Potent inhibitors of equine steroid isomerase EcaGST A3-3

Helena Lindström, Aslam M. A. Mazari, Yaman Musdal, Bengt Mannervik*

Department of Biochemistry and Biophysics, Arrhenius Laboratories, Stockholm University, Stockholm, Sweden

* bengt.mannervik@dbb.su.se

Abstract

Equine glutathione transferase A3-3 (EcaGST A3-3) belongs to the superfamily of detoxication enzymes found in all higher organisms. However, it is also the most efficient steroid double-bond isomerase known in mammals. *Equus ferus caballus* shares the steroidogenic pathway with *Homo sapiens*, which makes the horse a suitable animal model for investigations of human steroidogenesis. Inhibition of the enzyme has potential for treatment of steroid-hormone-dependent disorders. Screening of a library of FDA-approved drugs identified 16 out of 1040 compounds, which at 10 μM concentration afforded at least 50% inhibition of EcaGST A3-3. The most potent inhibitors, anthralin, sennoside A, tannic acid, and ethacrynic acid, were characterized by IC₅₀ values in the submicromolar range when assayed with the natural substrate Δ⁵-androstene-3,17-dione.

Introduction

Steroid hormones are involved in a plethora of physiological processes in mammals, ranging from regulation of blood pressure to reproduction [1]. Progesterone is a sex steroid hormone contributing to embryogenesis and maintaining pregnancy, it also plays an important role as an intermediate in metabolic pathways to other endogenous steroids [2]. Testosterone primarily exerts androgenic and anabolic effects in males [3]. These steroid hormones are mainly produced in testis, ovary, adrenal gland and placenta. However, some steroid hormones and their derivatives are also active in the nervous system where they are implicated in a variety of diverse physiological and pathophysiological conditions such as cognition, aggression, reproductive behavior, ageing, Alzheimer’s disease, Parkinson’s disease, and brain injury [4–6]. Steroid hormones synthesized in the brain and the nervous system are called “neurosteroids”, even though their chemical structures are identical to those of the cognate compounds produced in other tissues.

The series of steroid biosynthesis reactions is catalyzed by a variety of enzymes including members of the cytochrome P450 superfamily as well as multiple isoforms of 3β-hydroxysteroid dehydrogenase (3βHSD) and 17β-hydroxysteroid dehydrogenase (17βHSD) [7]. Formation of Δ⁴-pregene-3,20-dione (Δ⁴-PD, progesterone) and the proximate precursor of testosterone, Δ⁴-androstene-3,17-dione (Δ⁴-AD), are catalyzed by 3βHSD. This obligatory step includes an alcohol dehydrogenation followed by a Δ²-Δ⁴ double-bond isomerization. In *Homo sapiens* and *Equus ferus caballus*, the isomerization reaction has been shown to be even...
more efficiently catalyzed by another enzyme, GST A3-3, belonging to the alpha class of the glutathione transferase (GST, EC 2.5.1.18) superfamily (Fig 1). A porcine glutathione transferase has also been demonstrated to catalyze this reaction, albeit with lower efficiency [8–10].

Metabolites synthesized downstream of the GST-catalyzed isomerization are steroid hormones and neurosteroids with crucial functions in reproduction and in various aspects of well-being, and it is essential that they are kept at the adequate physiological concentrations. Overproduction of Δ⁴-AD and Δ⁴-PD under pathophysiological conditions could be suppressed by pharmacological intervention using GST inhibitors [11]. In the search for novel inhibitors we have screened a library of 1040 compounds, FDA-approved for various purposes.

Another important aspect of our investigation is the possible occurrence of unsuspected inhibitory side effects on steroid hormone production that may occur when the FDA-approved drugs are used for other pharmacological targets. The results of our present investigation could accordingly facilitate prediction of such adverse side effects.

**Materials and methods**

**Materials**

Δ⁵-AD was obtained from Steraloids Inc. (Newport, RI). The US Drug collection consisting of a set of 1040 FDA-approved compounds dissolved in DMSO was purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT). All other chemicals were purchased from Sigma-Aldrich and Merck.

EcaGST A3-3 [10], HsaGST A3-3 [12] and HsaGST M2-2 [13] were cloned, expressed and purified as described previously. Protein concentration was determined by means of the Bradford assay [14].

**Enzyme inhibition assays**

Δ⁵-AD was dissolved in methanol, CDNB in ethanol, and the compounds from the US Drug collection in DMSO. The final solvent concentration in the reaction system was kept at 5% (v/v) as a maximum.
The conjugation of GSH with the electrophilic substrate CDNB was used as a biochemical assay for general activity measurements. Screening of the US Drug library for inhibition was done in triplicates in 96-well plates in a Thermo Scientific Multiskan GO spectrophotometer. The conjugation activity at 30°C was monitored at 340 nm for 1 min in 0.1 M sodium phosphate buffer at pH 6.5 and 10 μM inhibitor concentration in a final volume of 300 μL. Initial concentrations of GSH and CDNB were 1 mM.

To obtain IC<sub>50</sub> values of the most potent inhibitors identified in the screening, enzymatic activity with CDNB as well as with Δ<sup>5</sup>-androstene-3,17-dione (Δ<sup>5</sup>-AD) was monitored spectrophotometrically with a series of inhibitor concentrations. Measurements with Δ<sup>5</sup>-pregnene-3,20-dione (Δ<sup>5</sup>-PD) were not made due to solubility difficulties.

Assay conditions for inhibition measurements of enzyme activity with CDNB were the following: EcaGST A3-3 15 nM, initial concentrations of GSH and CDNB 1 mM, inhibitor concentrations varying from 0.029 μM to 15 μM, in 100 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> at pH 6.5. Assay conditions for inhibition measurements of enzyme activity with Δ<sup>5</sup>-AD were the following: EcaGST A3-3 0.8 nM, rather than the higher concentration used with CDNB, initial concentrations of GSH 1 mM and of Δ<sup>5</sup>-AD 0.1 mM, inhibitor concentrations varying from 0.0037 μM to 15 μM, in 25 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> at pH 8.0.

For determination of inhibition modality and K<sub>i</sub> values of the most potent inhibitors, ethacrynic acid concentration was kept at 0.25 μM in the reaction with CDNB and anthralin concentration was kept at 0.09 μM in the reaction with Δ<sup>5</sup>-AD. GSH concentration was kept at 1 mM while the other substrate (CDNB or Δ<sup>5</sup>-AD) concentration was varied.

The reactions were followed spectrophotometrically for 1 min at 30°C using a Shimadzu UV-2501 PC spectrophotometer (Shimadzu Inc.). All experiments included triplicate measurements in each data point.

Due to the high activity of EcaGST A3-3 with steroid substrates the enzyme had to be diluted to nanomolar concentrations. With these low concentrations, adsorption of the enzyme to the inner walls of test tubes previously caused irreproducible measurement results [15]. Sarstedt micro-tubes 1.5 ml EASY-CAP (ref. no. 72.690.550) were found to give consistent results, and were subsequently used after numerous trials with tubes from various manufacturers.

### Data analysis

Molar absorption coefficients used for calculations were ε<sub>248</sub> = 16.3 mM<sup>-1</sup> cm<sup>-1</sup> for the isomerization of Δ<sup>5</sup>-AD, and ε<sub>340</sub> = 9.6 mM<sup>-1</sup> cm<sup>-1</sup> for the conjugation of CDNB.

IC<sub>50</sub> values of the most potent inhibitors were obtained by plotting percentage remaining enzyme activity (y) versus log of at least seven inhibitor concentrations using the nonlinear regression option of the GraphPad Prism version 7 software. The default option of Hill slope = 1 was not chosen since many of the curves were better fit to the equation with a variable Hill slope:

\[
y = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + 10^{a-x/n}}
\]

where \(y_{\text{max}}\) and \(y_{\text{min}}\) denote the highest and the lowest activity, respectively, \(a = \log(\text{IC}_{50})\), \(x = \log([I])\), and \(n\) is the Hill coefficient.

The equation for competitive inhibition fitted by non-linear regression was

\[
v = \frac{V_{\text{max}}[S]}{K_{\text{M}} \left(1 + \frac{[I]}{K_i}\right) + [S]}
\]

where [S] is the substrate concentrations and [I] is the inhibitor concentration.
Each data point is given as mean and standard deviation of triplicate measurements.

Results

Screening of the US drug library for inhibition of GST activity

The chemical compound library was screened for inhibitory effects on EcaGST A3-3 activity using the universal GST substrate 1-chloro-2, 4-dinitrobenzene (CDNB). Screening at 10 μM inhibitor concentration revealed 16 compounds giving at least 50% inhibition (Fig 2) and 13 compounds giving inhibition between 30% and 50% (Table 1). Sennoside A, tannic acid, and ethacrynic acid were the strongest inhibitors yielding an inhibition of 100%.

Determination of IC50 values with two alternative substrates

IC50 values of the eleven most potent inhibitors were determined using CDNB as substrate by varying the inhibitor concentration from 0.029 μM to 15 μM. In addition, IC50 values of these inhibitors with the natural substrate Δ5-AD were determined by varying the inhibitor concentration from 0.0037 μM to 15 μM (Fig 3 and Table 2). Ethacrynic acid, hexachlorophene and tannic acid were the most potent inhibitors of EcaGST A3-3 with CDNB, yielding IC50 values of 0.18, 0.32 and 0.33 μM, respectively. Enzymatic activity with Δ5-AD was most effectively inhibited by anthralin (IC50 = 0.085 μM), sennoside A (IC50 = 0.11 μM), and tannic acid (IC50 = 0.22 μM). In general, the inhibitory effects were similar irrespective of the substrate used in the assay. However, it is noteworthy that anthralin and sennoside A were both approximately 25 times more potent when used with Δ5-AD as substrate.

**Table 1**

| Compound                      | Inhibition (%) | Structure |
|-------------------------------|----------------|-----------|
| Sennoside A                   | 100            | ![Structure](image1.png) |
| Tannic acid                   | 100            | ![Structure](image2.png) |
| Ethacrynic acid               | 100            | ![Structure](image3.png) |
| Chlorophyllide Cu-complex Na salts | 98±2.2       | ![Structure](image4.png) |

**Table 2**

| Compound                      | Inhibition (%) | Structure |
|-------------------------------|----------------|-----------|
| Anthralin                     | 96±1.8         | ![Structure](image5.png) |
| Hexachlorophene               | 96±1.1         | ![Structure](image6.png) |
| Merbromin                     | 95±18          | ![Structure](image7.png) |
| Erythrosine sodium            | 79±5.4         | ![Structure](image8.png) |
| Aurothioglucone               | 78±28          | ![Structure](image9.png) |
| Nisoldipine                   | 75±2.3         | ![Structure](image10.png) |
| Bilthionate sodium            | 64±22          | ![Structure](image11.png) |
| Clioquinol                    | 64±21          | ![Structure](image12.png) |
| Pyrithione zinc               | 64±2.5         | ![Structure](image13.png) |
| Meclrocycline sulfosalicylate | 53±12          | ![Structure](image14.png) |
| Clofazimine                   | 52±14          | ![Structure](image15.png) |
| Prynium pamoate               | 50±6.0         | ![Structure](image16.png) |

Fig 2. The most potent inhibitors (≥50% inhibition at 10 μM) from the US drug library screened with EcaGST A3-3. Enzymatic activity was measured with 1 mM GSH and 1mM CDNB with and without 10 μM inhibitor in sodium phosphate buffer at pH 6.5 and 30˚C.

https://doi.org/10.1371/journal.pone.0214160.g002
The highest IC_{50} value in Table 2 determined for EcaGST A3-3 with CDNB as substrate was that of erythrosine sodium (IC_{50} = 8.1 μM), and with Δ5-AD as substrate merbromin gave the highest value (IC_{50} = 2.5 μM).

Conspicuously, the majority of the inhibition curves for the most potent inhibitors were not hyperbolic with respect to inhibitor concentration, but showed Hill coefficients close to 2 (Fig 3). This apparent positive cooperativity was noted both with CDNB and Δ5-AD as substrate.

### Comparison of inhibitory effects on different GSTs

For understanding the effects of pharmacological interventions based on GST inhibitors, it is important to know the selectivity among the numerous GSTs. The percentage inhibition of the 11 most potent inhibitors of EcaGST A3-3 was compared to the corresponding values for HsaGST A3-3 and HsaGST M2-2, as well as for HsaGST P1-1 [12] and HsaGST S1-1 [16] investigated previously. The inhibitory effects on the equine and the human GST A3-3 were similar, such that they both differ from the effects of some inhibitors on human GSTs M2-2, P1-1, and S1-1 (Table 3). For example, 10 μM ethacrynic acid gave 94–100% inhibition of EcaGST A3-3 and HsaGST A3-3, but had low or negligible effects on GSTs P1-1, S1-1, and M2-2. All values were determined with CDNB, which is a common substrate for all the enzymes.

A more stringent analysis was based on IC_{50} values. Table 4 summarizes IC_{50} data determined with the most potent EcaGST A3-3 inhibitors and the values obtained for the other GSTs. In addition, the IC_{50} values of the most potent inhibitors of GSTs M2-2 and S1-1 are listed. The latter inhibitors have negligible effect on equine and human GST A3-3. The most potent inhibitors of HsaGST A3-3 with CDNB were hexachlorophene, tannic acid and chlorophyllide Cu-complex Na salt with the IC_{50} values 0.16 μM, 0.21 μM and 0.33 μM, respectively, values similar to those obtained for EcaGST A3-3.

The strongest inhibitors of the enzymatic activity of HsaGST M2-2 with CDNB were sulfasalazine (IC_{50} = 0.30 μM), suramin (IC_{50} = 0.62 μM), and hydroxyzine pamoate (IC_{50} = 0.76 μM). These compounds did not cause inhibition above 30% of EcaGST A3-3. The lowest IC_{50} values for HsaGST S1-1 were 0.18 μM and 0.3 μM for erythrosine sodium and suramin, respectively.

### Table 1. Compounds from the US drug library screened with EcaGST A3-3 giving 30% to 50% inhibition. Enzymatic activity was measured with 1 mM GSH and 1mM CDNB with and without 10 μM inhibitor in sodium phosphate buffer at pH 6.5 and 30˚C.

| Compound                  | Inhibition (%) |
|---------------------------|----------------|
| Sanguinarine sulfate      | 47±19          |
| Metaproterenol            | 42±24          |
| Exemestane                | 41±12          |
| Chloramphenicol palmitate | 40±1           |
| Estropipate               | 37±23          |
| Clarithromycin            | 36±7           |
| Mebendazole               | 35±15          |
| Nalbuphine hydrochloride  | 35±11          |
| Lovastatin                | 33±15          |
| Aminolevulinic acid       | 32±11          |
| Hydrochloride             | 31±17          |
| Alverine citrate          | 31±24          |
| Fluvastatin sodium        | 31±24          |
| Potassium p-aminobenzoate | 30±16          |

https://doi.org/10.1371/journal.pone.0214160.t001
Potent inhibitors of equine steroid isomerase EcaGST A3-3

Anthralin androstenedione

Log [anthralin], μM

%age remaining activity

Sennoside A androstenedione

Log [sennoside A], μM

%age remaining activity

Tannic acid androstenedione

Log [tannic acid], μM

%age remaining activity

Ethacrynic acid androstenedione

Log [ethacrynic acid], μM

%age remaining activity

Anthralin CDNB

Log [anthralin], μM

%age remaining activity

Sennoside A CDNB

Log [sennoside A], μM

%age remaining activity

Tannic acid CDNB

Log [tannic acid], μM

%age remaining activity

Ethacrynic acid CDNB

Log [ethacrynic acid], μM

%age remaining activity
Determination of inhibition modalities

To evaluate inhibition modalities and $K_i$ values of the two most potent inhibitors of EcaGST A3-3, the effect of ethacrynic acid and anthralin was measured at varying concentrations of the alternative substrates CDNB and $\Delta^5$-AD, respectively. Both inhibitors exhibited competitive behavior, illustrated by the convergence at the Y-axis in the double reciprocal plots (see Fig 4). The graphs are based on the Michaelis-Menten model in the version describing linear competitive inhibition, and the equation was fitted by nonlinear regression to the entire data set obtained with and without inhibitor. Ethacrynic acid yielded a $K_i$ value of 0.14 $\mu$M and the $K_i$ of anthralin was 0.09 $\mu$M (Table 5).

Discussion

EcaGST A3-3 is the most efficient steroid double-bond isomerase known in mammals [10]. In the biosynthesis of steroid hormones, EcaGST A3-3 catalyzes the double-bond isomerization reaction from $\Delta^5$-PD to $\Delta^4$-PD as the last step in the synthesis of progesterone (i.e. $\Delta^4$-PD). Similarly, it catalyzes the transformation of $\Delta^5$-AD into $\Delta^4$-AD, where $\Delta^4$-AD is the ultimate precursor of testosterone. Among the main functions of progesterone is maintenance of pregnancy, whereas testosterone is the primary male sex hormone. In the nervous system, as in other tissues, other hormones are synthesized downstream of the steroid double-bond isomerization reaction catalyzed by EcaGST A3-3. Steroid hormones and their derivatives also play important roles as neurosteroids and are implicated in a number of physiological and pathophysiological processes [17,18]. For example, progesterone promotes myelination and dendritic growth, allopregnanolone increases hippocampal neurogenesis and estradiol regulates synaptic plasticity [19]. Thus, EcaGST A3-3 and the corresponding human HsaGST A3-3

Table 2. IC$_{50}$ values of the most potent inhibitors of EcaGST A3-3. Activities were tested with the alternative substrates CDNB (1 mM, in 100 mM sodium phosphate buffer) and $\Delta^5$-AD (0.1 mM, in 25 mM sodium phosphate buffer) in the presence of 1 mM GSH at 30˚C. Inhibitor concentrations varied between 0.029 $\mu$M and 15 $\mu$M at pH 6.5 (for CDNB) and between 0.0037 $\mu$M and 15 $\mu$M at pH 8.0 (for $\Delta^5$-AD). The four most potent inhibitors with either substrate are highlighted. With some inhibitors high absorbance or other physical factors gave anomalous values.

| Inhibitor              | IC$_{50}$ (CDNB)  | IC$_{50}$ ($\Delta^5$-AD) |
|------------------------|-------------------|--------------------------|
| Ethacrynic acid        | 0.18±0.067        | 0.25±0.10                |
| Hexachlorophene        | 0.32±0.036        | 0.44±0.22                |
| Tannic acid            | 0.33±0.018        | 0.22±0.034               |
| Aurothioglucose        | 0.85±0.12         | Anomalous curve          |
| Bithionate Na          | 1.9±0.52          | 1.6±0.31                 |
| Chlorophyllide Cu-complex Na salt | 2.1±0.27 | Anomalous curve |
| Anthralin              | 2.1±0.27          | 0.085±0.0048             |
| Merbromin              | 2.6±0.63          | 2.5±0.48                 |
| Senoside A             | 2.8±0.28          | 0.11±0.0082              |
| Erythrosine Na         | 8.1±2.4           | Anomalous curve          |
| Nisoldipine            | Anomalous curve   | Anomalous curve          |
could exert significant functions in the synthesis of neurosteroids and are potential pharmaceutical targets not only in reproductive disorders, but also in pathologies associated with the nervous system.

The US Drug collection is an assembly of 1040 diverse natural and synthetic FDA-approved compounds including chemotherapeutics, vasodilators, herbicides, diuretic, anti-inflammatory, antibacterial and antifungal agents. In the present study this set was investigated for inhibition of EcaGST A3-3. The rationale for this screening is that already approved compounds could proceed to clinical use on a faster route compared to de novo developed drug candidates. Conversely, the compounds already in use might exert undesired, not foreseen, side effects on EcaGST A3-3.

Among the tested compounds, 16 compounds inhibited EcaGST A3-3 reaction with the universal substrate CDNB by at least 50% (Fig 2). The 11 most potent inhibitors were subjected to further investigation for determination of the IC50 values with CDNB and the natural substrate Δ5-AD. In the reaction with CDNB, the four most potent of these compounds demonstrated IC50 values in the sub-micromolar range; six were in the low micromolar range and one generated an anomalous dose-response curve. With Δ5-AD, five were in the sub-micromolar range and two in low micromolar range, while four generated anomalous dose-response curves probably due to assay disturbances (Table 2). It should be noted that the strongest inhibitors were not equally potent with the alternative substrates CDNB and Δ5-AD.

Among the tested compounds, 16 compounds inhibited EcaGST A3-3 reaction with the universal substrate CDNB by at least 50% (Fig 2). The 11 most potent inhibitors were subjected to further investigation for determination of the IC50 values with CDNB and the natural substrate Δ5-AD. In the reaction with CDNB, the four most potent of these compounds demonstrated IC50 values in the sub-micromolar range; six were in the low micromolar range and one generated an anomalous dose-response curve. With Δ5-AD, five were in the sub-micromolar range and two in low micromolar range, while four generated anomalous dose-response curves probably due to assay disturbances (Table 2). It should be noted that the strongest inhibitors were not equally potent with the alternative substrates CDNB and Δ5-AD.

The well-characterized diuretic ethacrynic acid [20] was identified as a strong inhibitor of EcaGST A3-3 with both CDNB and Δ5-AD, being the most potent of all the inhibitors with CDNB. Interactions of ethacrynic acid with GSTs have been investigated previously, and the compound has been found to be both a substrate to and an inhibitor of GSTs, inhibiting several human GSTs [12,16,21–24].

Tannic acid was another of the four most potent inhibitors of activities with both CDNB and Δ5-AD. Like ethacrynic acid, this compound has been identified as a GST inhibitor in previous studies. It has been suggested to have potential for use as an anticancer agent in

| Inhibition (%) | EcaGST A3-3 | HsaGST A3-3 | HsaGST M2-2 | HsaGST P1-1 | HsaGST S1-1 |
|----------------|-------------|-------------|-------------|-------------|-------------|
| Sennoside A    | 100         | 90          | n. i.       | < 4         | 30          |
| Tannic acid    | 100         | 100         | 86          | < 4         | 100         |
| Ethacrynic acid| 100         | 94          | 0           | 25          | 32          |
| Chlorophyllide Cu-complex Na salt | 98 ±2.2 | 84          | 100         | 51          | 100         |
| Anthralom      | 96 ±1.8     | 88          | 80          | 22          | 84          |
| Hexachlorophene| 96 ±1.1     | 89          | 98          | 34          | 61          |
| Merbromin      | 95 ±18      | 98          | 85          | 44          | 85          |
| Erythrosine Na | 79 ±5.4     | 80          | 27          | < 4         | 100         |
| Aurothioglucone| 78 ±28      | 96          | 85          | < 4         | 24          |
| Nisoldipene    | 75 ±2.3     | 70          | 35          | 14          | 42          |
| Bithionate Na  | 64 ± 22     | 47          | 73          | 10          | 47          |

* [12]  
* [16]  
n. i. = inhibition lower than 30%,  

https://doi.org/10.1371/journal.pone.0214160.t003
Table 4. Comparison of IC_{50} values of the most potent inhibitors of EcaGST A3-3, HsaGST A3-3 and HsaGST M2-2 with published values for HsaGST P1-1 and HsaGST S1-1. Data are based on CDNB activities with GSH and CDNB concentrations constant at 1 mM in 0.1 M sodium phosphate buffer at pH 6.5 and 30°C.

| IC_{50} (μM) | EcaGST A3-3 | HsaGST A3-3 | HsaGST M2-2 | HsaGST P1-1 | HsaGST S1-1 |
|----------------|-------------|-------------|-------------|-------------|-------------|
| Ethacrynic acid | 0.18±0.067  | 0.44        | -           | 4.9         | 44          |
| Hexachlorophene | 0.32±0.036  | 0.164       | 1.5         | 9.7         | 8.9         |
| Tannic acid    | 0.33±0.018  | 0.21        | 1.2         | -           | 0.35        |
| Aurothioglucose| 0.85±0.12   | 0.91        | 12          | -           | -           |
| Bithionate Na  | 1.9±0.52    | 0.74        | -           | -           | 12          |
| Chlorophyllide Cu-complex Na salt | 2.1±0.27 | 0.334 | - | 2.3 | 1.7 |
| Anthralin      | 2.1±0.27    | 0.72        | -           | -           | 24          |
| Merbromin      | 2.6±0.63    | 0.664       | 4.9         | 3.1         | 3.3         |
| Sennoside A    | 2.8±0.28    | 0.98        | -           | -           | 39          |
| Erythrosine Na | 8.1±2.4     | 2.2         | -           | -           | 0.18        |
| Nisoldipine    | -           | 4.1         | -           | -           | 29          |
| Sulfasalazine  | -           | -           | 0.30        | -           | -           |
| Suramin        | -           | -           | 0.62        | -           | 0.3         |
| Hydroxyzine pamoate | -     | -        | 0.76        | -           | -           |
| Pyrvinium pamoate | -     | -        | 1.2         | -           | 17          |
| Pyrantel pamoate | -         | -         | 1.4         | -           | -           |
| Oxantel pamoate | -           | -           | 1.7         | -           | -           |
| Benzoypas      | -           | -           | 2.5         | -           | -           |
| Piroglinizone  | -           | -           | 5.9         | -           | -           |
| Sulindac       | -           | -           | 23          | -           | -           |

\(^a\)\[12\]  
\(^b\)\[16\]

- = low inhibition in the initial screening of the library, no IC_{50} value determined.
* = anomalous curve.

https://doi.org/10.1371/journal.pone.0214160.t004

Fig 4. Substrate-saturation curves (A) and double reciprocal plot (B) of the most potent inhibitor anthralin with EcaGST A3-3 at varied concentrations of Δ^5-AD. Data points are represented as means and standard deviations (SD) of triplicate measurements. The reaction was followed spectrophotometrically for 1 min at 1 mM GSH in the absence (blue) and presence (red) of 0.09 μM anthralin.

https://doi.org/10.1371/journal.pone.0214160.g004
treatment of cholangiocarcinoma [25] and to have a role as scavenger of active carcinogen metabolites, as well as to modulate the enzymes involved in the activation of xenobiotics and/or detoxication pathways [26].

The two strongest inhibitors with Δ5-AD were anthralin and sennoside A (Table 2). Anthralin is an antipsoriatic [27] and sennoside A is used as a cathartic [28]. These compounds have not been subjected to extensive studies involving GSTs and have not been detected as strong GST inhibitors previously.

The inhibition profiles obtained with the two alternative substrates exhibit similarities. Two of the four most potent inhibitors with either substrate are the same: ethacrynic acid and tannic acid. Interestingly, however, the two top inhibitors of Δ5-AD, anthralin and sennoside A, are found in the lower half of Table 2, where the inhibitors are listed in decreasing potency of CDNB inhibition.

The reason for the high affinity of sennoside A and anthralin can be sought in their structural similarities with the steroid substrates (Figs 1 and 2). Anthralin constitutes a part of sennoside A, and the anthracene portion of both compounds have a resemblance to Δ5-AD and Δ5-PD. This structural resemblance is found also in many other potent compounds (Fig 2). What distinguishes anthralin and sennoside A from the rest of the compounds is the presence of the adjacent ketone- and hydroxy-groups on the anthracene structure. Possibly, these functional groups are favorably positioned to interact with polar elements in the hydrophobic H-site of EcaGST A3-3. The low IC50 value of anthralin might also be partly due to entropic effects. The rigid molecule has no rotational energy, lowering the entropy loss upon formation of the EcaGST A3-3–anthralin complex and contributing to a lower dissociation constant [29].

In general, it cannot be taken for granted that enzyme inhibitors are exerting their action by binding to the active site. Even competitive inhibition can be accomplished by allosteric binding, provided that the inhibitor induces a conformational change of the enzyme that prevents the binding of the substrate. Given the structural similarity of the inhibitors to the steroid substrate, however, the competitive binding likely involves the active site in the present investigation.

Dose-response curves of the most potent inhibitors of EcaGST A3-3 with Δ5-AD are shown in Fig 3, with the corresponding curves for the reaction with CDNB for comparison. A more thorough examination of the curves reveals Hill coefficients near 2 for all inhibitors shown except that of ethacrynic acid with Δ5-AD as substrate (Fig 3). One explanation for the corresponding steep curves in the semi-logarithmic plots could be Cooperativity between the subunits of the homodimeric enzyme. Similarly, in the case of HsaGST P1-1 the presence of positive as well as negative cooperativity has been unveiled with certain substrates and inhibitors [30–34].

Originally, the subunits of soluble GSTs were found to be kinetically independent in studies based on inhibition of homo- and hetero-dimeric GSTs and measurements of their activity with various substrates [35,36]. Apparently, the experimental conditions, including the choice of substrates and inhibitors, determine whether cooperativity is displayed. A detailed investigation of the Alpha class GST A1-1, homologous to GST A3-3, demonstrated that the substrate used in the assay determined whether the enzyme displays half-of-the-sites or all-of-the-sites

Table 5. Steady-state kinetic parameters of the most potent inhibitors of EcaGST A3-3.

| Inhibitor     | Substrate | K_m (μM) | K_i (μM) | Inhibition modality |
|---------------|-----------|----------|----------|---------------------|
| Ethacrynic acid | CDNB      | 1260 ± 82 | 0.141 ± 0.0071 | Competitive          |
| Anthralin     | Δ5-AD     | 10.8 ± 0.46 | 0.0930 ± 0.0078 | Competitive          |
reactivity [37]. Apparently, the ligand bound to the enzyme governs the display of cooperativity. In inhibition studies of GST P1-1 involving ethacrynic acid unusual kinetic behavior was induced [33], and the effect of ethacrynic acid observed in the present study warrants further examination. However, this aspect is beyond the scope of this paper. Nevertheless, it should be emphasized that Hill coefficients were near 1.0 for the less potent inhibitors in the present investigation.

Inhibition data of EcaGST A3-3 and four human enzymes with CDNB are compared in Table 4. Many of the most potent inhibitors of EcaGST A3-3 are also potent with the other GSTs. Apart from this similarity is the striking difference of HsaGST M2-2 from the other enzymes. The most potent inhibitors of HsaGST M2-2 are not found among the strong inhibitors of other enzymes (with the exceptions of suramin and pyrvinium pamoate with HsaGST S1-1). A closer inspection of the molecular structures of the most potent HsaGST M2-2 inhibitors reveals that the structures do not resemble steroid hormones. Instead, they exhibit similarity to o-quinones, GST M2-2 substrates derived from dopamine and dopa [38,39], suggesting that the inhibitors act through competitive binding at the H-site, also in the case of HsaGST M2-2.

The inhibition profile of EcaGST A3-3 acquired from our measurements with its natural steroid substrates reveals that some of the substances in clinical use may exert side effects on equine steroid biosynthesis and thus might affect reproductive and other functions in the horse. Although measurements were not made with Δ⁵-PD in the current investigation, it should appear obvious that Δ⁵-PD double-bond isomerization is inhibited in an analogous manner as the Δ⁵-AD isomerization. Similarities between EcaGST A3-3 and HsaGST A3-3 inhibition profiles suggest that these side effects might also affect human steroid biosynthesis in a comparable manner. Because Homo sapiens and Equus ferus caballus share the Δ⁵-steroidogenic pathway for testosterone biosynthesis, whereas rodents use the Δ⁴-steroidogenic pathway [40], the horse appears to serve as a better animal model than rodents for investigations of therapeutic applications of GST A3-3 inhibitors.

Conclusions
We have identified FDA-approved compounds already in clinical and other use as potent submicromolar-range inhibitors of EcaGST A3-3. Given the important role this enzyme appears to play in steroidogenesis, it can be a potential pharmaceutical target in treatment of disorders in the reproductive and nervous systems. Conversely, the identification of potent inhibitors may explain or predict possible undesired side effects on steroidogenesis exerted by the named compounds when they are administered for other purposes.

Acknowledgments
We thank Professor Nancy Ing, Texas A & M, for initiating the research on EcaGST A3-3 and Dr. Birgitta Sjödin for valuable discussions.

Author Contributions
Conceptualization: Bengt Mannervik.
Formal analysis: Helena Lindström, Aslam M. A. Mazari, Yaman Musdal.
Funding acquisition: Bengt Mannervik.
Investigation: Helena Lindström, Aslam M. A. Mazari, Yaman Musdal.
Methodology: Bengt Mannervik.
**Project administration:** Helena Lindström, Aslam M. A. Mazari, Yaman Musdal.  
**Supervision:** Bengt Mannervik.  
**Validation:** Helena Lindström, Aslam M. A. Mazari, Yaman Musdal, Bengt Mannervik.  
**Visualization:** Helena Lindström.  
**Writing – original draft:** Helena Lindström, Bengt Mannervik.  
**Writing – review & editing:** Helena Lindström, Bengt Mannervik.  

**References**

1. Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, et al. International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev. 2006 Dec 1; 58 (4):782–97. https://doi.org/10.1124/pr.58.4.9 PMID: 17132855  
2. Chatuphonprasert W, Jarukamjorn K, Ellinger I. Physiology and pathophysiology of steroid biosynthesis, transport and metabolism in the human placenta. Front Pharmacol. 2018; 9:1027. https://doi.org/10.3389/fphar.2018.01027 PMID: 30258364  
3. Lee OD, Tillman K. An Overview of testosterone therapy. Am J Mens Health. 2016 Jan 1; 10(1):68–72. https://doi.org/10.1177/1557988314556671 PMID: 25398416  
4. Perrin JS, Hervé P-Y, Leonard G, Perron M, Pike GB, Pitiot A, et al. Growth of white matter in the adolescent brain: role of testosterone and androgen receptor. J Neurosci. 2008 Sep 17; 28(36):9519–24. https://doi.org/10.1523/JNEUROSCI.1212-08.2008 PMID: 18799693  
5. Reddy DS. Neurosteroids: endogenous role in the human brain and therapeutic potentials. Prog Brain Res. 2010; 186:113–37. https://doi.org/10.1016/B978-0-444-53630-3.00008-7 PMID: 21094889  
6. Liu A, Margail I, Zhang S, Labombarda F, Coquera B, Delespierre B, et al. Progesterone receptors: a key for neuroprotection in experimental stroke. Endocrinology. 2012 Aug; 153(8):3747–57. https://doi.org/10.1210/en.2012-1136 PMID: 22635676  
7. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev. 2004 Dec 1; 25(6):947–70. https://doi.org/10.1210/er.2003-0030 PMID: 15583024  
8. Johansson A-S, Mannervik B. Human glutathione transferase A3-3, a highly efficient catalyst of double-bond isomerization in the biosynthetic pathway of steroid hormones. J Biol Chem. 2001 Aug 31; 276 (35):33061–5. https://doi.org/10.1074/jbc.M104536200 PMID: 11418619  
9. Fedulova N, Raffalli-Mathieu F, Mannervik B. Porcine glutathione transferase Alpha 2–2 is a human GST A3-3 analogue that catalyses steroid double-bond isomerization. Biochem J. 2010 Oct 1; 431 (1):159–67. https://doi.org/10.1042/BJ20100839 PMID: 20673251  
10. Lindström H, Peer SM, Ing NH, Mannervik B. Characterization of equine GST A3-3 as a steroid isomerase. J Steroid Biochem Mol Biol. 2018 Apr; 178:117–26. https://doi.org/10.1016/j.jsbmb.2017.11.011 PMID: 29180167  
11. Raffalli-Mathieu F, Orre C, Stridsberg M, Hansson Edalat M, Mannervik B. Targeting human glutathione transferase A3-3 attenuates progesterone production in human steroidogenic cells. Biochem J. 2008 Aug 15; 414(1):103–9. https://doi.org/10.1042/BJ20080397 PMID: 18426392  
12. Musdal Y, Hegazy UM, Aksoy Y, Mannervik B. FDA-approved drugs and other compounds tested as inhibitors of human glutathione transferase P1-1. Chem Biol Interact. 2013 Sep 5; 229:91–9. https://doi.org/10.1016/j.cbi.2013.06.003 PMID: 23799903  
13. Johansson A-S, Bolton-Grob R, Mannervik B. Use of silent mutations in cDNA encoding human glutathione transferase M2-2 for optimized expression in Escherichia coli. Protein Expr Purif. 1999 Oct 1; 17 (1):105–12. https://doi.org/10.1006/prep.1999.1117 PMID: 10497975  
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:249–54. PMID: 942051  
15. Fedulova N, Mannervik B. Experimental conditions affecting functional comparison of highly active glutathione transferases. Anal Biochem. 2011 Jun 1; 413(1):16–23. https://doi.org/10.1016/j.ab.2011.01.041 PMID: 21295006  
16. Mazari AMA, Hegazy UM, Mannervik B. Identification of new inhibitors for human hematopoietic prostaglandin D2 synthase among FDA-approved drugs and other compounds. Chem Biol Interact. 2015 Mar 5; 229:91–9. https://doi.org/10.1016/j.cbi.2015.01.014 PMID: 25603235
17. Guennoun R, Labombarda F, Gonzalez Deniselle MC, Liere P, De Nicola AF, Schumacher M. Progesterone and allopregnanolone in the central nervous system: response to injury and implication for neuroprotection. J Steroid Biochem Mol Biol. 2015 Feb 1; 146:48–61. https://doi.org/10.1016/j.jsbmb.2014.09.001 PMID: 25196185

18. Schverer M, Lanfumey L, Baulieu E-E, Froger N, Villey I. Neurosteroids: non-genomic pathways in neuroplasticity and involvement in neurological diseases. Pharmacol Ther. 2018 Nov 1; 191:190–206. https://doi.org/10.1016/j.pharmthera.2018.06.011 PMID: 29953900

19. Rossetti MF, Cambiasso MJ, Holschbach MA, Cabrera R. Oestrogens and progestagens: synthesis and action in the brain. J Neuroendocrinol. 2016 Jul 1; 28(7):n/a-n/a.

20. Molnar J, Somberg JC. The clinical pharmacology of ethacrynic acid. Am J Ther. 2009 Jan 1; 16(1):86–92. https://doi.org/10.1097/MJT.0b013e318195e460 PMID: 19142159

21. Hansson J, Berhane K, Castro VM, Junghennius U, Mannervik B, Ringborg U. Sensitization of human melanoma cells to the cytotoxic effect of melphalan by modulating drug efflux pathways. J Hepatol. 2007 Feb 1; 46(2):222–9. https://doi.org/10.1016/j.jhep.2006.08.012 PMID: 17069924

22. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem. 1974 Nov 25; 249(22):7130–9. PMID: 4436300

23. Mannervik B, Alin P, Guthenberg C, Jenson H, Tahir MK, Warholm M, et al. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci U S A. 1985 Nov; 82(21):7202–6. PMID: 3864155

24. Ploemen JHTM, Bogaards JJP, Veldink GA, van Ommeren B, Jansen DHM, van Bladeren PJ. Isoenzymic selective irreversible inhibition of rat and human glutathione S-transferases by ethacrynic acid and two brominated derivatives. Biochem Pharmacol. 1993 Feb 9; 45(3):633–9. PMID: 8442764

25. Naus PJ, Henson R, Bleeker G, Wehbe H, Meng F, Patel T. Tannic acid synergizes the cytotoxicity of chemotherapeutic drugs in human cholangiocarcinoma by modulating drug efflux pathways. J Hepatol. 2001 Apr 1; 34(4):688–92. https://doi.org/10.1016/S0168-8278(01)00052-8 PMID: 11367373

26. Krajka-Kuźniak V, Baer-Dubowska V. The effects of tannic acid on cytochrome P450 and phase II enzymes in mouse liver and kidney. Toxicol Lett. 2003 Jul 20; 143(2):209–16. PMID: 12749824

27. Peus D, Beyerle A, Pott M, Meves A, Pittelkow MR, Rittner H, et al. Anti-psoriatic drug anthralin activates JNK via lipid peroxidation: mononuclear cells are more sensitive than keratinocytes. J Invest Dermatol. 2000 Apr 1; 114(4):688–92. https://doi.org/10.1046/j.1523-1747.2000.00934.x PMID: 10733674

28. Kon R, Yamamura M, Matsunaga Y, Kimura H, Minami M, Kato S, et al. Laxative effect of repeated Daiokanzoto is attributable to decrease in aquaporin-3 expression in the colon. J Nat Med. 2018 Mar 1; 72(2):493–502. https://doi.org/10.1007/s11418-018-1174-1 PMID: 29380109

29. Fersht A. Structure and Mechanism in Protein Science: a Guide to Enzyme Catalysis and Protein Folding. p. 72. World Scientific Publishing Co. Pte. Ltd.; 2007.

30. Ricci G, Bello ML, Caccuri AM, Pastore A, Nuccetelli M, Parker MW, et al. Site-directed mutagenesis of human glutathione transferase P1-1: mutation of Cys-47 induces a positive cooperativity in glutathione transferase P1-1. J Biol Chem. 1995 Jan 20; 270(3):1243–8. PMID: 7836386

31. Bello ML, Nuccetelli M, Chiessi E, Lahm A, Mazzetti AP, Battistoni A, et al. Mutations of gly to ala in human glutathione transferase P1-1 affect helix 2 (G-site) and induce positive cooperativity in the binding of glutathione11. Huber R, editor. J Mol Biol. 1998 Dec 18; 284(5):1717–25. PMID: 9878382

32. Ricci G, Caccuri AM, Lo Bello M, Parker MW, Nuccetelli M, Tachibana P, et al. Glutathione transferase P1-1: self-preservation of an anti-cancer enzyme. Biochem J. 2003 Nov 15; 376(Pt 1):71–6. https://doi.org/10.1042/BJ20030860 PMID: 12877654

33. Hegazy UM, Musdal Y, Mannervik B. Hidden allostery in human glutathione transferase P1-1 unveiled by unnatural amino acid substitutions and inhibition studies. J Mol Biol. 2013 May 13; 425(9):1509–14. https://doi.org/10.1016/j.jmb.2013.01.038 PMID: 23399543

34. Bocedi A, Fabrini R, Bello ML, Caccuri AM, Federici G, Mannervik B, et al. Evolution of negative cooperativity in glutathione transferase enabled preservation of enzyme function. J Biol Chem. 2016 Dec 23; 291(52):26739–49. https://doi.org/10.1074/jbc.M116.749507 PMID: 27815499

35. Tahir MK, Mannervik B. Simple inhibition studies for distinction between homodimeric and heterodimeric isoenzymes of glutathione transferase. J Biol Chem. 1986 Jan 25; 261(3):1048–51. PMID: 3944080

36. Danielson UH, Mannervik B. Kinetic independence of the subunits of cytosolic glutathione transferase from the rat. Biochem J. 1985 Oct 15; 231(2):263–7. PMID: 4062896

37. Lien S, Gustafsson A, Andersson A-K, Mannervik B. Human glutathione transferase A1-1 demonstrates both half-of-the-sites and all-of-the-sites reactivity. J Biol Chem. 2001 Sep 21; 276(38):3559–605. https://doi.org/10.1074/jbc.M103789200 PMID: 11468282
38. Segura-Aguilar J, Baez S, Widersten M, Welch CJ, Mannervik B. Human class Mu glutathione transferases, in particular isoenzyme M2-2, catalyze detoxication of the dopamine metabolite aminochrome. J Biol Chem. 1997 Feb 28; 272(9):5727–31. PMID: 9038184

39. Dagnino-Subiabre A, Cassels BK, Baez S, Johansson A-S, Mannervik B, Segura-Aguilar J. Glutathione transferase M2-2 catalyzes conjugation of dopamine and dopa-o-quinones. Biochem Biophys Res Commun. 2000 Jul 21; 274(1):32–6. https://doi.org/10.1006/wwbr.2000.3087 PMID: 10903891

40. Conley AJ, Bird IM. The role of cytochrome P450 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the Δ5 and Δ4 pathways of steroidogenesis in mammals. Biol Reprod. 1997 Apr 1; 56(4):789–99. PMID: 9096858