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Pharmacological characterization of the ATP-dependent low K_m guanosine 3′,5′-cyclic monophosphate (cGMP) transporter in human erythrocytes

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Abstract

The efflux pump for cGMP has been shown to be an ATP-energized multiorganic anion transporter. The present study was performed to extend the knowledge of the pharmacological characteristics of this efflux pump. Inside-out vesicles prepared from fresh blood were incubated with [^{3}H]-cGMP (1 μM) with or without various concentrations of competitors for 120 min at 37°. The tested compounds could be divided in four groups: one with high affinity (K_i < 5 μM), a second with moderate affinity (K_i: 5–50 μM), a third with low affinity (K_i: 0.1–5 mM) and the fourth with extremely low or no affinity at all. With the mean K_i-values given in parenthesis, the high affinity group consisted of mifepristone (0.2 μM), zaprinast (0.35 μM), dipyridamole (0.35 μM), estradiol 3-β-glucuronide (0.42 μM), genistein (0.43 μM), estradiol 17-β-glucuronide (0.47 μM), onapristone (1.3 μM), progesterone (1.7 μM) and sildenafil (3.6 μM). The inhibitors with medium affinity were estradiol (8 μM), sulfinpyrazone (13 μM), daunorubicin (23 μM), megestrol acetate (26 μM), doxorubicin (28 μM), 6-thioguanine (28 μM) and 6-thioguanosine-5′-monophosphate (32 μM). The low affinity group comprised 6-TIMP (220 μM), 6-methylmercaptopurine (MMP) (220 μM), vincristine (270 μM), medroxyprogesterone (680 μM), para-aminohippurate (PAH) (1.9 mM) and taurocholate (2.2 mM). No or minimal effect was seen in the presence of 6-mercaptopurine (6-MP), methotrexate, 9-(2-phosphonylmethoxyethyl)adenine and mitoxantrone. The cGMP transporter had a unique pharmacological profile, different from that of MRP1, but with some characteristics in common with MRP4 and MRP5.

Keywords: cGMP; Purines; Steroids; Cytotoxic drugs; Phosphodiesterase inhibitors; Transport

1. Introduction

Guanosine 3′,5′-cyclic monophosphate (cGMP) is transported out of cells by an organic anion pump, dependent on ATP [1], ATP-hydrolysis [2] and cGMP-stimulated ATPase activity [3]. The cGMP transporter protein possesses at least three different types of binding sites where drugs and other compounds may interact: (a) the ATP-binding site of the ATPase, (b) the binding site which recognize cGMP and activates transport, (c) one or more sites with ability to modulate transporter activity. We have characterized the ATP-binding site [4] and found that well-known ATPase modulators gave an inhibition pattern typical of M-type ATPases [5]. Several established membrane transport modulators inhibit cGMP transport, such as probenecid, verapamil and progesterone [2]. In addition, cGMP analogues are potent blockers of the efflux pump [6]. Both MRP4 [7] and MRP5 [8] have been reported to transport cGMP. The aim of the present study was to extend the pharmacological characteristics of the cGMP pump in order to distinguish it from other organic anion transport system.

2. Materials and methods

2.1. Chemicals

The following substances were used: [^{3}H]-cGMP (specific activity 16 Ci/mmol) and [^{3}H]-methotrexate (specific activity 6.6 Ci/mmol) from Amersham International; daunorubicin hydrochloride, dipyridamole, doxorubicin

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; MRP, multidrug resistance protein; 6-MP, 6-mercaptopurine; Medroxyprogesterone, 6-α-methyl-17-α-hydroxyprogesterone; MMP, 6-methylmercaptopurine; PAH, para-aminohippurate; PDE, cyclic nucleotide phosphodiesterase; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; 6-TG, 6-thioguanine; 6-TGMP, 6-thioguanosine-5′-monophosphate; 6-TIMP, 6-thioinosine-5′-monophosphate.

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hydrochloride, estradiol, estradiol 3-β-glucuronide, estradiol 17-β-glucuronide, genistein, reduced glutathione (GSH), megestrol acetate, 6-α-methyl-17-α-hydroxyprogesterone (medroxyprogesterone), methotrexate, MMP, mitoxantrone hydrochloride, progesterone, sulfinpyrazone, vincristine sulphate, taurocholate, 6-MP, 6-thioguanine (6-TG), zaprinast from Sigma–Aldrich; PAH from Fluka; mifepristone from Roussel Uclaf; onapristone from Schering AG, sildenafil citrate from Pfizer Ltd., 6-thio-guanosine monophosphate, 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and 6-thioinosine 5′-monophosphate (6-TIMP) from Peter Wielinga, The Netherland Cancer Institute. All other chemicals were of analytical grade.

2.2. Preparations of inside-out vesicles

Inside-out vesicles were prepared from freshly sampled blood as described previously [9,10].

2.3. Uptake studies

The experiments were performed at 37° with 50–300 μg protein in 400 μL in a medium comprising 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, 10 mM $MgCl_2$, pH 7.4, including 1 μM [^{3}H]-cGMP with or without 1 mM ATP plus the different concentration of test substances. DMSO was used to dissolve the substances which were insolvable in the incubation medium. An identical DMSO concentration was included in the control samples without competitors. The final DMSO concentration (<0.5%) does not affect the [^{3}H]-cGMP uptake [2]. The uptake of radioligand was terminated by addition of 10 mL ice-cold 30 mM NaF/0.5 mM Tris–HCl. After centrifugation at 16,300 *g* the vesicles were washed once with another 10 mL of the buffer and after a final centrifugation mixed with 1 mL ice-cold water and frozen over night at −20°. The samples were thawed and transformed to Eppendorf tubes and centrifuged at 15,000 *g* for 15 min. The radioactivity was determined in 0.8 mL supernatant together with 10 mL Ultima Gold^{XR} Packard in a 1900^{TR} Packard liquid scintillation analyzer.

2.4. Protein concentrations

Protein concentration were determined by the Coomassie blue method with reagents from Bio-Rad laboratories and BSA from Sigma as standards.

2.5. Data analysis

The data from the concentration inhibition experiments were analyzed according to Chou [11] to obtain EC_{50}-values which were transformed to K_i-values according to Cheng and Prusoff [12].

2.6. Descriptive statistics

Results are presented as mean value ± SEM.

3. Results

3.1. Methotrexate

In the present study, we found that unlabelled methotrexate inhibited uptake of [^{3}H]-methotrexate (Fig. 1). Analysis of these data gave a K_i-value of 0.9 ± 0.3 μM

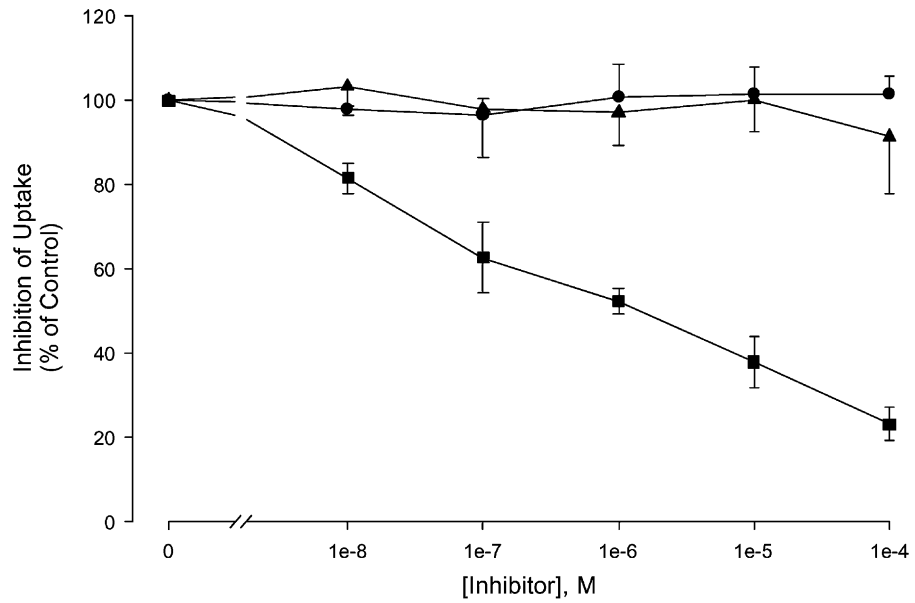

Fig. 1. ATP-dependent uptake of 3.8 μM [^{3}H]-MTX and effects of unlabelled 0.01–100 μM MTX, $n = 3$ (■) and 0.01–100 μM cGMP, $n = 3$ (●), and of 1 μM [^{3}H]-cGMP and effects of unlabelled 0.01–100 μM MTX, $n = 4$ (▲). Results are presented as percentage of control (radiolabelled substance in absence of unlabelled substance) and given as mean ± SEM.

($n = 3$). cGMP was unable to prevent uptake of [^{3}H]-methotrexate and unlabelled methotrexate had no effect on the uptake of [^{3}H]-cGMP (Fig. 1).

3.2. Vincristine

Since, GSH is required for resistance against the vinca alkaloids in cells overexpressing MRP1 and MRP2, the effect of vincristine was tested alone and together with GSH in the present study. Vincristine (0.01–100 μM) was co-incubated with [^{3}H]-cGMP in the absence and presence of GSH. The affinity did not change. The K_i-values were 270 ± 70 μM ($n = 5$), 280 (220–340 μM) ($n = 2$) and 250 (230–260 μM) ($n = 2$) in absence of GSH and in the presence of 1 and 10 μM GSH, respectively. The fact that GSH itself inhibits cGMP transport at millimolar concentrations [9,10] forwarded an experiment where GSH (5 mM) was present both in controls ([^{3}H]-cGMP without vincristine) and together with vincristine ([^{3}H]-cGMP with 0.01–100 μM vincristine). This experimental setup did not change the sensitivity to vincristine ($K_i = 250 \pm 40$ μM, $n = 3$).

3.3. Anthracyclines

Anthracycline antibiotics are also substrates for members of the multidrug resistance protein (MRP)-family. Daunorubicin and doxorubicin were equally potent in their inhibition of [^{3}H]-cGMP uptake with K_i-values of 23.4 ± 3.3 and 27.9 ± 0.8 μM ($n = 3$), respectively. In contrast, the synthetic anthracycline mitoxantrone showed no effect on cGMP transport (data not shown).

3.4. Estradiol and estradiol glucuronides

Estrogen glucuronides have been shown to interact with MRP-mediated transport. In the present study, estradiol and estradiol glucuronides inhibited cGMP uptake. The glucuronides were equally potent with K_i-values of 0.42 ± 0.11 μM ($n = 3$) and 0.47 ± 0.08 μM ($n = 4$) for estradiol 3-β-glucuronide for estradiol 17-β-glucuronide, respectively. Estradiol was markedly less potent ($K_i = 8.3 \pm 2.1$ μM, $n = 3$).

3.5. Purine analogues

Based on the possibility of structure related effects, several purine analogues were tested. PMEA and 6-MP showed no or minimal inhibition whereas MMP was an inhibitor of intermediate potency and 6-TG the most potent inhibitor. The order of potency was 6-TG = 6-TGMP > 6-TIMP = MMP ⩾ 6-MP ⩾ PMEA (Table 1).

3.6. Progestins and anti-progestins

In the present study, progestins and anti-progestins inhibited cGMP uptake. The order of potency was: mifepristone > onapristone = progesterone > megestrol acetate > medroxyprogesterone (Table 2).

Table 1
Inhibition of ATP-dependent uptake of [^{3}H]-cGMP by purine analogues[a]

Analogue	K_i (μM)
6-TG	28 ± 4
6-TGMP	32 ± 5
6-TIMP	220 ± 50
MMP	220 ± 130
6-MP	225000 ± 36000
PMEA	No inhibition

[a] The results are presented as K_i (mean value ± SEM, $n = 3$).

Table 2
Inhibition of ATP-dependent uptake of [^{3}H]-cGMP by progestins and anti-progestins[a]

Progestin/antiprogestin	K_i (μM)
Mifepristone	0.20 ± 0.02
Onapristone	1.3 ± 0.6
Progesterone	11.7 ± 0.5
Megestrol acetate	126 ± 14
Medroxyprogesterone	680 ± 160

[a] The results are presented as K_i (mean value ± SEM, $n = 3$).

3.7. Cyclic nucleotide phosphodiesterase (PDE)5 inhibitors

The present study shows that selective inhibitors of the cGMP specific phosphodiesterase (PDE5) also block ATP-dependent uptake of cGMP to human erythrocyte inside-out vesicles. The transport was inhibited with the following K_i-values ($n = 3$): zaprinast; 0.35 ± 0.03, dipyridamole; 0.35 ± 0.06 and sildenafil; 3.6 ± 0.3 μM.

3.8. Other compounds

Other compounds were tested for their ability to interact with the cGMP transporter. Sulfinpyrazone is a typical organic anion transport inhibitor which inhibited uptake of [^{3}H]-cGMP with a K_i of 12.7 ± 1.8 μM ($n = 6$). PAH which is widely used as a model substrate for organic anion transport in proximal tubule epithelia, was a markedly less potent inhibitor ($K_i = 1.9 \pm 0.9$ mM, $n = 3$). Genistein which is an inhibitor of tyrosine kinase, has been shown to decrease drug efflux in several MRP-overexpressing cells. In the present study, genistein proved to be a very potent inhibitor ($K_i = 0.43 \pm 0.11$, $n = 5$). Monovalent bile salts like taurocholate, are substrate for some organic anion transporters, but taurocholate was a weak inhibitor of the cGMP transporter (K_i: 2.2 ± 0.9 mM, $n = 3$).

4. Discussion

MRP1 has been detected in human erythrocyte membranes [13]. Consistent with the fact that methotrexate is a

substrate for human MRP1 [14–17], we found that unlabelled methotrexate inhibited the ATP-dependent uptake of [^{3}H]-methotrexate in human erythrocyte inside-out vesicles. However, no mutual interaction between cGMP and methotrexate existed. This supports our previous conclusion that the low K_m-cGMP transporter is different from MRP1 [9,10]. Vincristine and antracyclines are transported by MRP1 in presence, but not in the absence of glutathione [18,19]. The findings that doxorubicin and daunorubicin were able to inhibit cGMP transport without GSH and that the presence of GSH did not affect K_i-values for vincristine are also evidence for two different transport systems. Finally, estradiol 17-glucuronide and estradiol 3-glucuronide had virtually identical affinity for the cGMP transporter, in contrast to MRP1 where the affinities of these two glucuronides are very different [20].

MRP5 has also been detected in human erythrocyte membranes and transports cGMP with high affinity [8], virtually identical to that observed for the low K_m-cGMP transport [2,6]. The present study showed similar affinities of PDE5 selective inhibitors as for MRP5 [8]. On the other hand, there are also observations that question the identity between MRP5 and the low K_m-cGMP transporter. Whereas, 17-glucuronide estradiol was a potent inhibitor of cGMP transport, no MRP5-mediated high affinity transport could be detected [8]. PMEA did not inhibit cGMP transport, but PMEA resistance has been found in MRP5 transfected cells [21].

In a recent report [7], it was shown that MRP4, like MRP5 [8], catalyzes the MgATP-energized transport of cGMP. It is at present not known whether human erythrocytes possess detectable amounts of MRP4. However, expression of MRP4 is low in most tissues under normal conditions [22]. Many observations suggest that MRP4 and the low K_m-cGMP transporter are different. In contrast to the cGMP pump, MRP4 [7] transports methotrexate, has lower affinity for cGMP ($\approx$10 μM), but markedly higher affinity for cAMP ($\approx$45 μM) than reported for low K_m-cGMP transport system [2,6]. The present data show that estradiol glucuronides have much higher affinity for the cGMP pump than for MRP4 [7]. Finally, the MRP4 substrate PMEA [23] was unable to inhibit cGMP transport.

Purines and purine analogue are potential substrates for, or blockers of the cGMP efflux pump. The minimal effect of 6-MP on cGMP uptake is consistent with the observation that MRP5-mediated resistance was due to metabolites and not the native substance [21]. A similar observation was recently reported for MRP4 [7]. Relatively small chemical modification of 6-MP, resulting in the metabolites, 6-TIMP and MMP, increase their affinity for the cGMP transporter markedly. The present observation that 6-TG and its metabolite 6-thioguanosine-5′-monophosphate (6-TGMP) were equally potent ($K_i \approx 30$ μM) is intriguing since the non-cyclic monophosphate, GMP, was unable to interfere with cGMP transport [6].

Little is known about progestins as substrates for or blockers of organic anion transporters. We reported that progesterone inhibited cGMP transport by high affinity in an apparently non-competitive manner [2]. The present study shows that progesterone and the anti-progestins, mifepristone and onapristone, were potent inhibitors of the low K_m-cGMP transporter. Chemical modification of progesterone resulting in megestrol acetate and medroxyprogesterone caused a 15- and 300-fold reduction in affinity, respectively. The impact of structure was also demonstrated within the group of anthracyclines since mitoxantrone had no effect on cGMP transport in contrast to doxorubicin and daunorubicin.

Glutathione conjugates have affinity for the low K_m-cGMP transporter [9,10]. Taurocholate and genistein were tested since they are potent modulator of *S*-dinitrophenyl-glutathione transport in human erythrocytes [24,25]. Taurocholate was a weak inhibitor. The mechanism beyond the very potent inhibition of the cGMP pump by genistein is unknown. Inhibition of tyrosine kinase activity is one possibility.

The low K_m-cGMP transporter is a member of a large family of organic anion transporters, based on the previous observation of probenecid as an inhibitor [2] and verified in the present study showing that sulfinpyrazone and PAH, a structural analogue of probenecid, were able to reduce the ATP-dependent cGMP uptake to inside-out vesicles. An interaction between cGMP and PAH has previously been reported for a probenecid-sensitive transporter in kidney cortex [26].

By means of pharmacological tools we have found a characteristic profile of the low K_m-cGMP transporter. It has features in common with both MRP4 and MRP5, but some characteristics appear to be distinctly different.

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