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Interaction of A₃ Adenosine Receptor Ligands with the Human Multidrug Transporter ABCG2

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Abstract

Various adenosine receptor nucleoside-like ligands were found to modulate ATP hydrolysis by the multidrug transporter ABCG2. Both ribose-containing and rigidified (N)-methanocarba nucleosides (C2-, N⁶- and 5'-modified), as well as adenines (C2-, N⁶-, and deaza modified), were included. 57 compounds out of 63 tested either stimulated (50) or inhibited (7) basal ATPase activity. Structure-activity analysis showed a separation of adenosine receptor and ABCG2 activities. The 7-deaza modification had favorable effects in both (N)-methanocarba nucleosides and adenines. Adenine **37c** (MRS7608) and (N)-methanocarba 7-deaza-5'-ethyl ester **60** (MRS7343) were found to be potent stimulators of ABCG2 ATPase activity with EC₅₀ values of 13.2 ± 1.7 and 13.2 ± 2.2 nM, respectively. Both had affinity in the micromolar range for A₃ adenosine receptor and lacked the 5'-amide agonist-enabling group (**37c** was reported as a weak A₃ antagonist, K_i 6.82 μM). Compound **60** significantly inhibited ABCG2 substrate transport (IC₅₀ 0.44 μM). Docking simulations predicted the interaction of **60** with 21 residues in the drug-binding pocket of ABCG2.

Graphical Abstract

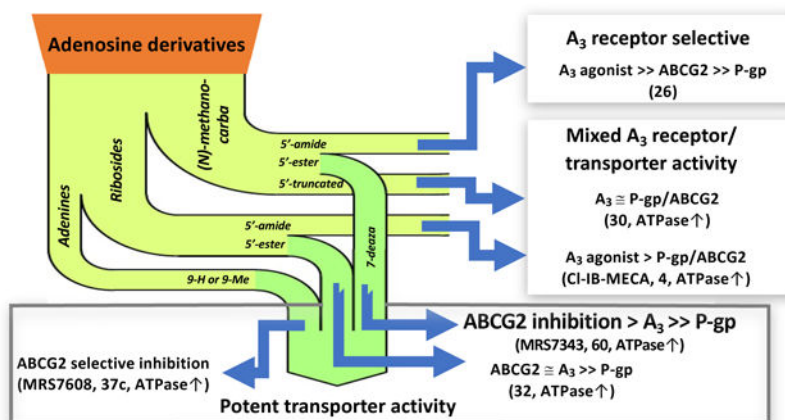
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Declaration of competing interests

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Keywords

ABC transporter; ABCG2; A₃ adenosine receptor; ATP hydrolysis; drug transport; multidrug resistance

1. Introduction

Cancer patients sometimes face the difficulty of continuing therapy due to the development of resistance to chemotherapeutic drugs. One of the main causes of multidrug resistance (MDR) is the efflux action of ATP-binding cassette (ABC) transporters that are expressed on the surface of cancer cells. The transporters pump anti-cancer drugs out of cells using the energy of ATP hydrolysis [1, 2]. Three main ABC transporters contribute to the development of MDR: ABCB1 (P-glycoprotein, P-gp), ABCG2, and ABCC1 [3]. Many chemically dissimilar anticancer drugs are substrates of these transporters. Also, P-gp and ABCG2 are expressed by normal cells, such as epithelial cells found in the intestine, kidney, liver, and placenta, and by endothelial cells of the blood-brain barrier in order to export toxic compounds from the body [1, 2, 4].

To understand the mechanism of ABC transporters and find ways to overcome MDR, the structures of ABC transporters have been thoroughly analyzed, especially those of P-gp and ABCG2. ABC transporters have a transmembrane domain (TMD) that contains substrate-binding sites, typically consisting of 12 transmembrane helices [5]. Other important structural features are the nucleotide-binding domains (NBDs), which contain the conserved Walker A and Walker B motifs, and a signature region upstream of the Walker B site [5]. The structure allowing communication between the TMD and the NBD is called the intracellular domain. Human P-gp is a full transporter with six TMDs and two NBDs [2, 6, 7], whereas ABCG2 is a half-transporter that needs to form a homodimer to be functional [8, 9]. Despite extensive structural analysis, the mechanism of the transport function of ABC transporters is not yet well understood.

Understanding the interaction between drug substrates and ABC transporters is considered important to determine the mechanism of transport function [10]. Recently, we investigated

whether A₃ adenosine receptor agonists can interact with ABC transporters. A₃ adenosine receptor agonists have been developed for the treatment of chronic diseases such as rheumatoid arthritis, psoriasis, chronic pain, and hepatocellular carcinoma [11–14]. In one clinical study, an agonist that is highly potent and selective for the A₃ receptor demonstrated efflux in a CACO-2 cell bidirectional transport model [12], suggesting a possible interaction with ABC transporters in the intestinal epithelium. In our previous study, we synthesized new nucleoside derivatives of A₃ adenosine ligands designed for P-gp interactions, indicated by a structure-activity relationship (SAR) analysis, and investigated the interaction of P-gp with some derivatives [15]. Those results suggested that A₃ adenosine receptor ligands modulate the function of P-gp.

In this study, we investigated whether A₃ adenosine receptor ligands, both adenine nucleoside and nucleobase derivatives, interact with ABCG2, another important ABC transporter, which modulates the absorption of drugs in the intestine. The interaction of A₃ receptor ligands with ABCG2 either as substrates or modulators can affect their bioavailability. We performed an analysis of the effect of these A₃ adenosine receptor ligands on the ATPase activity of ABCG2, with a total of 63 compounds. Of these, compound **60**, a 7-deaza-5'-ester derivative with low adenosine receptor affinity, was identified as a high-affinity ligand for ABCG2. This compound was also found to significantly inhibit substrate transport by ABCG2. Furthermore, we used docking simulations to study how compound **60** binds to the ABCG2 drug-binding site. Adenine derivative **37c** was also a potent transport inhibitor. In this way, we were able to identify certain A₃ adenosine receptor ligands as potential modulators of human ABCG2. These results could be useful for further study of the interactions between ABCG2 and drug substrates.

2. Results

2.1 SAR studies measuring ATPase activity reveal potential A₃ adenosine ligands as ABCG2 modulators

We investigated the interaction of A₃ adenosine ligands with the human multidrug transporter ABCG2 by determining their effect on the ATPase activity of the transporter. In this work, 63 compounds were tested at various concentrations (e.g., 100 nM, 1.0, 2.5, and 10 μM) using total membranes (membrane vesicles isolated after hypotonic lysis) from High-Five insect cells expressing ABCG2. Membranes of intracellular organelles and the plasma membrane are included. All adenosine ligands were tested for their ability to either stimulate or inhibit the ATPase activity of ABCG2. We tested this because most compounds that inhibit basal ATPase activity are most likely able to also inhibit its transport function. In addition, we reported all the results obtained in this section as a percentage of the ATPase activity of ABCG2, with values below or equal to 30% considered as having no effect on ATPase activity, which suggests no interaction with ABCG2.

In order to study their structure-activity relationships (SARs), A₃ adenosine receptor ligands were grouped into four structural categories: 1) N⁶ and C2-phenylethynyl modifications of (N)-methanocarpa 2-arylethynyl-purine derivative (5'-amides and 5'-alcohols) (Table 1), 2) N⁶, C2 and riboside modifications of adenosine derivatives, including both (N)-

methanocarba and ribose-containing ligands (Table 2), 3) N^6 and C2 modifications of adenine derivatives (Table 3), and 4) N^6 and C2 modification of (N)-methanocarba 5'-ester derivatives (Table 4). The (N)-methanocarba substitution of the ribose moiety is a rigid, fused ring system (bicyclo[3.1.0]hexane) that is intended to constrain this moiety conformationally, to enhance affinity and selectivity at the A_3 adenosine receptor [12–14]. Unlike the 5'-amide agonists, adenine derivatives interact with adenosine receptors as antagonists [16].

The synthetic routes to the newly prepared compounds are similar to our published nucleoside synthesis [12–20] and are shown in: Scheme 1A (**16**, **18** – **21**, prepared by a click reaction [19] of a nucleoside azide with an arylacetylene), Scheme 1B (**12**, **22** and **23**, were prepared by sequential nucleophilic substitution reaction with 3-azido-benzyl amine, followed by Sonogashira coupling with C2-arylethynyl group acidic hydrolysis of the isopropylidene group and a click reaction of the nucleoside azide derivatives with an arylacetylene). Scheme 2 (**61** – **64**, prepared from compound **70** [17] by nucleophilic amination with 3-phenylpropan-1-amine derivatives to give compounds **71**–**72** with good yield, which upon Sonogashira coupling (**73** – **76**) and subsequent acid hydrolysis afforded the desired nucleoside derivatives (**61** – **64**). Scheme S1, Supporting Information (**38**, **39**, prepared by sequential nucleophilic substitution reaction with an amine, installation of a C2-arylethynyl group).

First, we tested (N)-methanocarba nucleoside compound **5**, which was found in our earlier study to stimulate the ATPase activity of P-gp (ABCB1) [15]. This ligand consists of R^1 : N^6 -(3-chlorobenzyl) and R^2 : 3,4-difluorophenyl [15]. As shown in Table 1, compound **5** was observed to stimulate the basal ATPase activity of ABCG2 at 0.5 and 5 μ M concentrations. The extent of the stimulation was determined by testing concentrations ranging from 0.05 to 5 μ M, to provide an EC_{50} value of 0.08 ± 0.01 μ M (Figure 1A). We then decided to design derivatives of compound **5** with the goal of improving their affinity towards the transporter. For this reason, compounds **6** and **7** were synthesized by retaining the parent structure of compound **5** but replacing the R^1 : N^6 -(3-chlorobenzyl) with a hydrogen atom **6** or methyl group **7**. These adenosine ligands were observed to stimulate the ATPase activity of ABCG2 at higher concentrations, and the results indicate that the N^6 -(3-chlorobenzyl) group substantially increases the affinity of the adenosine receptor ligand toward ABCG2.

For the next series of compounds, we decided to synthesize methanocarba derivatives with R^1 retaining N^6 -(3-chlorobenzyl) but replacing the R^2 group (3,4-difluorophenyl) with a phenyl group **8**, a 4-fluorophenyl group **9**, and a 2-chlorophenyl group **10**. These compounds all stimulated the basal ATPase activity of ABCG2 at a 2.5 μ M concentration, confirming our previous observation that N^6 -(3-chlorobenzyl) helps to generate high affinity substrates of ABCG2. Furthermore, we synthesized compound **11** by retaining the parent structure of **5** but replacing R^1 (N^6 -(3-chlorobenzyl)) with N^6 -(3-azido-benzyl)), and R^2 remained 3,4-difluorophenyl. Our results showed stimulation of ATPase activity of ABCG2 by **11** at low concentrations, similar to compound **5**. However, when we modified methanocarba derivative **11** by replacing the 5'-amide with an adenosine-like 5'-hydroxyl group to obtain compound **12**, there was no change in the stimulatory effect. To increase the stimulatory effect of compound **5**, we synthesized compounds **13**, **14**, and **15** by replacing the N^6 -(3-

chlorobenzyl) at position R¹ with (3,4-difluorophenyl) cyclopropylamino and replacing the initial R² 3,4-difluorophenyl with a phenyl group. These compounds showed stimulation of basal ATPase activity of ABCG2 at concentrations in the nanomolar range, with EC₅₀ values of 16.0 ± 3.0 nM, 13.0 ± 3.0 nM, and 14.0 ± 5.0 nM, respectively (Table 5). Compounds **13**, **14** and **15** only differ structurally in terms of their stereochemistry.

In our previous work, we identified triazole derivative **17** as an adenosine receptor ligand that partially inhibited the basal ATPase activity of P-gp [15]. This (N)-methanocarba nucleoside was designed with the same parent structure of compound **5**, although at position R¹ the compound had an extended 3-substituted benzyl, and at R², a 3,4-difluorophenyl. For this reason, we decided to select compound **17** and its derivatives **16**, **18**, **19**, **20**, and **21** to investigate their effect on the basal ATPase activity of ABCG2 in total membranes of High-Five insect cells. Triazole compounds **16**, **17**, **18**, **19**, **20**, and **21** all have an extended 3-substituted benzyl at the R¹ position, but all differ structurally at the R³ position: **16** (3-fluorophenyl), **17** (3,4-difluorophenyl), **18** (3,5-difluorophenyl), **19** (2,4-difluorophenyl), **20** (2,3,4-trifluorophenyl), and **21** (4-trifluoromethylphenyl). Compounds **16**, **17**, and **20** all inhibit the basal ATPase activity of ABCG2, with IC₅₀ values of 2590 ± 40 nM, 2570 ± 30 nM, and 4010 ± 20 nM, respectively (Figure S1A, Figure 1B, and Table 5). In addition, compound **18**, which has 3,5-difluorophenyl at the R³ position shows partial inhibition of the ATPase activity of ABCG2. Compound **19** with 2,4-difluorophenyl at position R³ has no effect on the ATPase activity at 2.5 μM. Furthermore, we synthesized compound **21** with trifluoromethylphenyl at the R³ position, which exhibited partial inhibition of basal ATPase activity at a 10 μM concentration. To increase the inhibitory effect of compounds **16** and **17**, we decided to modify the ligands by replacing the 5'-CONHCH₃ on the methanocarba ring with CH₂OH to obtain compounds **22** and **23**. We speculated that modifying the ring could increase the hydrogen bonding with residues in the drug-binding pocket of ABCG2. However, compounds **22** and **23** had no effect on the basal ATPase activity of ABCG2.

Subsequently, we decided to synthesize additional adenosine ligands with the goal of improving the interaction with ABCG2 by modifying the analogs found to stimulate the ATPase activity. In Table 2 we show the effect of N⁶, C2 and riboside modifications of adenosine derivatives having either (N)-methanocarba or ribose rings on the ATPase activity of ABCG2. In this SAR study, 6-amino-truncated (N)-methanocarba compound **24**, with structural features similar to those of compound **6**, was designed by replacing the 3,4-difluorophenyl R² substituent with 2-chloro-thien-5-yl. This group was added to the structure in an effort to increase the potential ionic bonding, non-covalent sulfur interactions and π-π interactions of the nucleoside derivative with the drug-binding pocket of ABCG2. Compound **24** was observed to have no effect on the basal ATPase activity of ABCG2, indicating no interaction with ABCG2 (see Table 2). Furthermore, compounds **25** and **26** were designed by replacing the R¹, initially a hydrogen atom (**24**), with **25** (methylamino), and **26** (propylamino). The presence of these amino groups at the R¹ position increased the affinity of the ligands toward ABCG2 by stimulating the ATPase activity at a 2.5 μM concentration. In addition, we modified compound **25**, by replacing 5'-CONHCH₃ on the methanocarba ring with a COOMe to obtain compound **27** and observed an increase in affinity of the adenosine ligand towards ABCG2. Compound **28** was later synthesized

with structural features similar to those of compound **5**, but by using click chemistry we converted the alkyne at position R⁵ to a 1,2,3-triazole. This structural change at the R⁵ position of compound **28** was observed to switch the effect of ATPase activity from stimulation (compound **5**) to partial inhibition at a 10 μ M concentration. Additionally, we designed compound **29** by the introduction of an azide at the R⁵ position. This resulted in stimulation of the basal ATPase activity of ABCG2 at a 2.5 μ M concentration. Compound **30** was then synthesized with structural features like those of compound **5**, but with truncation of the 5'-CONHCH₃ group at the R⁴ position on the methanocarba ring system (i.e., replacement with a hydrogen atom). The results in Table 2 indicate that most of the adenosine derivatives stimulate the ATPase activity at lower concentrations, and we can switch the affinity of the ligand towards ABCG2 by replacing the alkyne at position R⁵ with a 1,2,3-triazole (see for example compound **28**).

In the last two decades, Food and Drug Administration guidelines for drug design have recommended the testing of investigational drugs to determine whether they are substrates of the ABCG2 multidrug transporter. For this reason, we tested *N*⁶-(3-iodobenzyl) compounds **31** and **4**, which are currently in phase 2 and 3 clinical trials [21]. These potent A₃ adenosine receptor agonists contain a modified ribose moiety at the R⁴ position rather than an (N)-methanocarba ring system as in compound **5**, and either a hydrogen (**31**) or chloro (**4**) at the R⁵ position rather than a 3,4-difluorophenylethynyl group. These compounds were observed to stimulate the basal ATPase activity of ABCG2, with EC₅₀ values of 70 \pm 30 nM (**31**) and 1420 \pm 190 nM (**4**) (Figures S1B and S1C). To investigate the effect of the ribose group on the interaction of the adenosine ligands with ABCG2, we designed compounds **32**, **33**, and **34** by replacing the methanocarba ring system of compounds **25**, **26**, and **27** with a ribose group at position R⁴. These adenosine derivatives with a ribose-containing group all stimulated the ATPase activity of ABCG2 at lower concentrations, indicating that a methanocarba ring that enforces a North conformation was not required for interaction with this transporter (see Table 2).

To further identify ligands with apparent high affinity based on their ability to stimulate the ATPase activity of ABCG2 at low nanomolar concentrations, we decided to select known adenine derivatives recently reported to have moderate binding affinity toward the human A₃ adenosine receptor [16]. These ligands were synthesized based on the *N*⁶ and C2 modifications of adenine derivatives, and their effect on the ATPase activity of ABCG2 was determined, and results can be seen in Table 3. All adenine derivatives, which are compounds **35**, **36**, **37a-c**, **38**, **39**, and **40**, were observed to stimulate the basal ATPase activity of ABCG2 at 100 nM and 1 μ M concentrations, unless otherwise indicated in the table legend. To investigate the extent of their stimulation, we decided to select compounds **35** and **37c** and test whether these ligands can stimulate the ATPase activity of ABCG2 in the nanomolar range. For this reason, we tested concentrations ranging from 1 nM to 250 nM, with EC₅₀ values of 50.0 \pm 10.0 nM for **35**, and EC₅₀ value of 13.2 \pm 1.7 nM for **37c** (Figure 1C).

Based on the results obtained concerning the interaction of compound **37c** with ABCG2, we then decided to design adenosine ligands with the goal of improving their affinity towards the transporter. Compounds **41**, **42**, **43**, and **44** were all modified by replacing the methyl

group at the R⁶ position of compound **27** with an ethyl group, yielding compound **41**. Compounds **42** (1-deaza), **43** (3-deaza), and **44** (7-deaza) are structurally like **41** but have a CH in place of a nitrogen atom on the ring system (see Table 4). As shown in Table 4, compounds **41**, **42**, and **43** all stimulated the basal ATPase activity of ABCG2 at a 1 μM concentration, although we observed no interaction with the transporter at a 100 nM concentration. In the case of compound **44**, we observed partial stimulation of ATPase activity at a 100 nM concentration. Compounds **45** - **52** were all designed by replacing the ethyl group at position R⁶ of **41** with (CH₂)₂CH₃ (**45**), CH(CH₃)₂ (**46**), (CH₂)₃CH₃ (**47**), (CH₂)₂-CH(CH₃)₂ (**48**), (CH₂)₂-c-Hex (**49**), CH₂Ph (**50**), (CH₂)₂Ph (**51**), and (CH₂)₃Ph (**52**). Most of these compounds had no effect on the basal ATPase activity of human ABCG2 at a concentration of 100 nM, suggesting that these adenosine ligands have low affinity towards ABCG2. On the other hand, compounds **45**, **50**, and **52** all showed partial stimulation of the ATPase activity of ABCG2. Moreover, compound **53** was synthesized by replacing the 2-chloro-thien-5-yl at the R⁵ position of (compound **27**) with 2-fluoro-thien-5-yl, and the methyl group at the R⁶ position with ethyl, in an effort to increase its ABCG2 affinity. However, the results shown in Table 4 indicate that compound **53** had no effect on the ATPase activity of ABCG2 at a 100 nM concentration.

In the previous series of adenosine ligands tested, we identified compound **37c** to be a high affinity ligand that stimulated the basal ATPase assay of ABCG2 in the nanomolar range with an EC₅₀ value of 13.2 ± 1.7 nM. To improve on the affinity of ligands toward ABCG2, we decided to structurally design compound **54** by replacing the hydrogen atom at position R⁴ of **37c** with the methanocarpa ring system. Compound **54** was observed to stimulate the basal ATPase activity of ABCG2 at both 100 nM and 1 μM concentrations. Afterward, we synthesized compound **55** which is structurally almost identical to **54**, differing only by the substitution of the hydroxyl group and methoxy group on the phenyl ring of position R¹. We observed stimulation of basal ATPase activity of ABCG2 at 100 nM (see results in Table 4). Furthermore, compounds **56** and **57** were synthesized by replacing the 2-fluoro-thien-5-yl-ethynyl group at position R⁵ of **44** with iodo in the case of **56**, and with a phenyl ethynyl group in **57**. These compounds all showed no interaction with the protein at a 100 nM concentration. Compounds **58** and **59** on the other hand, which had structural features similar to those of **57**, differing only at position R¹, also showed no effect on the ATPase activity of ABCG2.

The aim of structurally modifying these ligands is to identify adenosine analogs which can interact with ABCG2 at the nanomolar range. For this reason, compound **60** was designed by replacing the N⁶-cyclopropylmethylamino group of compound **59** at position R¹ with 2-phenylethylamino. Compound **60** was observed to stimulate the ATPase activity of ABCG2 at both 100 nM and 1 μM, with a percentage stimulation of 77% at a 100 nM concentration (Table 4). We then tested a range of concentrations of **60** from 1 nM to 250 nM and obtained an EC₅₀ value of 13.2 ± 2.2 nM, as shown in the graph in Figure 1D. To improve on the affinity, compound **61** was synthesized by replacing the phenylethylamino group of **60** at position R¹ with 3-phenylpropylamino. Subsequently, compound **62** was later designed by replacing the phenyl ring at position R² of **61** with a 2-chloro-thien-5-yl moiety to increase the ionic bonding or π-π interactions between the adenosine ligand and

residues in the drug binding pocket of ABCG2. Compounds **61** and **62** were observed to partially stimulate the ATPase activity of ABCG2 at a 100 nM concentration. In addition, compound **63**, which is structurally like **55** but differs by replacing the 2-chloro-thien-5-yl at position R⁵ of **55** with a phenyl ring and adding an extra carbon atom on the chain of the functional group at position R¹ of compound **55**. Furthermore, we designed compound **64**, which is structurally almost identical to **63** except that the phenyl ring of **63** was replaced with 2-chloro-thien-5-yl at position R² of **64**. From the results shown in Table 4, compound **63** was observed to partially stimulate the activity of ABCG2 at a 100 nM concentration. Compound **64**, on the other hand, had a percentage stimulation of 74% at 100 nM, and we later tested the concentration range from 1 nM to 250 nM, with an EC₅₀ value of 24.3 ± 4.9 nM, as shown in Table 5. The SAR results suggest that compounds **37c** and **60** are most likely high affinity ligands for ABCG2 based on modulation of ATPase activity at low nanomolar concentrations and could be useful candidates for pharmacological probes of the human A₃ adenosine receptor.

2.2 Selected A₃ adenosine ligands inhibit Mitoxantrone transport mediated by ABCG2

Based on structural-activity analysis, we selected A₃ adenosine receptor ligands that have a high affinity with ABCG2 for substrate transport assays to determine if these compounds affect the transport function of ABCG2. To reveal this, substrate transport assays were performed using flow cytometry. For these assays, R-5 cells expressing ABCG2 and pcDNA control cells were used. Mitoxantrone was used as a fluorescent substrate of ABCG2, as described in Materials and Methods.

As shown in Figure 2A, the fluorescence intensity of R-5 cells (blue) was lower than that of pcDNA cells (red) indicating mitoxantrone was pumped out by ABCG2 transporters expressed on the R-5 cell surface. Adding an A₃ adenosine ligand to R-5 incubating cells increased the fluorescence intensity, indicating that the compound inhibited mitoxantrone efflux by ABCG2. All selected A₃ adenosine ligands, as shown in Table 5, were able to inhibit mitoxantrone efflux completely (more than 80% inhibition) at a concentration of 10 μM. Of these compounds, compound **60** demonstrated the most significant inhibitory effect, with an IC₅₀ value of 0.44 ± 0.06 μM, as shown in Figures 2A and 2B. Therefore, compound **60** was determined to be a lead compound for further analysis.

2.3 Interaction of compound 60 with residues in the drug-binding pocket of ABCG2

To determine whether the lead A₃ adenosine receptor ligand (compound **60**) binds to the drug-binding pocket of human ABCG2, docking analysis was done using the cryo-EM structure of a human ABCG2 mutant bound to a substrate (PDBID: 6HCO)[22]. In this structure, a single molecule of estrone-3-sulfate (E₁S), an endogenous substrate of ABCG2, is bound in the central cavity of the drug-binding pocket. In our docking simulations we set 26 residues from each monomer in the binding cavity to have flexible side chains and docked the structure of compound **60**. We obtained 18 different docking poses with docking scores ranging from -12.9 kcal/mol to -11.9 kcal/mol. In the lowest energy pose, we identified 21 residues within 4.5 Å from the ligand, belonging to both monomers. Among these, eleven were found within 4.5 Å of E₁S (Figure 3), suggesting an overlap between the binding of both compound **60** and E₁S. The mostly hydrophobic residues in close proximity

are predicted to interact favorably with the hydrophobic substituents of **60**, including its R¹ and R⁵ groups. No polar interactions were observed. Compound **60** might also bind to the NBDs of ABCG2. However, based on its stimulatory effect on the ATPase activity of ABCG2, that would be very unlikely. This needs to be explored further.

2.4 Comparison of affinity for A₃ adenosine and other receptors

The affinities of the analogues at the A₃ adenosine receptor, the k-opioid receptor and the TSPO (translocator protein, also known as peripheral benzodiazepine receptor, on the outer mitochondrial membrane) are listed in Tables S1 – S4 (Supporting Information). Many of these receptor binding affinities were previously reported [12–14, 17–20, 23]. SAR analysis showed a separation of adenosine receptor affinity and ABCG2 activity. For example, the human A₃ adenosine receptor agonists of single digit nM affinity (**4**, **5**, **8** – **11**, **15**, **17**, **25** – **29**, **31** – **33**, **45**, **50**) showed variable effects on ABCG2. Among them: the following compounds stimulated ATP hydrolysis at multiple concentrations: **4**, **5**, **26**, **27**, **29**, **31** – **33**, **45**, **50**; the following compounds stimulated ATP hydrolysis at 2.5 μM, but not 10 μM: **8**, **10**, **15** and **25**. The following compounds inhibited ATP hydrolysis at both concentrations: **16** – **18** and **20**, but **19**, **21**, and **28** inhibited only at 10 μM. Among a separated subset of compounds that achieved 50% ATPase stimulation at 2.5 μM (**4**, **5**, **7** – **9**, **11** – **15**, **25** – **27**, **29** – **33**, **35**, **37** – **40**, **55**, **63** and **64**) or 50% inhibition at 2.5 μM (**16**), compounds **7**, **12** and **30**, **35**, **37** – **40**, **63** and **64** were weak in A₃ adenosine receptor binding (K_i 99 nM).

Compounds **56** – **64**, which had considerable ABCG2 activity, were 5'-ester derivatives that lacked the adenine 7 position nitrogen; thus, they are 7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine derivatives. One of the important results of this SAR study of the ABCG2 transporter is that the 7-deaza and 5'-ethyl ester analogues are preferred. It is noteworthy that a potent stimulator of ATP hydrolysis, **60**, a 7-deaza-5'-ester derivative, has low A₃ adenosine receptor affinity (K_i 0.483 μM for the human and >10 μM for the mouse homologues) [17]. Compound **60** was inactive at the human A₁ and A_{2A} receptors [17]. Furthermore, similar 5'-ester derivatives in the methanocarpa series have low efficacy as partial agonists at the A₃ adenosine receptor. This compound also bound to the human k-opioid receptor as a weak partial agonist with a binding K_i value of 0.21 μM [17].

The affinities of the adenine derivatives (Table 3) for ABCG2 were greater, in general, than for the adenosine receptor (Table S3, Supporting Information). Compound **37c**, a weak adenine antagonist of the A₃ receptor (K_i 6.82 μM at the human homologue) [16] was able to potently stimulate ATP hydrolysis, similar to the activity of **60**. Among the adenine derivatives studied (Table 3), the 9-H and 9-methyl analogues were comparable in their ABCG2 activity (cf. **37** and **40**). The adenine derivatives had highly variable human A₃ receptor affinity (Table S3, Supporting Information) but were consistently weak or inactive in other adenosine receptor subtypes [16]. Compound **37c** was inactive at the human A₁ and A_{2A} receptors. In this adenine series related to **37c**, as reported by Yu et al. [16], four analogues were found to be inactive as the mouse A₃ receptor. Thus, we found that some of the adenine derivative compounds were more selective for ABCG2 compared to adenosine receptors.

3. Discussion

Due to its involvement in the development of MDR, ABCG2 is one of the most thoroughly studied ABC transporters, similar to P-gp. Recently ABCG2 inhibitors of diverse chemical classes, including repurposed known drugs, have been reported for potential use as anti-cancer agents [24, 25]. To understand the mechanism of the transport function of ABCG2, it is important to understand interactions between the drug-binding site of ABCG2 and drug substrates [26, 27]. We previously found that A₃ adenosine derivatives interact with the drug-binding site of P-gp and can be important modulators of P-gp [15]. Therefore, we decided to extend this approach to ABCG2 and examined the functional effects of an even broader range of adenosine and adenine derivatives on the ATPase activity of this transporter. Synthetic nucleosides are recognized as a good source of useful and orally bioavailable pharmaceuticals, with many derivatives approved for treatment of certain diseases. Furthermore, the approach of conformationally constraining the ribose-like moiety by means of a bicyclic methanocarba substitution has been applied to a wide range of drug targets [17, 20, 28, 29].

In this study, we employed a structural-activity relationship approach [16] as a tool to develop a pharmacophore model for the interaction of A₃ adenosine receptor ligands with ABCG2. We tested the modulatory effect of the derivatives, a total of 63 compounds, on ABCG2-mediated ATP hydrolysis. Of these compounds, 57 compounds were found to modulate ABCG2 ATP hydrolysis, with 50 compounds stimulating and 7 compounds inhibiting the basal ATPase activity. These results contrast with our previous findings with P-gp, as most of the tested A₃ adenosine derivatives showed neither stimulation nor inhibition of basal ATPase activity of that transporter [15]. It seems that the A₃ adenosine ligands are more interactive and have greater affinity with ABCG2 than with P-gp.

Among the compounds that stimulate ABCG2 ATPase activity, adenine derivative **37c** and 5'-ethyl ester derivative **60** were identified to be high-affinity ligands for ABCG2 with EC₅₀ values of 13.2 ± 1.7 and 13.2 ± 2.2 nM, respectively (Table 5). Several other compounds stimulated ABCG2 ATPase activity at nearly the same level of potency (EC₅₀ values, nM), i.e., adenine derivative **35** (50.0 ± 10.0) and 5'-ethyl ester derivative **64** (24.3 ± 4.9). Compound **60** contained a 2-phenylethyl group at the N⁶ position, while the corresponding substituent of **64** was a modified 3-phenylpropyl group. N⁶-(2-Phenylcyclopropyl) derivatives **13** – **15** also potently stimulated ATPase activity. Both adenine derivatives **35** and **37c** contained the following substitutions: 9-H, N⁶-arylalkyl and C2-arylethynyl. Figure 4 is a schematic representation of how adenosine derivatives affect the ATPase activity of ABCG2. In summary, several compounds potently stimulated ATPase activity with weak adenosine receptor interaction (e.g., **37c** and **60**), while other examined nucleoside analogues were potent A₃ adenosine receptor agonists and weak ABCG2 and P-gp modulators (e.g., riboside 5'-methylamide **4** and (N)-methanocarba 5'-methylamide derivative **26**). Other compounds were relatively potent modulators of both ABCG2 and the A₃ adenosine receptor (e.g., riboside 5'-methylamide with a C2-arylethynyl group **32**, which was additionally very weak at P-gp). However, the 5'-truncated (N)-methanocarba derivative **30** inhibited the ATPase activity of ABCG2 and was additionally potent at stimulating P-gp ATPase activity (116% increase of ATPase activity at 2.5 μM [15]).

Substrate transport assays were performed for selected A₃ adenosine receptor ligands, and all tested compounds were able to completely inhibit mitoxantrone transport by ABCG2. Compound **60** had the greatest inhibitory effect on mitoxantrone transport, with an IC₅₀ value of 0.44 ± 0.06 μM (Figure 2 and Table 5). In addition, our docking data suggests that compound **60** can bind in the drug-binding cavity of ABCG2. Furthermore, the many residues within 4.5 Å allow extensive interactions between (N)-methanocarba 5'-ester derivative **60** and ABCG2, which seems to be essential for distinguishing substrates and inhibitors [30]. These results suggest that this lead compound strongly interacts with ABCG2 and plays an important role in the modulation of substrate transport and ATP hydrolysis.

In conclusion, we found that A₃ adenosine receptor ligands can exhibit a greater modulatory effect on ABCG2 activity than on P-gp activity. We were able to identify lead compound **60** as a promising candidate modulator of ABCG2. Our results could be useful for the development of highly potent ABCG2 inhibitors.

4. Materials and Methods

4.1 Chemicals

Dimethyl sulfoxide (DMSO), sodium butyrate compounds **31** and **4** and other chemicals, except the specified, fluorescent substrates used in this work, were obtained from Sigma-Aldrich (St. Louis, MO).

4.2 Chemical Synthesis

The adenosine and adenine derivatives (Tables 1 – 4) included many already published A₃ adenosine receptor agonists and antagonists (Tables S1 – S4, Supporting Information) [12–20, 24]. Many of the derivatives were based on a (N)-methanocarba substitution of the native ribose moiety, which utilizes a rigid bicyclic ring to fix a North-envelope conformation of the pseudo ribose ring [12, 17, 20]. Also, various substitutions (including truncation) of the N⁶, 5' and C2 positions were examined. Finally, adenine derivatives lacking the ribose moiety were also compared. The newly synthesized C2-arylethynyl compounds are: 5'-hydroxy (N)-methanocarba **12**, **22** and **23**; 5'-methylamide (N)-methanocarba **16**, **18** – **21**; and 5'-ester-7-deaza (N)-methanocarba **61** – **64** analogues. Table 1 includes C2-arylethynyl (N)-methanocarba analogues (both 5'-methylamide and 5'-hydroxy). Table 2 includes a variety of ribose and (N)-methanocarba analogues, including 4'-truncated compounds. Table 3 represents N⁶ substituted C2-arylethynyl adenine derivatives (both 9-H and 9-CH₃), with two 7-deazaadenines are included (**38**, **39**). Table 4 shows C2-arylethynyl (N)-methanocarba analogues containing 5'-esters. The details of the chemical synthesis of the new compounds and representative NMR and mass spectra results are provided in the Supporting Information.

4.3 Preparation of total membrane vesicles from High-Five insect cells

The membrane vesicles were prepared by hypotonic lysis of High-Five insect cells expressing the human multidrug transporter ABCG2 and differential centrifugation, as previously described [31, 32].

4.4 ATPase activity of ABCG2

Human ABCG2-mediated ATPase activity was measured using methods previously described [33]. Briefly, total membrane vesicles (10 µg protein/tube) were diluted with ATPase 2X buffer (50 mM MES-Tris (pH 6.8), 50 mM KCl, 10 mM MgCl₂, 5 mM NaN₃, 1 mM EGTA, 1 mM ouabain, and 2 mM DTT). One µL of an adenosine or adenine analog dissolved in dimethyl sulfoxide (DMSO) in the presence and absence of sodium orthovanadate was added to the ATPase assay mixture. DMSO was added as a control sample in this experiment. The mixtures were pre-incubated at 37°C for 2 min before the addition of 5 mM ATP to each test tube to start the reaction. After 20 min the reaction was stopped by addition of SDS, and the inorganic phosphate generated was measured colorimetrically. Three independent experiments in duplicate were carried out and the results are reported as mean ± SE.

4.5 Cell culture

Human embryonic kidney cells (HEK293) transfected with either empty pcDNA vector (named pcDNA cells) or pcDNA3 vector containing full-length ABCG2 (termed R-5 cells) were provided by Dr. Robert W. Robey, Laboratory of Cell Biology, National Cancer Institute. Cells were cultured in Eagle's Minimum Essential Medium (Corning, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mg/ml G418 (Invitrogen, Carlsbad, CA, USA), as described previously [34]. High-Five insect cells were obtained from ThermoFisher Scientific (Waltham, MA).

4.6 Cell surface expression of ABCG2 and transport assay

Cell surface expression and the effect of the test compounds on the transport function of ABCG2 and P-gp was determined using flow cytometry [34–36]. The BacMam baculovirus transduction system was used to express P-gp on HeLa cells for transport function assays of P-gp, as described previously [37, 38]. To determine the cell surface expression of ABCG2, R-5 cells were incubated in Iscove's Modified Dulbecco's Medium (IMDM) containing 5% FBS with phycoerythrin-labeled 5D3 antibody (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 1 hour. After incubation, cells were washed with cold IMDM and resuspended with cold PBS containing 1% bovine serum albumin (BSA). In transport assays, R-5 ABCG2-expressing cells were incubated in IMDM medium containing 5% FBS with fluorescent substrate, mitoxantrone for ABCG2 at a final concentration of 5 µM at 37°C for 45 min. Then the cells were washed with cold IMDM and resuspended with cold PBS containing 1% BSA. The cell surface expression of ABCG2 and the transport was measured by flow cytometry using a FACS CANTO II instrument with FACS Diva software (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR USA).

4.7 In silico docking of compounds in the drug-binding pocket of ABCG2

AutoDock Vina [39] software was used to dock compound **60** in the structure of the inward-open conformation of ABCG2 bound to estrone-3-sulfate and 5D3-Fab (PDBID: 6HCO) [22]. The MGLtools software package (Scripps Research Institute, La Jolla, CA, USA)

was used to prepare the pdbqt files. In our docking simulations we set 26 residues from each monomer in the binding cavity to have flexible side chains and docked the structure of compound **60**. These residues were N393, N398, V401, L405, I409, T413, N424, F431, F432, T435, N436, F439, S440, V442, S443, Y538, L539, T542, I543, V546, F547, M549, I550, L554, L555. The receptor grid centered at $x = 155$, $y = 155$, and $z = 149$, and a box with dimensions $44 \text{ \AA} \times 40 \text{ \AA} \times 50 \text{ \AA}$ was used. The exhaustiveness level for both proteins was set at 100 to ensure that the global minimum of the scoring function would be found considering the large box size and the number of flexible residues. Molecular models were analyzed using PyMOL, Version 1.7, Schrödinger, LLC [40, 41] (New York, NY, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

ABC transporters	ATP-binding cassette transporters
AR	adenosine receptor
MDR	multidrug resistance
TMD	transmembrane domain
NBD	nucleotide-binding domain

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Highlights

- Interaction of adenosine/adenine derivatives was more widespread with ABCG2 than P-gp
- Distinct SAR for ABCG2 ATPase stimulation compared to A₃ adenosine receptor affinity
- 7-Deaza (N)-methanocarba 5'-ester derivative **60** stimulated ATPase with EC₅₀ 13.2 nM
- 2-(5-Cl-thien-2-yl)ethynyl-adenine derivative **37c** stimulated ATPase with EC₅₀ 13.2 nM

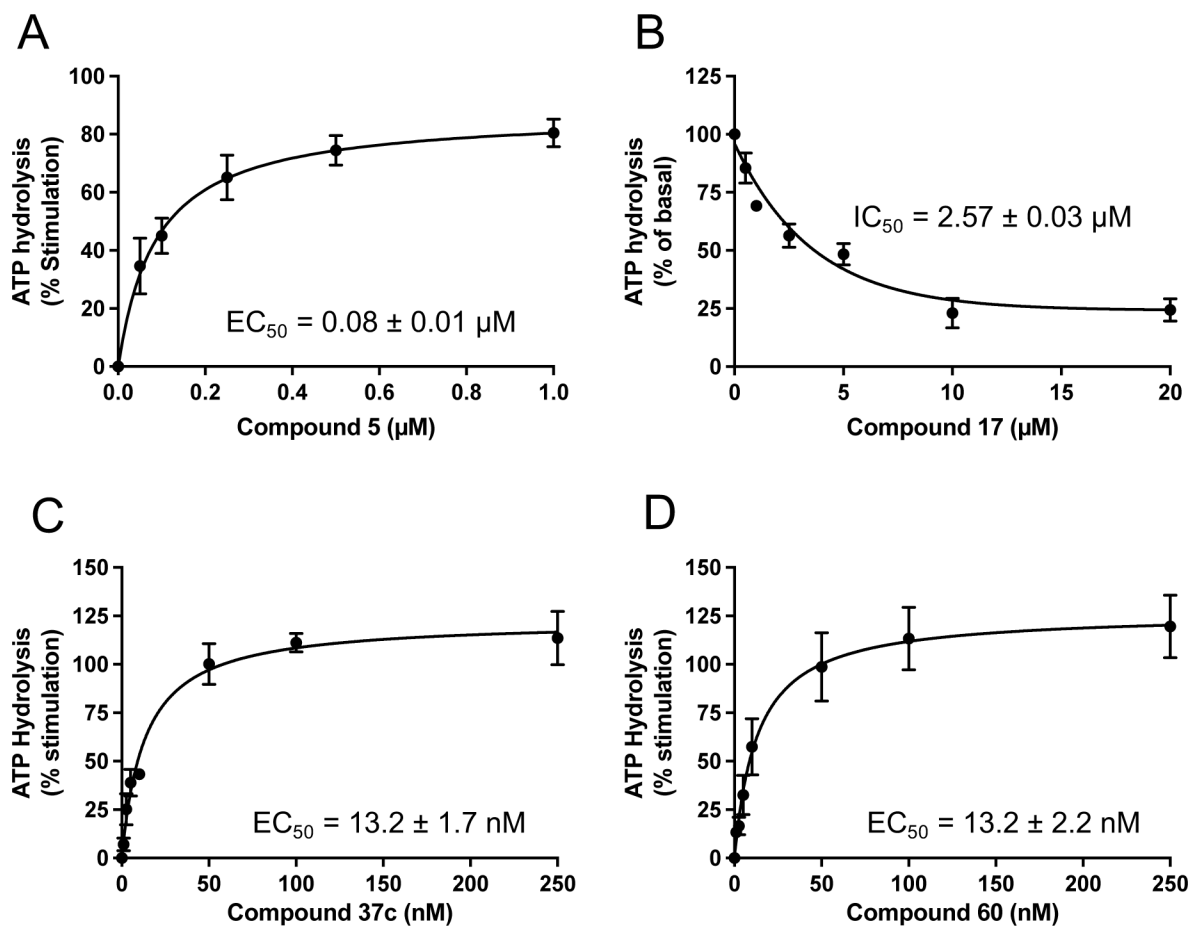


Fig. 1. Modulation of ABCG2 ATPase activity by A₃ adenosine receptor ligands. Results for four representative ligands, the potent A₃ agonist **5** (A), triazole-extended 6-benzylamino derivative **17** (B), adenine (A₃ antagonist) derivative **37c** (C), and (N)-methanocarpa-7-deazaadenosine 5'-ester derivative **60** (D) are shown. Each experiment was performed at least three times in duplicate. The EC₅₀ and IC₅₀ values were calculated using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

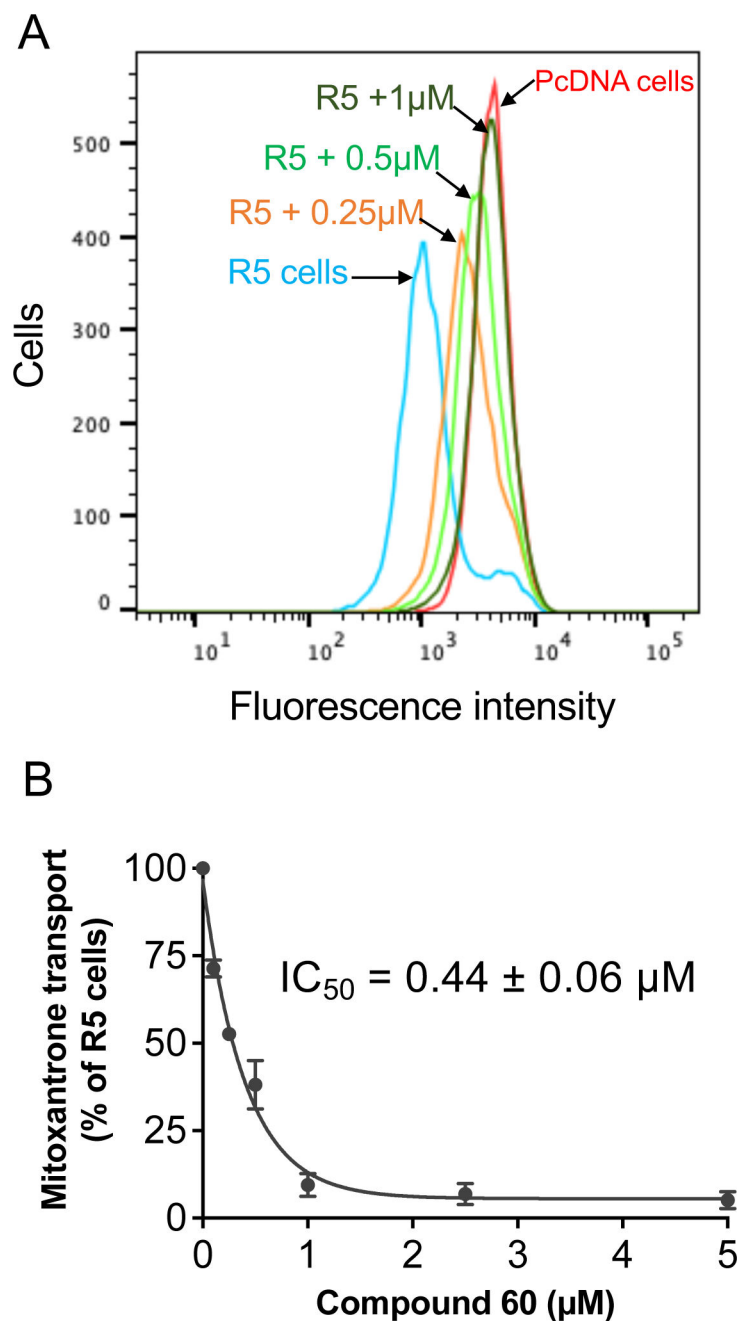


Fig. 2. Compound **60** inhibited mitoxantrone transport mediated by ABCG2. (A) Representative histogram showing the effect of compound **60** on mitoxantrone transport at indicated concentrations. Fluorescence intensity of pcDNA control cells is shown in red and R-5 cells expressing ABCG2 are shown in blue. The reversal effect of compound **60** for R-5 cells at 0.25, 0.5 and 1 μ M are shown in orange, light green, and green, respectively. (B) Concentration-dependent inhibition of mitoxantrone efflux by compound **60**. Mitoxantrone efflux by R-5 cells in the absence of compound **60** was taken as 100%, and the percentage of efflux at the indicated concentrations of compound **60** was calculated. Data points were

plotted as the mean \pm S.D. ($n = 3$). Compound concentration that produces 50% inhibition of mitoxantrone transport in R-5 cells is given as IC_{50} value, calculated using GraphPad Prism.

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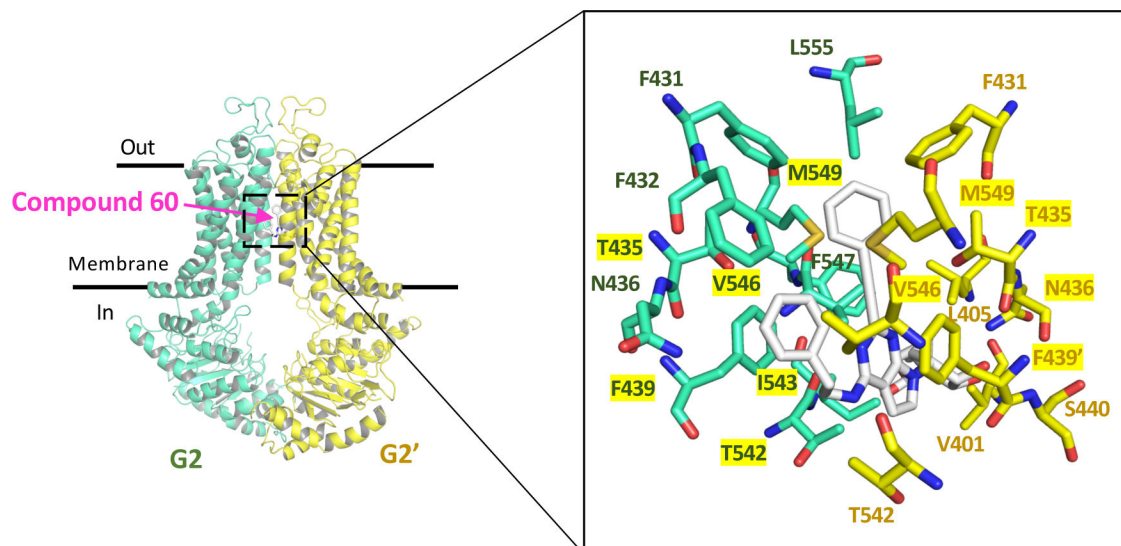


Fig. 3. Docking of (N)-methanocarba-7-deazaadenosine 5'-ester derivative **60** in the drug-binding pocket of human ABCG2. The cryo-EM structure of a human ABCG2 mutant bound to E₁S (PDBID: 6HCO) was used as a template for docking. On the left side, the structure of ABCG2 depicts the two monomers of (G2 and G2') in cyan and yellow cartoons and the binding site for the compound **60** is boxed. The close-up in the right panel depicts the expanded region showing the lowest energy docking pose for compound **60** and the residues in the drug-binding pocket interacting with it. A total of 21 residues were identified to be within 4.5 Å of compound **60**, and eleven of these (highlighted in yellow) were also within 4.5 Å of substrate E₁S.

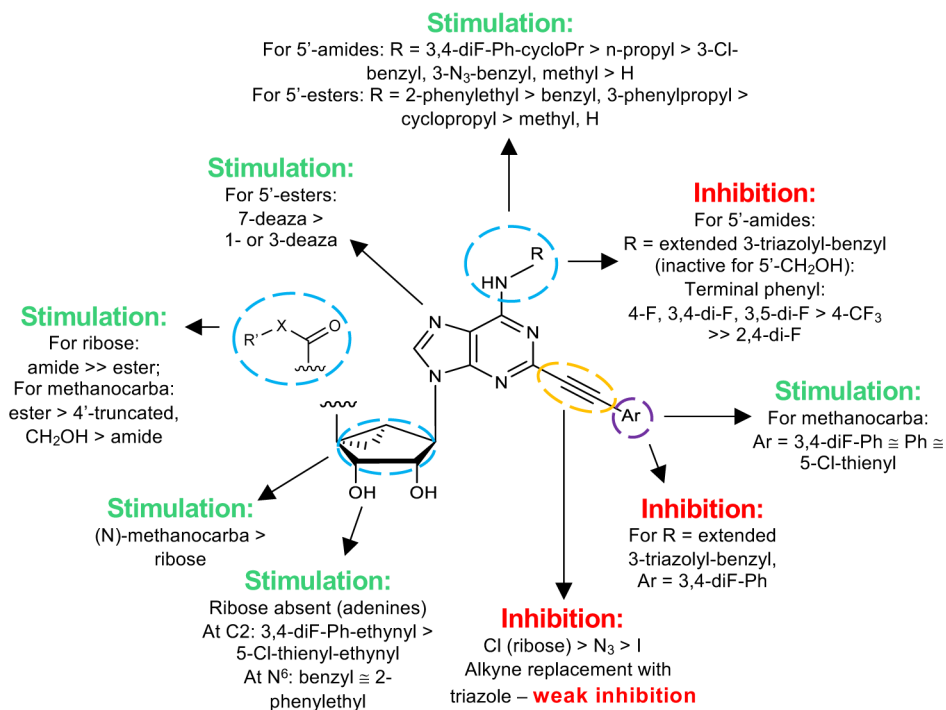
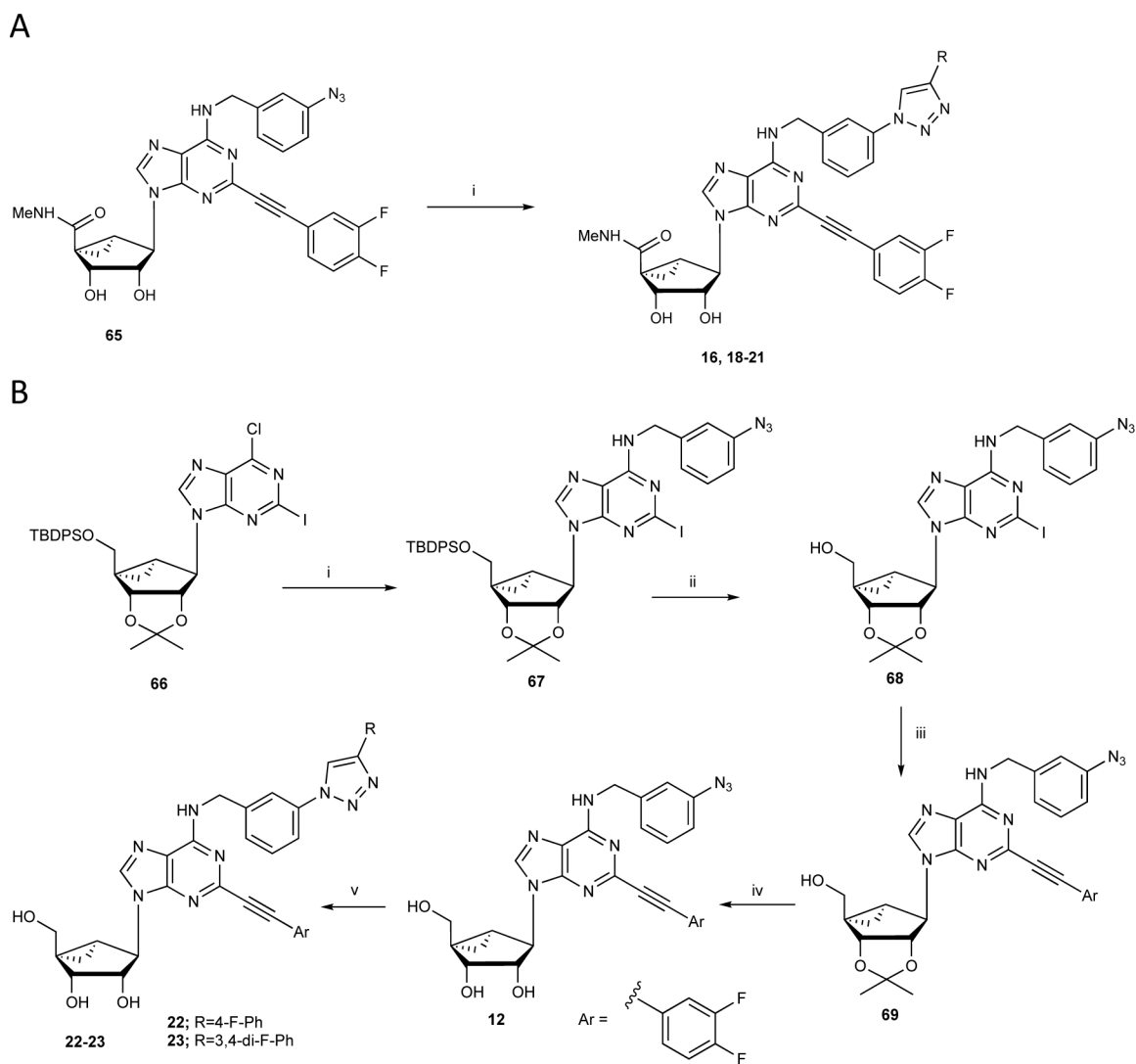
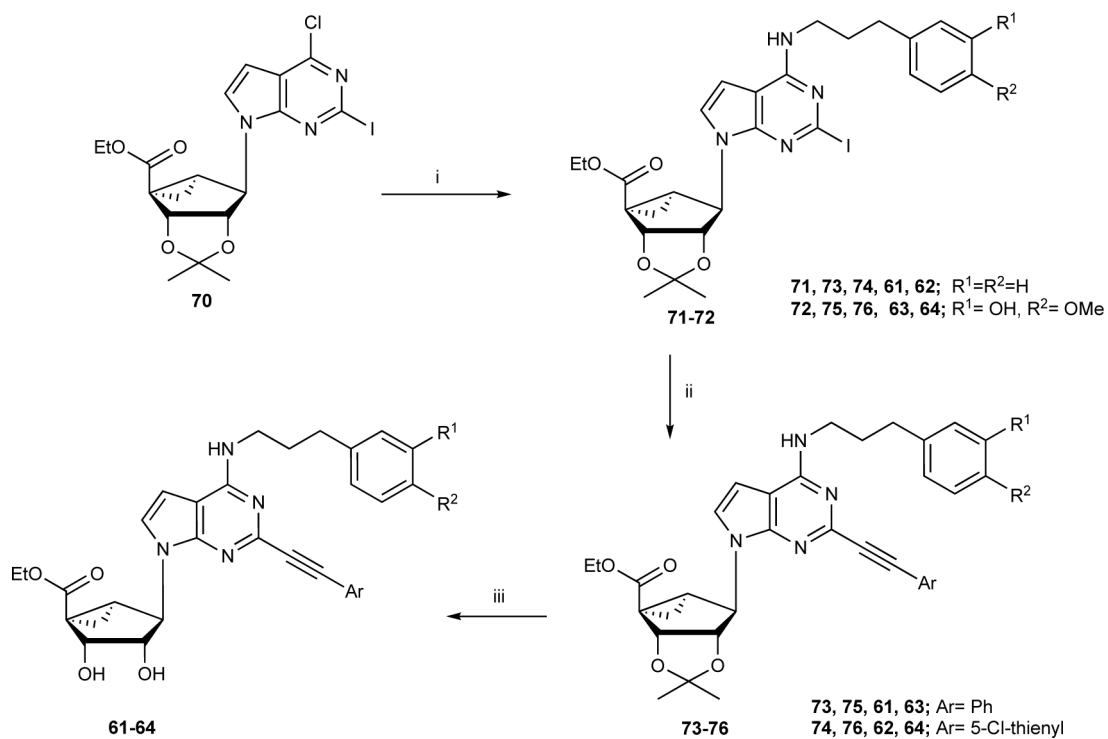


Fig. 4.
Schematic representation of the modulatory effect of tested nucleoside adenines on the ATPase activity of human ABCG2.

**Scheme 1.**

Synthesis of (N)-methanocarba nucleoside derivatives **12**, **16** and **18 – 23**. A) Reagents and conditions: (i) phenylacetylene, sodium ascorbate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, *t*-BuOH- H_2O , rt, 87–90%. B) (i) 3-azidobenzylamine, DIPEA, 2-propanol, 87%, (ii) TBAF, THF, rt, 91%; (iii) 3,4-di-F-phenylacetylene, $\text{PdCl}_2(\text{Ph}_3\text{P})_2$, CuI, Et_3N , DMF, 68%; (iv) 10% TFA, MeOH, 70°C, 89%; (v) phenylacetylene, sodium ascorbate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, *t*-BuOH- H_2O , rt, 87–88%.

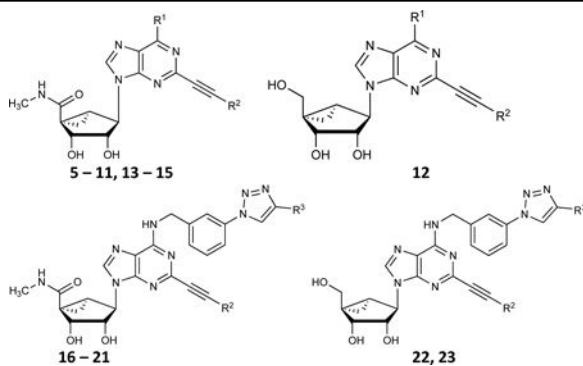
**Scheme 2.**

Synthesis of 7-deaza (N)-methanocarba nucleoside 5'-esters **61** – **64**. Reagents and conditions: (i) Ph(CH₂)₃NH₂, DIPEA, 2-propanol, 69–72%; (ii) arylalkyne, PdCl₂(Ph₃P)₂, CuI, Et₃N, DMF, rt, 67–72%; (iii) 10% TFA, MeOH, 70 °C, 88–91%.

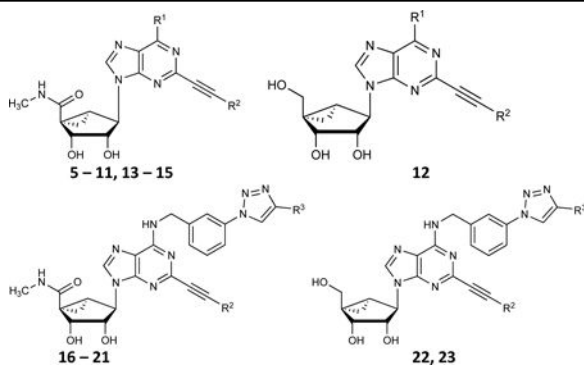
Table 1

Effects of N^6 and C2-phenylethynyl modifications of (N)-methanocarpa 2-arylethynyl-purine derivatives (5'-amides and 5'-alcohols) on the ATPase activity of human ABCG2

No.	R^1 or R^3	R^2	ATPase activity of ABCG2 % Stimulation or inhibition	
			$2.5 \mu\text{M}^b$	$10 \mu\text{M}^b$
<i>6-Benzylamino and 6-Alkyl derivatives</i>				
5			74.4±5.2 ↑ @ 0.5 μM	56.8±9.9 ↑ @ 5 μM
6	H		NE	64.2±11.5 ↑
7	CH ₃		49.6±9.0 ↑	72.6±15.1 ↑
8			51.3±19.1 ↑	NE
9			60.3±8.4 ↑	49.3±15.7 ↑



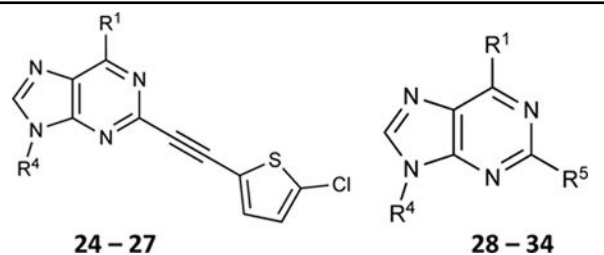
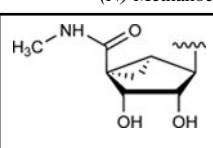
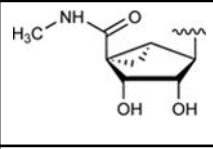
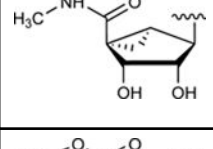
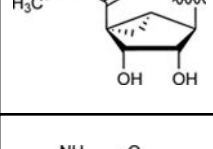
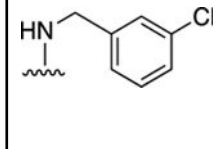
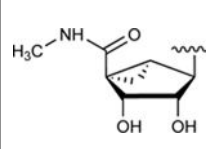
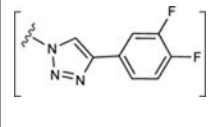
No.	R ¹ or R ³	R ²	ATPase activity of ABCG2 % Stimulation or inhibition	
			2.5 μM ^b	10 μM ^b
10			49.3±12.8 ↑	NE
11			55.5±11.9 ↑ @ 0.5 μM	78.3±8.7 ↑ @ 5 μM
12			61.9±9.8 ↑	NE
<i>6-Phenylcyclopropylamino derivatives</i>				
13			110.6±12.1 ↑	46.7±5.9 ↑
14			74.8±1.9 ↑	ND

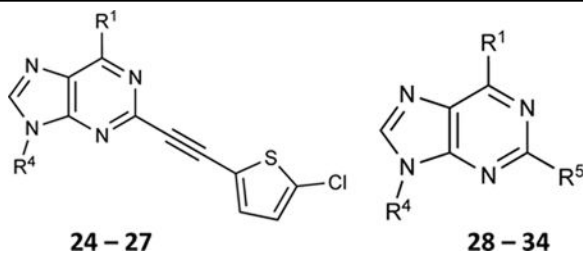


No.	R ¹ or R ³	R ²	ATPase activity of ABCG2 % Stimulation or inhibition	
			2.5 μM ^b	10 μM ^b
15			100.7±26.0 ↑	ND
<i>Triazole-Extended 6-Benzylamino derivatives</i>				
16			54.4±6.1 ↓	74.7±6.3 ↓
17			43.5±5.0 ↓	76.9±6.4 ↓
18			44.7±7.2 ↓	49.4±0.9 ↓
19			NE	57.1±6.3 ↓

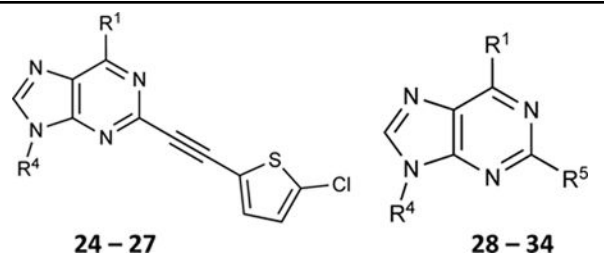
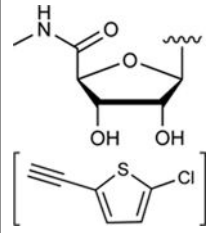
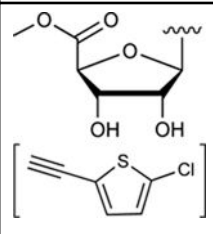
Table 2

Effect of N^6 , C2 and riboside modifications of adenosine derivatives (both (N)-methanocarba and ribose-containing) on the ATPase activity of ABCG2

				
No.	R ¹	R ⁴ [R ⁵]	ATPase activity of ABCG2 % Stimulation or inhibition	
			2.5 μM ^b	10 μM ^b
<i>(N)-Methanocarba derivatives</i>				
24	H		NE	NE
25	NHCH ₃		59.2±5.9 ↑	NE
26	NH(CH ₂) ₂ CH ₃		77.9±3.3 ↑	40.0±7.3 ↑
27	NHCH ₃		89.1±2.1 ↑	79.0±8.1 ↑
28		 	NE	36.6±0.9 ↓



No.	R ¹	R ⁴ [R ⁵]	ATPase activity of ABCG2 % Stimulation or inhibition	
			2.5 μM ^b	10 μM ^b
29			97.5±23.1 ↑	92.9±15.1 ↑
30			75.2±13.8 ↑	54.1±8.6 ↑
<i>Ribose derivatives</i>				
31			66.2±7.3 ↑	98.2±5.4 ↑
4			100.8±5.8 ↑	69.9±6.0 ↑
32	NHCH ₃		111.9±16.8 ↑	121.7±10.5 ↑

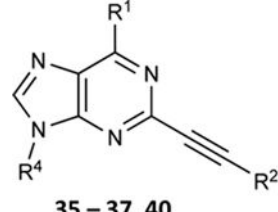
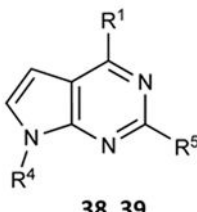
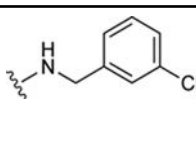
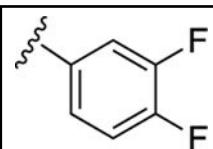
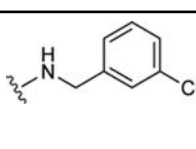
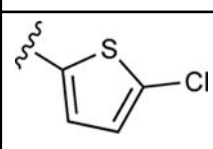
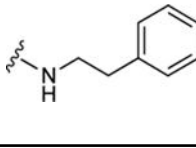
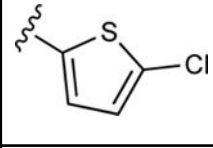
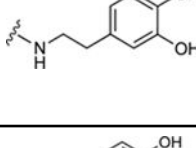
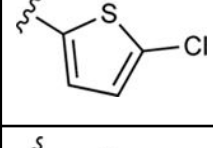
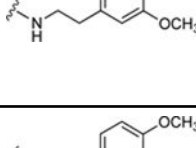
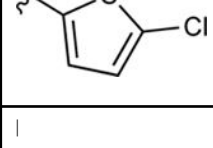
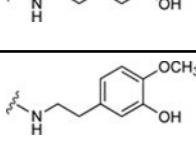
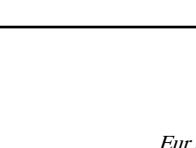
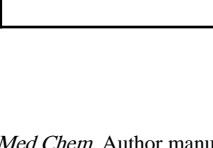
				
No.	R ¹	R ⁴ [R ⁵]	ATPase activity of ABCG2 % Stimulation or inhibition	
			2.5 μM ^b	10 μM ^b
33	NH(CH ₂) ₂ CH ₃		124.5±3.4 ↑	121.7±21.2 ↑
34	NHCH ₃		NE @ 100 nM	52.2±8.7↑ @ 1 μM

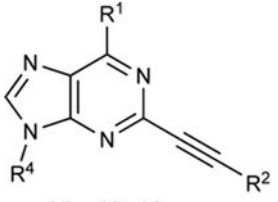
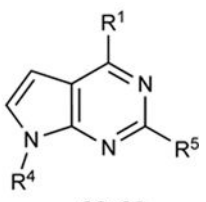
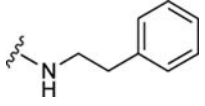
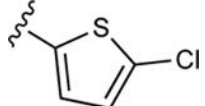
^a Modulation of the basal ATPase activity of ABCG2 by A₃ adenosine receptor ligands showing percentage stimulation or inhibition. Details are the same as given in the legend to Table 1.

^b Concentrations were 2.5 and 10 μM, unless otherwise indicated.

Table 3

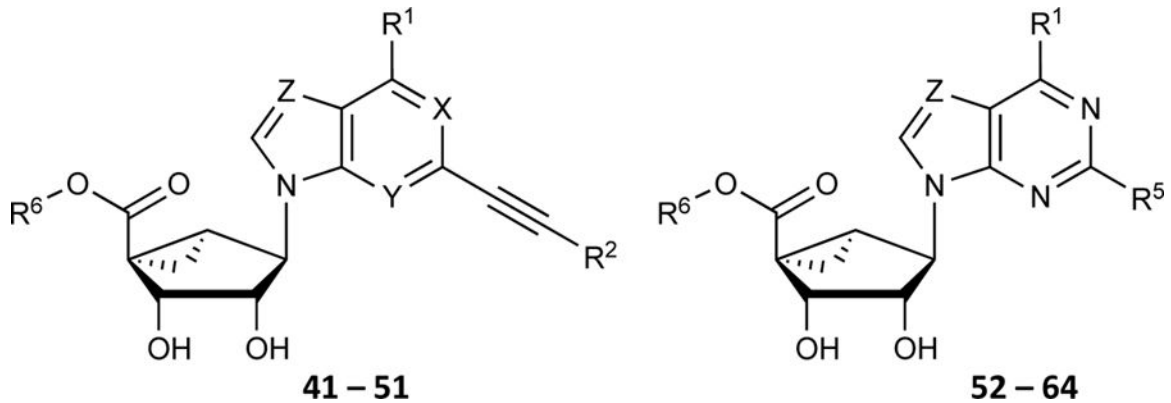
Effect of N^6 and C2 modifications of adenine and 7-deazaadenine derivatives on the ATPase activity of ABCG2. R^4 is either H or CH_3

 35 – 37, 40		 38, 39		
No.	R^1	R^2 or R^5	ATPase activity of ABCG2 % Stimulation or inhibition	
			100 nM ^c	1 μM ^c
$R^4 = H$				
35			165.2±10.1↑ @ 2.5 μM	163.5±4.9↑ @ 10 μM
36			40.5±3.8 ↑	63.7±2.3 ↑
37a			80.8±7.9 ↑	133.1±22.9 ↑
37b			95.9±8.1 ↑	110.5±6.7 ↑
37c			111.2±13.2 ↑	40.0±7.3 ↑
38			79.6±9.6 ↑	142.4±7.3 ↑
39			104.1±12.5 ↑	158.8±4.1 ↑

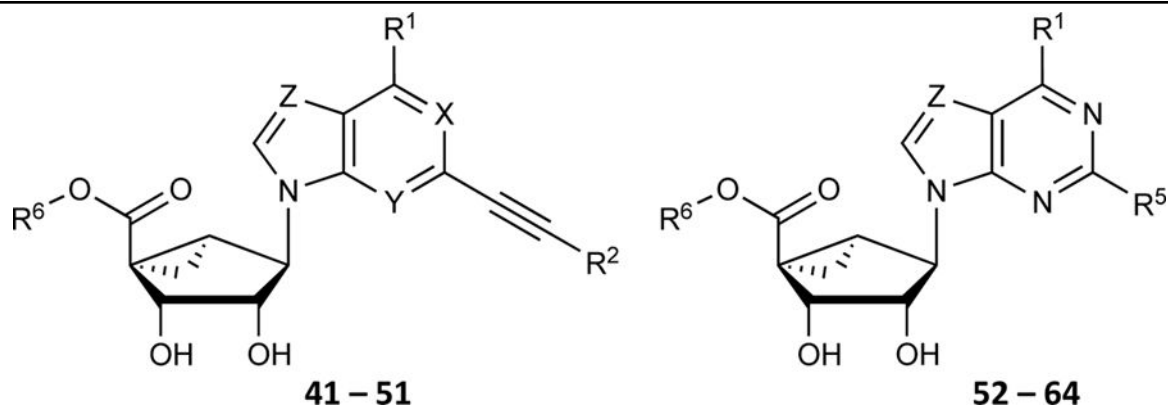
 35 – 37, 40		 38, 39		
No.	R ¹	R ² or R ⁵	ATPase activity of ABCG2 % Stimulation or inhibition	
			100 nM ^c	1 μM ^c
<i>R⁴ = CH₃</i>				
40			92.1±13.9 ↑	108.3±1.4 ↑

^aModulation of the basal ATPase activity of ABCG2 by A₃ adenosine receptor ligands showing percentage stimulation. Details are the same as given in the legend to Table 1.

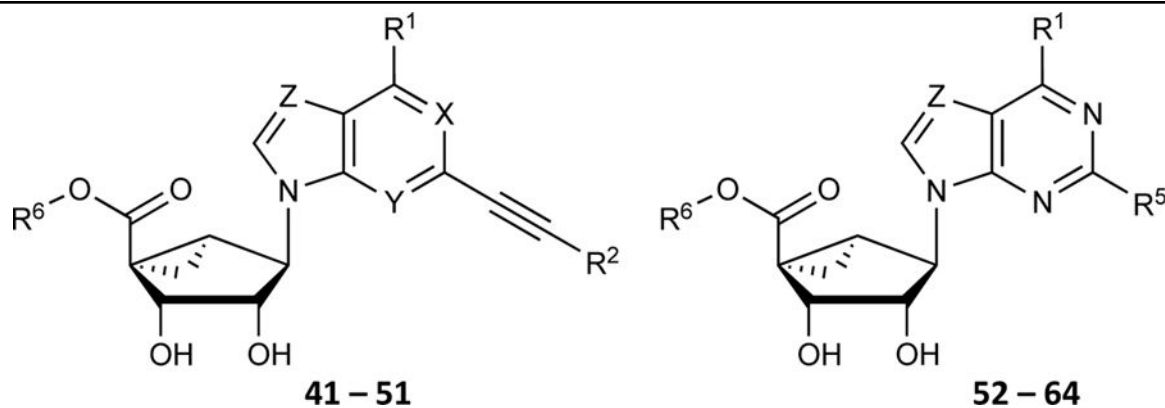
^cConcentrations were 100 nM and 1 μM, unless otherwise indicated.

Table 4Effect of N^6 and C2 modifications of (N)-methanocarba 5'-ester derivatives on the ATPase activity of ABCG2. R^2 is 2-chloro-thien-5-yl, unless noted. X, Y, Z = N, unless otherwise noted


No.	R^1	R^6 (and other)	ATPase activity of ABCG2 % Stimul or Inhibition	
			100 nM	1 μ M
<i>X, Y, Z as indicated</i>				
41	NHCH ₃	CH ₂ CH ₃	NE	92.9 ↑
42	NHCH ₃	CH ₂ CH ₃ (X = CH)	NE	50.3
43	NHCH ₃	CH ₂ CH ₃ (Y = CH)	NE	62.2
44	NHCH ₃	CH ₂ CH ₃ (Z = CH)	41.0±11.2 ↑	90.3
<i>R⁶ as indicated, X, Y, Z = N</i>				
45	NHCH ₃	(CH ₂) ₂ CH ₃	33.9±4.7 ↑	83.5 ↑
46	NHCH ₃	CH(CH ₃) ₂	NE	51.7
47	NHCH ₃	(CH ₂) ₃ CH ₃	NE	92.0
48	NHCH ₃	(CH ₂) ₂ -CH(CH ₃) ₂	NE	50.9
49	NHCH ₃	(CH ₂) ₂ -c-Hex	NE	50.8
50	NHCH ₃	CH ₂ Ph	50.3±0.8 ↑	105 ↑
51	NHCH ₃	(CH ₂) ₂ Ph	NE	64.0
52	NHCH ₃	(CH ₂) ₃ Ph	38.8±1.3 ↑	61.0
<i>R⁶ = CH₂CH₃, X, Y, Z = N</i>				



No.	R ¹	R ⁶ (and other)	ATPase activity of ABCG2 % Stim or Inhibition	
			100 nM	1 μM
58		R ⁵ =	NE	73.0 ↑
59		R ⁵ =	NE	79.8 ↑
60		R ⁵ =	77.3±10.9 ↑	104.0 ↑
61		R ⁵ =	37.9±5.1 ↑	82.8 ↑
62		R ⁵ =	45.5±10.4 ↑	64.3 ↑
63		R ⁵ =	55.9±10.7 ↑	84.9 ↑



No.	R ¹	R ⁶ (and other)	ATPase activity ABCG2 % Stim or Inhibition	
			100 nM	1 μM
64		R ⁵ =	74.2±9.1 ↑	79.7

^aModulation of the basal ATPase activity of ABCG2 by A₃ adenosine analogs showing percentage stimulation. Details are the same as given in the legend to Table 1.

Table 5

EC₅₀ and IC₅₀ values for ABCG2 ATPase activity and inhibitory effect on mitoxantrone efflux by A₃ adenosine receptor ligands

Compounds	ATPase activity EC ₅₀ or IC ₅₀ (nM)	Inhibition of mitoxantrone efflux	
		% of pcDNA control cells ^a , at 10 μM of compounds	IC ₅₀ (μM) ^b
5	EC ₅₀ = 80.0 ± 10.0	99.2 ± 12.0	NC ^c
13	EC ₅₀ = 16.0 ± 3.0	100.0 ± 6.3	NC
14	EC ₅₀ = 13.0 ± 3.0	97.4 ± 12.1	NC
15	EC ₅₀ = 14.0 ± 5.0	97.2 ± 11.9	NC
16	IC ₅₀ = 2590 ± 40	101.7 ± 17.0	NC
17	IC ₅₀ = 2570 ± 30	92.5 ± 13.5	NC
20	IC ₅₀ = 4010 ± 20	105.3 ± 3.0	NC
35	EC ₅₀ = 50.0 ± 10.0	81.4 ± 11.5	NC
37c	EC ₅₀ = 13.2 ± 1.7	110.4 ± 15.8	4.98 ± 1.69
60	EC ₅₀ = 13.2 ± 2.2	92.6 ± 9.2	0.44 ± 0.06
64	EC ₅₀ = 24.3 ± 4.9	109.1 ± 16.4	0.60 ± 0.08

^aFluorescence intensity of pcDNA control cells was taken as a 100% inhibition, and percentage of mitoxantrone efflux inhibition in R-5 cells by 10 μM of A₃ adenosine receptor ligands was calculated compared to pcDNA control cells.

^bCompound concentration that produces 50% inhibition of mitoxantrone efflux in R-5 cells is given as IC₅₀ value.

^cNC, Not calculated.