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Modulation of high affinity ATP-dependent cyclic nucleotide transporters by specific and non-specific cyclic nucleotide phosphodiesterase inhibitors

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ABSTRACT

Intracellular cyclic nucleotides are eliminated by phosphodiesterases (PDEs) and by ATP Binding cassette transporters such as ABCC4 and ABCC5. PDE5 and ABCC5 have similar affinity for cGMP whereas ABCC5 has much higher affinity for cGMP compared with cAMP. Since the substrate (cGMP) is identical for these two eliminatory processes it is conceivable that various PDE inhibitors also modulate ABCC5-transport. Cyclic GMP is also transported by ABBC4 but the affinity is much lower with a K_m 50–100 times higher than for that of ABBCC5. The present study aimed to determine K_i-values for specific or relative specific PDE5 inhibitors (vardenafil, tadalafil, zaprinast and dipyridamole) and the non-specific PDE inhibitors (IBMX, caffeine and theophylline) for ABCC5 andABCC4 transport. The transport of [^{3}H]-cGMP (2 μM) was concentration-dependently inhibited with the following K_i-values: vardenafil (0.62 μM), tadalafil (14.1 μM), zaprinast (0.68 μM) and dipyridamole (1.2 μM), IBMX (10 μM), caffeine (48 μM) and theophylline (69 μM). The K_i-values for the inhibition of the [^{3}H]-cAMP (2 μM) transport were: vardenafil (3.4 μM), tadalafil (194 μM), zaprinast (2.8 μM), dipyridamole (5.5 μM), IBMX (16 μM), caffeine (41 μM) and theophylline (85 μM). The specificity for ABCC5 we defined as ratio between K_i-values for inhibition of [^{3}H]-cGMP and [^{3}H]-cAMP transport. Tadalafil showed the highest specificity (K_i-ratio: 0.073) and caffeine the lowest (K_i-ratio: 1.2).

1. Introduction

The major pharmacotherapeutic progress during the last 50 years includes drugs which modulate signalling systems. One example is the drugs that influence cyclic nucleotide biokinetics with effects on the balance between synthesis and elimination (Beavo and Brunton, 2002). The cellular elimination of cyclic nucleotides comprises PDEs responsible for biotransformation and ABC-transporters which account for the cellular efflux of unmodified molecule. In humans 11 PDE families with different tissue distribution and substrate specificity exist (Francis et al., 2011). Significant progress has been made in developing specific inhibitors of PDEs to obtain more selective effects and minimise adverse effects. The feasibility of these enzymes as drug targets is exemplified by the commercial and clinical successes of the erectile dysfunction drugs, sildenafil, tadalafil and vardenafil. However, still non-specific PDE inhibitors are in clinical use (Savai et al., 2010).

During the last decades the mechanisms that regulate the intracellular levels of cGMP have been clarified. A finely tuned balance between synthesis, degradation and efflux with compartmentalisation of the biokinetics components (cyclases, phosphodiesterases and efflux pumps) exist (Cheepala et al., 2013). The signature substance of specific PDE5 inhibitors, sildenafil, prevents the high affinity cellular cGMP efflux (Sundkvist et al., 2002) by inhibition of ABCC5 (Jedlitschky et al., 2000). Cyclic GMP is transported by ABCC5 with high affinity (K_m-value ≈ 2 μM) (Jedlitschky et al., 2000), and clearly higher than ABCC4-mediated transport. It is possible that an early report of high affinity (K_m ≈ 10 μM) might have overestimated the ABCC4 affinity for cGMP by limiting the concentrations to 25 μM (Chen et al., 2001). Much higher K_m-values have been reported: 170–180 μM (Klokouzas et al., 2003; van Aubel et al., 2005) and a K_i-value of 280 μM (Orvoll et al., 2013). In agreement, Jedlitschky et al. (2000) reported that inhibition of ABCC5-mediated cGMP transport of cAMP was detected only at concentrations above 100 μM. On the other hand ABCC4 has a preference for cAMP in low concentrations with K_m-values 30–45 μM (Chen et al., 2001; Orvoll et al., 2013).

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Several reports exist on interactions between PDE inhibitors and ABC-transporters; inhibition of ABCC5 by zaprinast (Jedlitschky et al., 2000), ABCB1 by sildenafil, vardenafil and tadalafil (Shi et al., 2011; Ding et al., 2011) and ABCG2 by sildenafil (Shi et al., 2011). In the present paper the term selective has been used to distinguish between molecular targets (i.e. PDEs versus ABCC transporters) and specificity to distinguish between the individual enzymes or transporters within the same family. Recently, we reported that sildenafil and sildenafil analogues inhibit high affinity [^{3}H]-cGMP transport (Sager et al., 2012) and that, at least some of these, exhibit a specificity in their inhibition of ABCC4 and ABCC5 (Orvoll et al., 2013). To extend these observations the present study aimed to obtain K_i-values of specific and non-specific PDE inhibitors of ABCC4 and ABCC5.

2. Materials and methods

2.1. Chemicals

Cyclic GMP, cyclic AMP, zaprinast, dipyridamole, theophylline, theobromine, caffeine, IBMX (3-isobutyl-1-methylxanthine), ATP magnesium, acetyl thiocoline chloride and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma-Aldrich (Schnelldorf, Germany), tadalafil from Toronto Research Chemicals Inc. (Ontario, Canada), [^{3}H]-cGMP and [^{3}H]-cAMP from Perkin Elmer Inc. (Boston, MA, USA). Vardenafil was a gift from Bayer Health Care AG (Leverkusen, Germany). Other chemicals were of analytical grade.

2.2. Preparation of inside out vesicles

Fresh human EDTA blood was obtained from healthy donors (Department of laboratory medicine, University hospital of North Norway). The blood cells were separated from plasma by centrifugation and washed with 5 mM Tris–HCl, 113 mM KCl (pH 8.1) three times before lysis. The cells were lysed in 10 volumes of 5 mM Tris–HCl, 0.5 mM EGTA, 4 mM KCl (pH 8.1) and washed in the same buffer (20.000*g* for 20 min) until ghosts were milky white. Inside-out vesicles were prepared using a modification of the procedure originally described by Steck et al. (1970). The membrane vesiculation was initiated by adding 39 volumes of a hypertonic buffer (500 mM Tris–HCl, pH 8.2) to one volume of cell suspension and this process was completed after several hours. All the steps above were performed at 2–4 °C. After centrifugation the suspension was forced through a 27 gauge syringe needle to enhance homogenisation of the membranes. Inside-out vesicles were separated from right side out vesicles and ghosts by ultracentrifugation (100.000*g*) overnight using a density gradient from 1.048 g/ml to 1.146 g/ml (Histodenz, Sigma-Aldrich, Schnelldorf, Germany) in 5 mM Tris, 3 mM KCl and 0.3 mM EGTA. The uppermost band was collected, washed and resuspended in 1.47 mM KH_2PO_4, 81 mM K_2HPO_4 and 140 mM KCl (pH 7.6). Sidedness was verified using acetylcholinesterase accessibility with small modifications of the original method (Ellman et al., 1961).

2.3. Transport assay

Transport assays were performed by incubating the inside-out vesicles at 37°C with or without 2.0 mM ATP at various inhibitor concentrations. All assays were carried out in triplicates. Inhibition assays for transport of 2 μM [^{3}H]-cAMP or 2 μM [^{3}H]-cGMP were performed for dipyridamole (10 nM–1 mM), vardenafil (1 nM–1 mM), tadalafil (1 nM–100 μM), IBMX (1 nM–1 mM), zaprinast (1 nM–100 μM), caffeine (10 nM–1 mM), theophylline (10 nM–1 mM), theobromine (100 μM). After 60 min incubation in the presence of the appropriate inhibitor the transport was stopped by adding ice cold buffer with pH 7.6 (1.47 mM KH_2PO_4, 8.1 mM K_2HPO_4, 140 mM KCl). The inside-out vesicles suspension was filtered and washed with the use of nitrocellulose membranes (0.22 μm GSWP, Millipore, Billerica, MA) and the filters with inside-out vesicles were dissolved in ethylene acetate and the radioactivity was quantified (Packard 1900 TR Liquid Scintillation analyser), after addition of scintillation fluid (Ultima Gold XR, Packard, Groningen, The Netherlands).

2.4. Data analysis

The IC_{50} values were determined according to Chou (1976). These data were transformed to K_i-values according to Cheng and Prusoff (1973). Substrate concentration was 2 μM for both cyclic nucleotides whereas the K_m-values used were 2.6 μM and 30.8 μM for cGMP and cAMP, respectively, based on recently reported values (Orvoll et al., 2013).

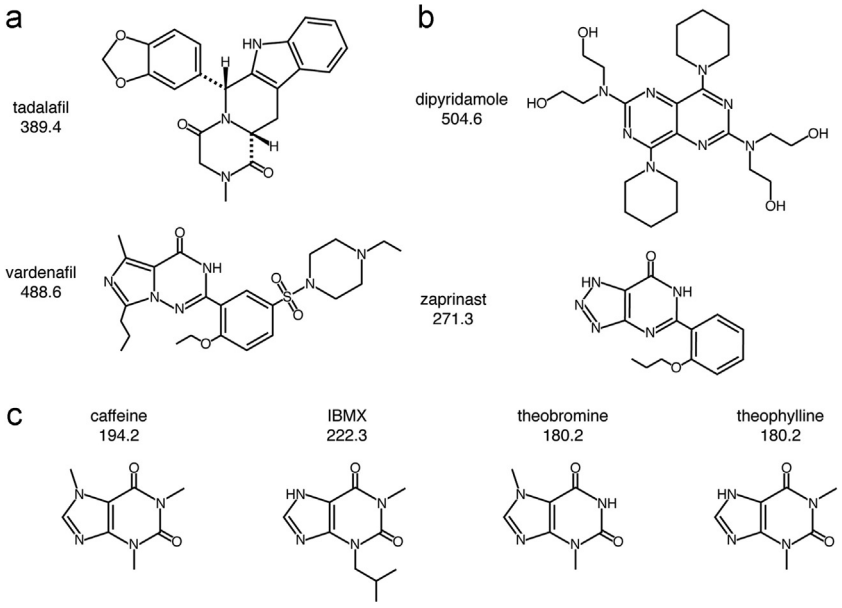

Fig. 1. Structures of specific PDE5 inhibitors (panel A), assumed specific PDE5 inhibitors (panel B) and non-specific PDE inhibitors (panel C).

3. Results

3.1. Vardenafil, tadalafil, zaprinast, dipyridamole and cyclic nucleotide efflux

The effect of PDE5 inhibitors on high affinity ATP-dependent cyclic nucleotide transport were previously characterised in the model with human erythrocyte inside-out vesicles for cGMP with sildenafil and zaprinast (Sundkvist et al., 2002) and for cAMP with sildenafil (Orvoll et al., 2013). This model was employed in the present study to compare the potency of the PDE5 inhibitors vardenafil, tadalafil, zaprinast and dipyridamole (Fig. 1 panel A and panel B). Fig. 2 panel A and Table 1 show the order of potency in [^{3}H]-cGMP transport inhibition: vardenafil ≈ zaprinast > dipyridamole»tadalafil. The transport of [^{3}H]-cAMP was inhibited by somewhat different order of potency: zaprinast ≥ vardenafil > dipyridamole»tadalafil (Fig. 2 panel B and Table 1). The ratio between K_i for [^{3}H]-cGMP and [^{3}H]-cAMP transport (K_i-ratio) shows the ability to discriminate between ABCC5 and ABCC4. The specificity towards ABCC5 showed the following order: tadalafil»vardenafil ≥ dipyridamole ≥ zaprinast (Table 1).

3.2. IBMX, caffeine, theophylline and cyclic nucleotide efflux

In order to compare affinity and specificity of non-specific PDE inhibitors IBMX, caffeine and theophylline were used in the transport assays. These compounds gave a concentration-dependent inhibition of the radiolabelled cGMP (Fig. 3 panel A) and cAMP (Fig. 3 panel B). The order of potency in [^{3}H]-cGMP transport inhibition was IBMX»caffeine > theophylline and for [^{3}H]-cAMP transport inhibition: IBMX > caffeine > theophylline (Table 2). The specificity for ABCC5 expressed as K_i-ratio was IBMX > theophylline > caffeine. In the introductory screening studies theobromine was included. The reduction of [^{3}H]-cGMP and [^{3}H]-cAMP transport with 100 μM of theobromine was 55.0 ± 4% and 51.2 ± 3.2%, respectively.

4. Discussion

Cyclic nucleotide phosphodiesterases have recently gained an increasing pharmaceutical interest, due to structure-based design of novel specific inhibitors, to the increasing understanding of the roles of individual PDEs, and to the development of refined strategies to target individual PDE variants (Maurice et al., 2014). Specific inhibitors of PDEs have been developed, and sildenafil was established as a specific PDE5 inhibitor with IC_{50}-values of 4–10 nM and the subsequent substances like vardenafil and tadalafil had IC_{50}-values of 0.14–1 nM and 1.8–10 nM, respectively (Francis et al., 2011). In this work we have used the term specific to distinguish between members of the PDE-family and members of the ABC-transporter subfamily C. To differ between entirely different molecular targets such as PDEs and ABC-transporters, we have employed the term selectivity.

We have employed human erythrocytes which express both ABCC4 (Klokouzas et al., 2003; Wu et al., 2005; de Wolf et al., 2007; Rius et al., 2008) and ABCC5 (Jedlitschky et al., 2000; Klokouzas et al., 2003; Wu et al., 2005; de Wolf et al., 2007). Cyclic GMP is transported out with high affinity by ABCC5 (Jedlitschky et al., 2000) and cAMP by ABCC4 with lower, but still high affinity (Chen et al., 2001). The impact of ABCC4 on cellular cAMP efflux was recently confirmed (Copsel et al., 2011). The high affinity efflux of these endogenous substances is specific since they are unable to mutually interact in physiologic concentrations (Orvoll et al., 2013). The specificity of ABCC4 and ABCC5 is regulated by the cAMP and cGMP in a concentration dependent manner (Orvoll et al., 2013). Apparently the transporters exist in a specific high affinity state that switch into a “multiorganic anion transporter state” at higher concentrations with similar and low affinity for cAMP and cGMP (Wielinga et al., 2003). The reported low affinity transport K_m-values for cAMP are 300–400 μM (Jedlitschky et al., 2000; Orvoll et al., 2013) and for cGMP (150–650 μM) (Jedlitschky et al., 2000; Klokouzas et al., 2003; van Aubel et al., 2005; Wittgen et al., 2012). In the inside-out vesicles model of human erythrocytes the ATP-dependent transport of cyclic nucleotides in low concentrations mainly represents specific and high affinity transport of cAMP by ABCC4 and cGMP by ABCC5.

Table 1
K_i-values for inhibition of [^{3}H]-cGMP and [^{3}H]-cAMP for the specific PDE5 inhibitors vardenafil and tadalafil and the previously assumed specific PDE5 inhibitors zaprinast and dipyridamole. IC_{50}-values were determined according to Chou (1976) and K_i was obtained according to Cheng and Prusoff (1973). The specificity for ABCC5 is expressed as a ratio between K_i for cGMP and cAMP transport (K_i-ratio).

Inhibitor	K_i (μM)[a]		K_i-ratio
	[^{3}H]-cGMP	[^{3}H]-cAMP	
Tadalafil	14.1 ± 1.6	194 ± 19	0.073
Vardenafil	0.62 ± 0.09	3.4 ± 0.07	0.18
Dipyridamole	1.2 ± 0.15	5.5 ± 0.29	0.21
Zaprinast	0.68 ± 0.06	2.8 ± 0.25	0.24

[a] Results are presented as mean ± S.E.M.

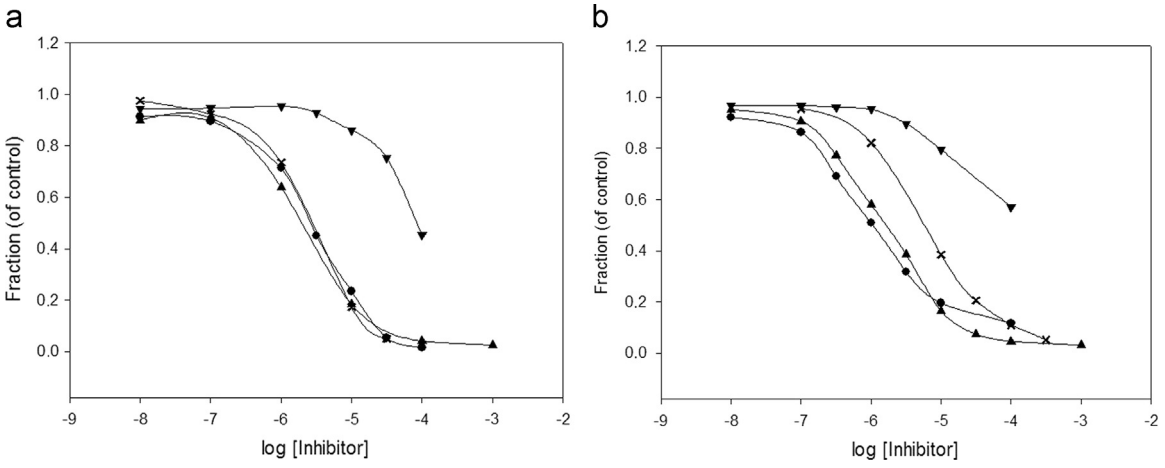

Fig. 2. Inhibition of radiolabelled cyclic nucleotides by specific PDE5 inhibitors vardenafil (▲) and tadalafil (▼) and assumed specific PDE5 inhibitors zaprinast (●) and dipyridamole (x). Panel A: [^{3}H]-cGMP and panel B: [^{3}H]-cAMP.

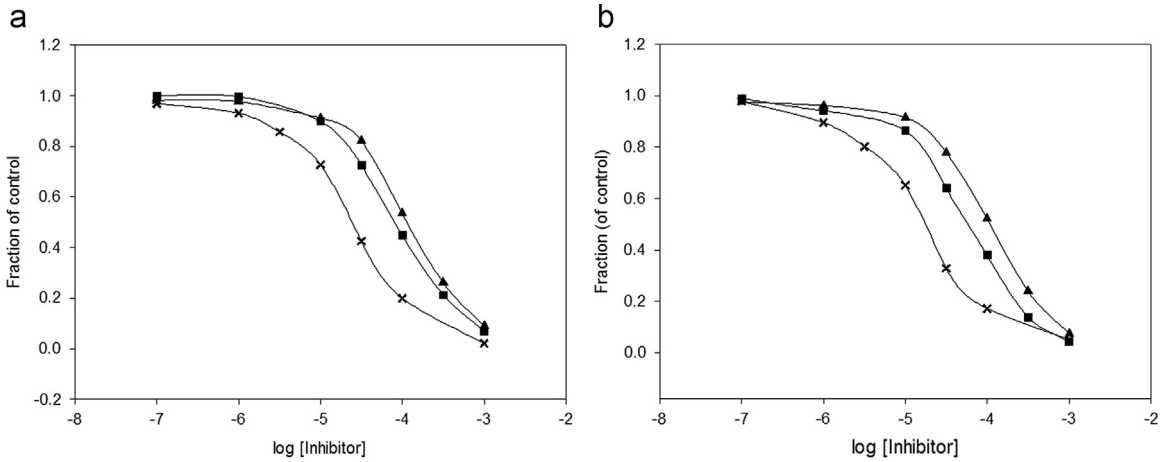

Fig. 3. Inhibition of radiolabelled cyclic nucleotides by non-specific PDE inhibitors IBMX (x), caffeine (■) and theophylline (▲). Panel A: [^{3}H]-cGMP and panel B: [^{3}H]-cAMP.

Table 2
K_i-values for inhibition of [^{3}H]-cGMP and [^{3}H]-cAMP for the non-specific PDE5 inhibitors dipyridamole, IBMX, theophylline and caffeine. IC_{50}-values were determined according to Chou (1976) and K_i was obtained according to Cheng and Prusoff (1973). The specificity for ABCC5 is expressed as a ratio between K_i for cGMP and cAMP transport (K_i-ratio).

Inhibitor	K_i (μM)[a]		K_i-ratio
	[^{3}H]-cGMP	[^{3}H]-cAMP	
IBMX	10.0 ± 0.8	16.2 ± 1.3	0.62
Caffeine	48.2 ± 1.2	40.7 ± 2.9	1.2
Theophylline	68.7 ± 1.5	85.4 ± 2.6	0.80

[a] Results are presented as mean ± S.E.M.

Table 3
K_i-values for cGMP transport by ABCC5 and cGMP hydrolysis by PDE5. Present results and reported values. Most studies report IC_{50}-values for PDE5 inhibition without substrate concentration and K_m value for cGMP hydrolysis.

	ABCC5 K_i (μM)	PDE5 K_i (μM)
Sildenafil	0.24 (Jedlitschky et al, 2000)	0.0020 (Moreland et al., 1998)
	3.6 (Sundkvist et al, 2002)	0.0027 (Ballard et al., 1998)[a]
	1.2 (Sager et al, 2012)	0.001 (Turko et al., 1999)
		0.0147(Kim et al., 2001)
		0.0022(Wang et al., 2001)[a]
Vardenafil	0.62	0.0045 (Kim et al, 2001)
		0.0023 (Cahill et al., 2012)
Tadalafil	14.1	0.0019 (Cahill et al, 2012)
Zaprinast	0.68	0.65 (Ballard et al, 1998)[a]
	0.35 (Sundkvist et al, 2002)	0.25 (Moreland et al, 1998)
		0.13 (Turko et al, 1999)
		0.22(Wang et al, 2001)[a]
Dipyridamole	1.2	0.46 (Wang et al, 2001)[a]
	0.35 (Sundkvist et al, 2002)	
IBMX	10	3.6 (Wang et al, 2001)[a]
Caffeine	48	250–1000 (Francis et al, 2011)[b]
Theophylline	69	250–1000 (Francis et al, 2011)[b]

[a] K_i calculated based on IC_{50} according to Cheng and Prusoff (1973).
[b] K_i estimated based on relative potency to vardenafil.

Table 3 shows K_i-values obtained in the present study and reported in or calculated based on data in previous publications for cGMP transport by ABCC5 and cGMP hydrolysis by PDE5. Sildenafil inhibits ABCC5-mediated cGMP efflux with a K_i of 0.7 μM (Jedlitschky et al., 2000). Later observations suggested that the affinity was somewhat less with K_i-values of 1.2–3.6 μM (Sundkvist et al., 2002; Sager et al., 2012). However, a 100–1000 fold lower affinity for ABCC5 shows that sildenafil has a distinct selectivity for PDE5. This also applies to vardenafil and tadalafil but the present study showed that vardenafil (K_i=0.6 μM) was markedly stronger inhibitor than tadalafil (K_i=14.1 μM) of ABCC5-mediated cGMP transport.

It has been a common belief that zaprinast (Francis et al., 2011) and dipyridamole (Lugnier, 2006) were specific PDE5 inhibitors but this notion should be reconsidered. Zaprinast also inhibits PDE1 and PDE6, inhibits adenylyl cyclase and modulate intracellular calcium (Francis et al., 2011). However, it is interesting that a similar affinity exist for the inhibition by zaprinast of PDE5 (K_i of 0.13–0.8 μM) (Francis et al., 2011) and ABCC5 with K_i of 0.49 μM (Sundkvist et al., 2002) and 0.64 μM in present study. The IC_{50} values for dipyridamole inhibition of cAMP–PDE and cGMP–PDE were 13 and 0.3 μM, respectively (Lugnier et al., 1986). Later, dipyridamole was reported to interact with PDE2, PDE4, PDE6; PDE7, PDE8, PDE10 and PDE11 (Lugnier, 2006; Bender and Beavo, 2006). Other reported effects are inhibition of nucleoside transporters in erythrocytes (Schaper, 2005) and inhibition of ABCC5 mediated cGMP transport with K_i of 0.35 μM (Sundkvist et al., 2002) and 1.2 μM in the present study. The dipyridamole K_i-values for PDE5 and ABCC5 are similar.

In the present work we studied the specificity of high affinity transport. Whilst tadalafil displayed a lower inhibitory effect on the ABCC transporters, it had a much higher specificity for ABCC5 compared to ABCC4 (K_i-ratio ≈ 0.07). For vardenafil, dipyridamole and zaprinast the ABCC5 specificity existed with respective ratios of 0.18, 0.21 and 0.24. In comparison, the K_i-ratio for sildenafil was 0.45, calculated from previous reported IC_{50} values (Orvoll et al., 2013). However, the ABC-transporter specificity is a challenge illustrated by the facts that sildenafil, in addition to ABCC4 and ABCC5, inhibits ABCB1- and ABCG2-transport (Shi et al., 2011), vardenafil and tadalafil interact with ABCB1 (Ding et al., 2011). On the other hand, this is also the hallmark of this multiorganic anion transporter family.

IBMX is a non-specific inhibitor of cyclic nucleotide PDEs with a K_i of 2–10 μM for PDE5 (Francis et al., 2011). Similar inhibition of high affinity cyclic nucleotide transport was evident with K_i-values of 10 and 16 μM for [^{3}H]-cGMP and [^{3}H]-cAMP, respectively. This indicates that IBMX tends to be ABCC5-specific. Caffeine and theophylline show little selectivity among PDEs 1–11 with IC_{50} values ranging from 100 to 1000 mM (Francis et al., 2011). These substances appear to be non-selective (PDE5 versus ABCC4/5) and non-specific (ABCC5 versus ABCC4).

In addition to the inhibition of PDEs and ABC-transporters, theophylline and caffeine antagonise the effects of adenosine on adenosine receptors, where they have the modest affinity with K_i-values 1–25 μM and 10–100 μM for theophylline and caffeine, respectively (Muller and Jacobson, 2011). The present study shows affinities of caffeine for ABCC4 and ABCC5 of similar magnitude as for the adenosine receptors. The future will show whether this observation adds ABC-transporters to the list of potential molecular targets for caffeine. Plasma caffeine levels 8 h after ingestion (100 mg) were reported to be 10–15 μM (Tanaka et al., 2014) but intracellular concentrations were not reported. Caffeine, a methylxanthine, enters the brain by both simple diffusion and saturable, carrier-mediated transport (McCall et al., 1982) and interacts with equilibrative nucleoside transporter transporters (ENT), more recently classified as SLC29 (Lang et al., 2004). Obvious candidates for caffeine transport are SLC28 and SLC29 (Cano-Soldado and Pastor-Anglada, 2012) but caffeine is not mentioned as substrate or inhibitor for these transporters in the Concise Guide to Pharmacology 2013/14: Transporters (Alexander et al., 2013). However, the caffeine inhibition of ABCC4 and ABCC5, localised at the blood side (Hartz and Bauer, 2011), will diminish substrate efflux from the brain, including caffeine itself.

In conclusion, PDE5 specific inhibitors such as sildenafil, vardenafil and tadalafil are selective since affinity for ABCC4/5 is exceedingly lower compared to that of PDE5. The present data also demonstrate specificity towards ABCC5 compared to ABCC4. Finally, we show that caffeine and theophylline inhibit ABCC4/5 transport with K_i-values that are relevant after intake of these substances and apparently with higher affinity than inhibition of PDE5 (Table 3).

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