Inhibition of ABCC5-mediated cGMP transport by progesterone, testosterone and their analogues

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ABSTRACT

The biodynamics and biokinetics of sex hormones are complex. In addition to the classical steroid receptors (nuclear receptors), these hormones act through several non-genomic mechanisms. Modulation of ABC-transporters by progesterone represents a non-genomic mechanism. In the present study, we employed inside out vesicles from human erythrocytes to characterize high affinity cGMP transport by ABCC5 (member 5 of the ATP-Binding Cassette subfamily C). Progesterone and testosterone inhibited the transport with respective K_i of 1.2 ± 0.3 and 2.0 ± 0.6 μmol/L. We used virtual ligand screening (VLS) to identify analogues to progesterone and testosterone. A large number of substances were screened *in silico* and the 19 most promising candidates were screened *in vitro*. Each substance was tested for a concentration of 10 μmol/L. The range of cGMP transport reduction was 21.5% to 86.2% for progesterone analogues and 8.6% to 93.8 % for testosterone analogues. Three of the most potent test compounds (TC) of each analogue class, in addition to progesterone and testosterone, were characterized for concentrations from 1 nanomol/L to 1 mmol/L. The progesterone analogues showed following K_i-values (μmol/L): TC-08: 0.61, TC-16: 0.66 and TC-15: 9.3. The K_i-values (μmol/L) for the testosterone analogues were: TC-18: 0.10, TC-07: 0.67 andTC-05: 2.0. The present study shows that VLS may be a versatile tool in the development of membrane transport modulating agents (MTMAs).

1. Introduction

According to the free hormone hypothesis, the lipophilic character of the steroid hormones enables the unbound fraction to enter target cells by passive diffusion. However, it has been suggested [1] that the bound fraction of steroids, and especially conjugated steroids, might utilize transmembrane SLC- carriers for cellular uptake. The SLCO transporter superfamily includes 11 human SLCOs [2]. The SLCOs have transport characteristic compatible with active transport, but the mechanisms are not known in detail, but evidence for an anion-exchange transport exists [2]. Members of the SLC22-family are also involved in cellular uptake of endogenous organic anions [3]. Both transporter affinities for substrates and cellular transporter expression are determinative for the respective specificity and tissue selectivity.

In addition to passive cellular efflux, primary active pumps such as ABC-transporters contribute to cellular uptake and efflux of steroids.

Progesterone is a substrate for ABCB1 with slow transport [4] and responsible for the reversal of multidrug resisteance (MDR) [5]. Progesterone reduced cGMP efflux from intact HEK293 cells overexpressing ABCC4 [6]. The observation that testosterone levels in some of the seminiferous tubules of the testis may reach approximately 100 times the levels in the systemic circulation [7] is consistent with active transport. ABCC1 and ABCG2 are expressed at the luminal side of testis capillaries, whereas Sertoli cells mainly express ABCB1, ABCC1 and ABCC4 [8–10]. These transporters are important for the blood testis barrier (BTB) since they protect he male reproductive system against toxic xenobiotics [11].

We have previously reported that progesterone inhibits cGMP efflux by ABCC5 [12,13] (for reference [13], see note in Addendum) with high affinity (K_i: 1.7–2.2 μmol/L). This is a non-genomic effect of progesterone, in addition to several others [14,15]. As far as we know, the interaction between testosterone and ABCC5 has not been reported

before. In the present study, analogues of progesterone and testosterone were identified with molecular modeling and virtual ligand screening (VLS), and their effects on ABCC5-mediated cGMP transport were characterized *in vitro*.

2. Materials and methods

2.1. Software

The ICM program [16], version 3.6−1, was used for homology modeling, compound docking and substructure search. The program package included the ICM VLS add-on and access to Molcart, a database of chemical structures for approximately 40000 commercially available compounds.

2.2. Homology modeling and virtual ligand screening (VLS)

A homology model of ABCC5 [17] was used for the present study. The methods employed in the s present work are described in a recent publication [18]. Energy-based torsional sampling was used to generate additional conformations of the ligand binding area of ABCC5 to investigate putative ligand binding modes in the highly flexible transporter protein. This computational technique generates more "drug gable" conformations of ligand binding pockets [19].

The putative analogues of progesterone and testosterone were identified with the ICM VLS technology, which provides excellent tools for accurate individual ligand-protein docking. Table 1 shows the compounds with a potential to modulate ABCC5 transport. The substances were synthesized and purchased from Pharmeks Interbioscreen (Institutsky Prospect, Chernogolovka, Russia).

2.3. Preparation of inside-out vesicles (IOVs) and transport assay

The isolation and lysis of human erythrocytes, vesicle formation, ghost membranes and separation of IOVs from both right-side-out vesicles and ghosts, were performed essentially as described previously [20]. The sidedness in the fraction containing IOVs was verified using acetylcholinesterase accessibility [21,22].

2.4. Transport assay

The procedure employed for transport studies has been described in detail earlier [20]. The uptake of [³H]-cGMP (2 μmol/L) to IOVs was terminated after 60 min incubation at37°. The difference between total transport (with 2 mmol/L Mg^{2+}-ATP) and non-specific transport (without 2 mmol/L Mg^{2+}-ATP) represents the specific transport of cGMP. In the inhibition studies, progesterone, testosterone and their putative analogues, in addition to sildenafil, were present in concentrations from 1 nanomol/Lto1 mmol/L.

Comparison of two different ultrafiltration methods showed virtually identical results. In the first assay, a 12-well manifold was employed [20]. The second assay involved 96-well incubation-plates and manifolds. [³H]-cGMP, Mg^{2+}-ATP, steroid analogues, potassium- phosphate buffered saline (pH 8.1) and IOVs were added to the wells with the plates placed on wet ice (0−4°). After centrifugation for 30 s at 200 *g* (Omnifuge, 2.0 RS, Heraeus Sepatech, Germany), the plates were incubated for 60 min in a water bath at 37°. The transport was terminated by placing the plates on wet ice. Prior to the filtration, the filter paper (nitrocellulose membrane sheet 0.2 μm, Bio-Rad Laboratories, Germany) was soaked in potassium-phosphate buffered saline before assembly of the manifold. After filtration, the filters were washed with ice-cold potassium-phosphate buffered saline and dried for 20 min at 30°. Each well with the dried filter, was added 30 μL scintillation fluid (MicroScint, Perkin Elmer, Waltham, MA, USA). The radioactivity was quantified by scintillation counting using a Packard TopCount NXT (Packard, Downers Grove, IL, USA).

Table 1

Putative ABCC5 inhibitors (TC-01- TC-19) from the VLS procedure that were ordered from Interbioscreen (Institutsky Prospect, 7a, 142432 Chernogolovka, Russia), with test compound code, IUPAC name.

Test compound code (TC)	Product code	IUPAC name
TC-01	#423	2-((8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((furan-2-ylmethyl)amino)-4-oxobu tanoate
TC-02	#424	2-(4-(2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)-3-methylpentanoic acid
TC-03	#425	2-((8R,9S,10R,13S,14S,17R)-17-acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl4-((2-methoxy-2-oxo-1-phenylethyl)amino)-4-oxobutanoate
TC-04	#426	2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((2-methoxy-2-oxo-1-phenylethyl)amino)-4-oxobutanoate
TC-05	#427	2-((8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((3-(4-hydroxyphenyl)-1-methoxy-1-oxopro pan-2-yl)amino)-4-oxobutanoate
TC-06	#428	5-(4-(2-((8R,9S,10R,13S,14S,17R)-17-acetoxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)pentanoic acid
TC-07	#429	(8R,9S,10R,13S,14S,17S)-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl4-((3,4-dihydroxyphenethyl)amino)-4-oxobutanoate
TC-08	#430	2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((4-fluorophenethyl)amino)-4-oxobutanoate
TC-09	#431	2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((2-methoxy-2-oxo-1-phenylethyl)amino)-4-o xobutanoate
TC-10	#432	4-(4-(2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)-3-phenylbutanoic ac id
TC-11	#433	2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl-4-oxo-4-((pyridin-4-ylmethyl)amino)butanoate
TC-12	#434	4-((4-(2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)methyl)benzoic acid
TC-13	#435	

2

Table 1 (*continued*)

Test compound code (TC)	Product code	IUPAC name
		2-(2-(4-(2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)acetamido)-3-(1H-indol-3-yl)propanoic acid
TC-14	#436	2-(2-(4-(2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)acetamido)-3-(1H-indol-3-yl)propanoic acid
TC-15	#437	2-((8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl-4-oxo-4-((1-phenylethyl)amino)butanoate
TC-16	#438	2-((8S,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3,11-dioxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 4-((3-(1H-indol-3-yl)-1-methoxy-1-oxopropan-2-yl)amino)-4-oxobutanoate
TC-17	#439	4-((4-(2-((8R,9S,10R,13S,14S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)methyl)benzoic acid
TC-18	#440	2-(2-(4-(((8R,9S,10R,13S,14S)-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)oxy)-4-oxobutanamido)acetamido)-3-(1H-indol-3-yl)propanoic acid
TC-19	#441	2-(4-(2-((8R,10R,13S,17R)-17-hydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-4-oxobutanamido)-3-(5-hydroxy-1H-indol-3-yl)propanoic acid

2.5. Analysis of experimental data

IC_{50}-values were obtained according to Chou [23] and K_i-values calculated according to Cheng and Prusoff [24], using substrate concentration (2 μmol/L cGMP) and the K_m-value (2.7 μmol/L) for cGMP, obtained under similar experimental conditions [20].

3. Results

3.1. Virtual ligand screening (VLS)

The *in silico* screening identified 19 putative analogues (Table 1). Some of these substances were characterized as analogues to progesterone and some to testosterone (Table 2). In the present study, the docking score (ICM) obtained for the interaction between the analogues and the ABCC5 binding site, ranged from -33.57 to -38.35. The drug likeness ranged from -0.0377 to 1.4982.

3.2. Pharmacological screening

The *in vitro* screening was performed with the 19 compounds shown in Table 2. Their ability to inhibit high affinity [3H]-cGMP and ATP-dependent transport, was tested with 10 μmol/L analogue. Table 2 shows that the reduction was 21.5–86.2 % for progesterone analogues and 8.6–93.8 % for testosterone analogues. The most potent inhibitors (n = 6) showed orders of potency for the progesterone analogues: TC-08 ≥ TC-16 > TC-15 and for the testosterone analogues: TC-07 > TC-18 >

Table 2

Progesterone (P) and testosterone (T)analogues – VLS-results on drug likeness, docking (ICM-score) and screening of test compounds (TC) 1 – 19 for their ability (in a concentration of 10 μM) to inhibit ATP-dependent [3H]-cGMP uptake to hRBC IOVs. Three time independent series in duplicate (Mean $\pm$ SD).

Test compound codes	Drug likeness	ICM-score	Analogue to	Transport (% of control)
TC-01 (#423)	1.26268	−34.0394	P	82.5 ± 6.5
TC-08 (#430)	1.08898	35.5277	P	21.5 ± 6.6 *
TC-09 (#431)	1.37145	−35.7915	P	43.1 ± 7.6
TC-10 (#432)	1.29281	−34.5299	P	86.2 ± 5.9
TC-11 (#433)	1.43892	−34.7817	P	77.2 ± 5.3
TC-12 (#434)	1.29676	−35.4267	P	76.7 ± 4.9
TC-13 (#435)	0.25981	−35.9009	P	75.7 ± 11.7
TC-14 (#436)	1.02529	−37.4281	P	56.5 ± 5.4
TC-15 (#437)	1.49841	−38.3509	P	41.0 ± 5.1 *
TC-16 (#438)	0.382176	−38.7701	P	23.7 ± 4.6 *
TC-17 (#439)	1.02194	−41.3668	P	71.5 ± 5.4
TC-19 (#441)	0.424506	−39.5453	P	56.5 ± 8.1
TC-02 (#424)	1.18021	−33.277	T	79.4 ± 2.6
TC-03 (#425)	1.18277	−33.5606	T	58.9 ± 5.5
TC-04 (#426)	1.37079	−33.5668	T	45.4 ± 10.0
TC-05 (#427)	0.981743	−33.9976	T	38.3 ± 7.0 *
TC-06 (#428)	1.02383	34.1923	T	93.8 ± 7.3
TC-07 (#429)	0.981427	−34.2954	T	25.9 ± 4.8 *
TC-18 (#440)	-0.0376617	−36.5219	T	8.6 ± 2.9 *

* Analogues used for concentration effect studies.

TC-05.

3.3. Concentration-dependent inhibition

The IC_{50}-values were obtained from the concentration-dependent inhibition curves (Fig. 1) and the K_i-values calculated as described in the methods. Sildenafil was used as positive control for inhibition of cGMP efflux. The IC_{50}- and K_i- values for sildenafil, obtained in the present stud were 4.72 ± 0.39 and 2.67 ± 0.22 μmol/L, respectively. Progesterone and testosterone were employed as reference inhibitors for their respective analogues. Table 3 shows that progesterone and testosterone were nearly equipotent. The orders of inhibitory potency for progesterone and for testosterone analogues were TC-08 ≥ TC-16 > > TC-15 and TC-07 > TC-18 > TC-05, respectively.

4. Discussion

The present study has focused on the inhibition of ABC5-mediated transport of cGMP by progesterone, testosterone and their analogues. ABCC5, which is ubiquitously expressed, and localized to the basolateral membrane of some polarized epithelial cells and to the apical membrane in others, such as the brain capillary endothelial cells. ABCC5, like ABCC4, is capable of dual-membrane localization [24]. The ABCC5 expression is high in some tissues (*i.e.* cardiac and smooth vascular muscles) and in some organs (*i.e.* brain and kidney) [25]. In the IOV model with human erythrocytes, the ABCC5-mediated cGMP transport was composed of one high and a second low affinity component [26]. This transport system was identified as ABCC5 [27], and verified by others [28,29]. The reduction of ABCC5 membrane concentration paralleled a marked lowering of cGMP transport in proteoliposomes [30] and in pituitary GH3 cells after silencing ABCC5 [31]. In the last mentioned study, the cAMP transport was unperturbed. The authors suggested that two pumps existed for cyclic nucleotides in pituitary cells and that that ABCC5 operated as a cGMP-selective transporter.

In traditional biochemical experiments, we showed that 100 μmol/L cAMP reduced the high affinity transport of cGMP with only 15 % [12]. In a more recent study [20], a steep fall in cGMP transport was seen for cAMP concentrations from100 to 1000 μmol/L. This gave an estimated IC_{50}-value of 695 μmol/L. We have proposed that ABCC5 has a specific
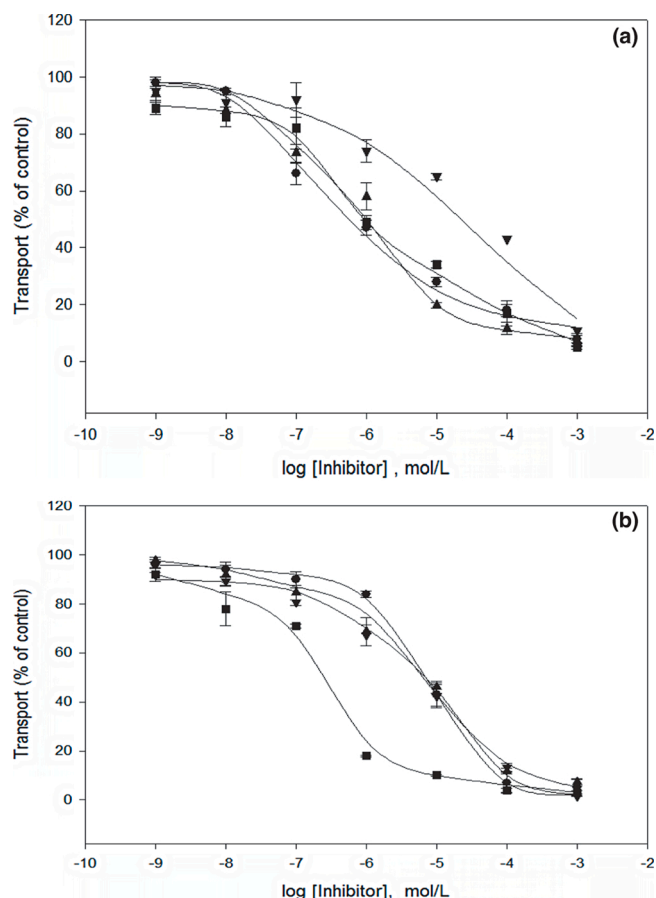
Fig. 1. Active transport of [^{3}H]-cGMP in presence of progesterone and analogues (panel A) and testosterone and analogues (Panel B). The results are presented as mean ± SD (n = 3). Panel A: Progesterone (●), TC-08 / #430 (▲), TC-15 (▼), TC-16 (■). Panel B: Testosterone (●), TC-05 (▲) TC-07 (▼), TC-18 (■).

Table 3

IC$_{50}$- and K$_i$-values of progesterone analogues (P) and testosterone analogues (T) for their inhibition of ATP-dependent [^{3}H]-cGMP high affinity uptake to hRBC IOVs. Progesterone and testosterone were employed as reference substances. The numbers represent three time-independent series in duplicate (Mean ± SD).

Compound	Analogue to	IC$_{50}$ (μmol/L)	K$_i$ (μmol/L)
Progesterone	–	2.16 ±0.59	1.22 ± 0.34
TC-08 (#430)	P	1.09 ±0.09	0.61 ±0.05
TC-15(#437)	P	16.4 ± 2.63	9.28 ±1.48
TC-16 (#438)	P	1.17 ± 0.30	0.66 ± 0.17
Testosterone	–	3.50 ± 1.12	1.97 ± 0.63
TC-05 (#427)	T	3.57 ± 0.82	2.02 ± 0.46
TC-07 (#429)	T	1.19 ± 0.082	0.672 ± 0.046
TC-18 (#440)	T	0.177 ± 0.016	0.100 ± 0.009

high affinity site for cGMP and a second common nonspecific low affinity site for the two cyclic nucleotides [32].

The present study of specific high affinity cGMP transport, showed similar progesterone K$_i$-value as those reported before with the IOV model [12,13]. Progesterone modulation of ABCC5 transport extends the list of non-genomic effects [15]. Two of the analogues (TC-08 and TC-16) were clearly more potent than progesterone whereas the third (TC-15) was much less potent. The present results extend our experience with VLS as a valuable tool for predicting putative drugs such as analogues to sildenafil [17] and vardenafil [18].

Modern male reproductive physiology (androgenesis and physiology) started with the discovery and synthesis of testosterone [33].

However, the impact of testosterone on clinical condition like metabolic syndrome and type-2 diabetes has received much attention in the recent years [34]. Furthermore, the role of testosterone for female health, bone and muscle physiology, mood disorders and cognitive health have also come into focus [35].

Testosterone inhibited ABCC5 transport with a similar K$_i$-value as progesterone. As far as we know, no previous report of ABCC5-mediated transport of testosterone exists. It is likely that testosterone is a slow moving substrate for ABCC5 as progesterone is for ABCB1 [4]. ABCC5 has low expression in human specialized testicular tissues [25], but is present in the testicular vascular smooth muscle cells.

The effect of sildenafil on erectile dysfunction was initially only ascribed to the inhibition of PDE5 [36]. However, the observation that sildenafil inhibited ABCC5-mediated cGMP efflux [27], made the cellular pharmacodynamics of sildenafil more complex. Since ABCC5 is highly expressed in corpus cavernous [37], testosterone (K$_i$ ≈ 2.0 μmol/L) may have a sildenafil-like effect (K$_i$ ≈ 2.7 μmol/L) on cellular extrusion of cGMP. With a focus on this non-genome action, the two testosterone analogues TC-07 (K$_i$ ≈ 0.7 μmol/L) and TC-18 (K$_i$ ≈ 0.1 μmol/L), would be markedly more potent than sildenafil. Progesterone, testosterone and their analogues should also be tested for their ability to inhibit PDE5in order to clarify their pharmacodynamics roles.

Nongenomic testosterone effects by other ABC-transporters have been firmly documented in adult human testis. Leydig cells express ABCB1, ABCC1 and ABCC4 [38,39]. ABCB1 and ABCG2 are present at the luminal side of testis capillaries, whereas Sertoli cells mainly express ABCB1, ABCC1 and ABCC4 [8–10]. The present study shows an additional non-genomic effect of testosterone.

Serum protein binding has an important regulatory role in the steroid action [40]. The free hormone hypothesis implies that only the unbound fractions of steroids are available for cellular uptake. This idea is too simplistic [41]. The observations that progesterone and /or testosterone are substrates for the active membrane pumps like ABCB1 [5,9], ABCC4 [6], ABCC5 [6,13] plus the present work, and ABCG2 [9]. These transporters make it possible for both unbound and bound hormone to enter tissues and cells. In clinical pharmacokinetics, this corresponds to substances defined as high extraction drugs. Such transport is characterized by high extraction, and depends on the rate of substrate presentation and not the extent of protein binding [42].

Members of the SLC-families are identified as transporters for metabolites of progesterone and testosterone, for review [43].This is of special interest in tissues with peripheral metabolism (other than liver tissue) [35] because biotransformation reduces concentrations of progesterone and testosterone and thereby facilitate transport by the gradient that occurs.

In this study, we have employed a cell model (hRBC IOVs) to characterize interaction between ABCC5 and hormones (progesterone and testosterone) in addition to analogues of these hormones (putative therapeutic agents). However, future studies with primary cell models such as known target cells for progesterone and testosterone, are needed to extend the scientific impact in biomedicine and clinical medicine. The interaction between the analogues and nuclear receptors [44,45] in addition to non-genomic mechanisms such as membrane receptors [15, 46], should also be characterized in future studies.

5. Addendum

We regret having overlooked errors in Table 2 [13] during proof reading. The correct K$_i$-values are 1.7 ± 0.5 μmol/L and 26 ± 14 μmol/L for progesterone and megestrol acetate, respectively.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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