL) was added and the reaction mixture was stirred at rt for 3 days. The reaction was filtered and the filtrate was concentrated. The residue was purified by silica gel chromatography using 10 % ethyl acetate in hexanes as eluent to afford the white solid product **6f** (1.90 g, 63% yield). The silyl ether **6f** (1.90 g) was then removed using p-toluenesulphonic acid (1.16 g, 2 eq.) in DCM (14 mL), acetone (14 mL), methanol (0.4 mL) and water (0.3 mL) at rt for 16 h. The reaction was quenched with aq. sodium bicarbonate and extracted with ethyl acetate (2 x 60 mL). The combined organic layer was dried with sodium sulfate, filtered and concentrated. The residue was purified by silica gel chromatography using 10 - 30 % acetone in DCM as eluent to afford [(13S,17S)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,-17-decahydrocyclopenta[a]phenanthren-17-yl](2S)-2-[methyl-(4-sulfamoylbenzoyl)amino]propanoate as white solid **7f** (1.47 g, 94% yield): ^{1}H NMR (δ, $CDCl_3$ 300 MHz): 7.96 (d, 2H, *J*= 8.1 Hz), 7.99 (d, 2H, *J*= 8.1 Hz), 7.13 (d, 1H, ArH, *J*= 8.1 Hz), 6.62 (dd, 1H, ArH, *J*= 2.7, 8.4 Hz), 6.54 (d, 1H, ArH, *J*= 2.7 Hz), 5.30 (s, 1H), 4.95 (s, 2H, NH_2), 4.80 (m, 1H), 3.00 (s, 1H, CH_3, rotamer), 2.90 (s, 2H, CH_3, rotamer), 0.85 (s, 2H, CH_3, rotamer), 0.83 (s, 1H, CH_3, rotamer). IR (cm^{-1}): 3353, 3257, 2924, 2862, 1731, 1617. $[\alpha]_D^{24}$ = -10^o (c=0.5, 1,4-dioxane).

[(13S,17S)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-17-yl] 2-methyl-2-[(4-sulfamoylbenzoyl)amino]propanoate was synthesized according to method A as white solid **7c** (0.61 g): ^{1}H NMR (δ, DMSO-d_6 300 MHz): 8.98 (s, 1H), 8.78 (s, 1H), 7.97 (d, 2H, ArH, *J*= 8.4 Hz), 7.89 (d, 2H, ArH, *J*= 8.1 Hz), 7.48 (s, 2H, NH_2), 7.02 (d, 1H, ArH, *J*= 8.7 Hz), 6.48 (dd, 1H, ArH, *J*= 2.4, 8.1 Hz), 6.41 (d, 1H, ArH, *J*= 2.1 Hz), 4.55 (t, 1H, *J*= 8.4 Hz), 1.47 (s, 6H, di-CH_3), 0.66 (s, 3H, CH_3). IR (cm^{-1}): 3504, 3355, 3168, 3067, 2922, 2867, 1727, 1648. $[\alpha]_D^{24}$ = +48^o (c=0.5, 1,4-dioxane).

[(13S,17S)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-17-yl] (2S)-3-methyl-2-[(4-sulfamoylbenzoyl)amino]butanoate was synthesized according to method A as white solid **7d** (1.90 g): ^{1}H NMR (δ, 5 $CDCl_3$ 300 MHz): 7.97 (d, 2H, ArH, *J*= 8.7 Hz), 7.91 (d, 2H, ArH, *J*= 8.4 Hz), 7.13 (d, 1H, ArH, *J*= 8.1 Hz), 6.81 (d, 1H, *J*= 8.4 Hz), 6.63 (dd, 1H, ArH, *J*= 2.7, 8.4 Hz), 6.56 (d, 1H, ArH, *J*= 2.1 Hz), 5.01 (s, 2H, NH_2), 4.79 (m, 3H), 1.04 (dd, 6H, *J*= 2.7, 6.9 Hz), 0.87 (s, 3H, CH_3). IR (cm^{-1}): 3338, 3252, 2967, 2924, 1722, 1710, 1657. $[\alpha]_D^{24}$ = +40^o (c=0.6, 1,4-dioxane).

[(13S,17S)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-17-yl] (2S)-3-phenyl-2-[(4-sulfamoylbenzoyl)amino]propanoate was synthesized according to method A as white solid **7e** (0.44 g): ^{1}H NMR (δ, DMSO-d_6 300 MHz): 9.04 (d, 1H, NH, *J*= 7.5 Hz), 9.00 (s, 1H, OH), 7.94 (d, 2H, ArH, *J*= 8.4 Hz), 7.89 (d, 2H, ArH, *J*= 8.1 Hz), 7.49 (s, 2H, NH_2), 7.30 (m, 5H), 7.02 (d, 1H, ArH, *J*= 8.4 Hz), 6.49 (dd, 1H, ArH, *J*= 1.8, 8.1 Hz), 6.42 (d, 1H, ArH, *J*= 1.8 Hz), 4.67 (m, 2H), 0.68 (s, 3H, CH_3). IR (cm^{-1}): 3351, 2924, 2868, 1722, 1650. $[\alpha]_D^{24}$ = +42^o (c=0.5, 1,4-dioxane).

(2S)-((13S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,-16,17-decahydro-6H-cyclopenta[a]-phenanthren-17-yl) 1-(4-sulfamoylbenzoyl)pyrrolidine-2-carboxylate was synthesized according to method A as white solid **7g** (0.51 g): ^{1}H NMR (δ, $CDCl_3$ 300 MHz): 7.96 (d, 2H, ArH, *J*= 7.25 Hz), 7.66 (d, 1.6H *rotamer*, ArH, *J*= 7.28 Hz), 7.47 (d, 0.4H *rotamer*, ArH, *J*= 7.28 Hz), 7.40 (s, 1H, ArOH), 7.10 (d, 1H, ArH, *J*= 8.43 Hz), 6.64 (d, 1H, ArH, *J*= 8.31 Hz), 6.58 (s, 1H, ArH), 6.04 (bs, 2H, NH_2), 4.81 (t, 1H, 17-CH), 4.67 (m, 1H, CH), 0.84 (s, 3H, CH_3). IR (cm^{-1}): 3378, 3218, 2925, 1738, 1615, 1498. $[\alpha]_D^{23}$ = -12^o (c=0.5, 1,4-dioxane).

Method C: The steroid **5g** (0.7 g) and diisopropylethylamine (DIEA, 1 mL) in THF (20 mL) were chilled to -50 ^{o}C, followed by the addition of m-chlorosulfonyl-benzoyl chloride (0.9 mL). The resulting yellow mixture was slowly warm to 0 ^{o}C over 40 min., and 28% ammonium hydroxide (7 mL) was then added. The resulting mixture was then warmed to rt over 30 min., the reaction was diluted with aq. ammonium chloride, extracted with ethyl acetate (3 x 25 mL), the combined organic was dried under sodium sulfate, filtered and concentrated. This residue was purified by silica gel chromatography using 15 % acetone in dichloromethane to afford the white solid **6h** (0.53 g, 67% yield). The compound **6h** (0.52 g, 1 eq.) was treated with tetrabutylammonium fluoride trihydrate (TBAF, 0.32 g, 1.2 eq.) in THF (30 mL) at rt for 60 min. The reaction was quenched with aq. ammonium chloride, extracted with ethyl acetate (3 x 25 mL), the combined organic was dried under sodium sulfate, filtered and concentrated. This residue was purified by silica gel chromatography using 5 % acetone in 1:1 hexanes:ethyl acetate

to (2S)-((13S,17S)-3-hydroxy-13-methyl-7,8,9,11,12,13,14,15,-16,17-decahydro-6H-cyclopenta[a]-phenanthren-17-yl) 1-(3-sulfamoyl-benzoyl)pyrrolidine-2-carboxylate (0.31 g, 73 % yield) as white solid **7h:**): ^{1}H NMR (δ, $CDCl_3$ 300 MHz): 8.12 (s, 1H, ArH), 7.99 (d, 1H, ArH, *J*= 7.71 Hz), 7.76 (d, 1H, ArH, *J*= 7.62 Hz), 7.57 (t, 1H, ArH, *J*= 7.7 Hz), 7.37 (s, 1H, ArOH), 7.11 (d, 1H, ArH, *J*= 8.64 Hz), 6.65 (d, 1H, ArH, *J*= 5.76 Hz), 6.58 (s, 1H, ArH), 5.85 (bs, 2H, NH_2), 4.80 (t, 1H, 17-CH, *J*=8.29 Hz), 4.66 (m, 1H), 0.84 (s, 3H, CH_3). IR (cm^{-1}): 3341, 3249, 2924, 2866, 1727, 1612, 1499**.** $[\alpha]_D^{23}$ = -16^o (c=0.5, 1,4-dioxane).

5.2. Biological Evaluation

In Vitro Studies

5.2.1 Carbonic anhydrase II (CAII) inhibition assay

The catalytic activity of human CA II was monitored by the hydrolysis of the non-physiological but commercially available ester, 4-NPA (Sigma-Aldrich, St. Louis, MO).

The assay solution (100 uL) containing 1 uM purified CA II in 50 mM, pH 7.5 MOPS, 33 mM Na2SO4, 1.0 mM EDTA buffer was dispensed into flat-bottom 96-well plate. Each compound plate was tested in triplicate. Compounds were allowed to equilibrate with the enzyme for 15 min at room temperature. On each plate, the CA II inhibitor and acetazolamide, was added in duplicate (10 uM final concentration) as a positive control (100% inhibition), and 100% DMSO was included as a negative control (0% inhibition). The reaction was initiated by addition of 10 uL of 5 mM 4-NPA solution in 10% DMSO, 90% assay buffer. The absorbance of each well was measured at 348 nm every 12 to 15 s for 5 min with SpectraMax Plus 384 microplate spectrophotometer, running SoftMax Pro software. Initial substrate concentration was 450 uM 4-NPA and final volume of 111 uL per well and 18 uM final compound concentration[11]. Each data points obtained is an average of triplicate per compound tested.

5.2.2. Esterase activity assay

The amount of estradiol (E2) released was measured by ELISA kit (Calbiotech, Spring Valley, CA; cat#ES180S) based on the principle of competitive binding between E2 in the test specimen and E2-enzyme conjugate for a constant amount of anti-estradiol conjugated polyclonal antibody. Anti-E2 coated wells were incubated with E2 standards, controls, samples and E2 enzyme conjugates. A fixed amount of HRP- labelled E2 competes with the endogenous E2 in the standard or samples. E2 peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of E2 in the specimen increases and that will be measured spectrophotometrically at 450nm. The mean absorbance value for each specimen to determine the corresponding concentration of E2 in pg/mL against the standard curve prepared from known estradiol standard solutions provided[12]. Each compound was run in duplicate and reported as a percent average.

5.2.3. In vivo uterine weight increase studies

The rat investigations were done with some modifications of the procedure described elsewhere[10]. Ovariectomized adult female Wistar rats, 2 weeks after ovariectomy were used in the experiments. The systemic estrogenicity was evaluated. Animal were treated with the test substance from day 1-3, sacrificed on day 4 of the study. The number of rats per group was 5. The weight of the uterus was measured and reported as a percentage of weight increase compared to control. Ethinyl estradiol and estradiol were used as positive controls.

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