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Tyrphostin RG14620 selectively reverses ABCG2-mediated multidrug resistance in cancer cell lines

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Abstract

The multidrug resistance (MDR) phenotype associated with the overexpression of ATP-binding cassette (ABC) drug transporters ABCB1, ABCC1 and ABCG2 is a major obstacle in cancer chemotherapy. Numerous epidermal growth factor receptor (EGFR) inhibitors have previously been shown capable of reversing MDR in ABCG2-overexpressing cancer cells. However, most of them are not transporter-specific due to the substantial overlapping substrate specificity among the transporters. In this study, we investigated the interaction between ABCG2 and tyrphostin RG14620, an EGFR inhibitor of the tyrphostin family, in multidrug-resistant cancer cell lines. We found that at nontoxic concentrations, tyrphostin RG14620 enhances drug-induced apoptosis and restores chemosensitivity to ABCG2-overexpressing multidrug-resistant cancer cells. More importantly, tyrphostin RG14620 is selective to ABCG2 relative to ABCB1 and ABCC1. Our findings were further supported by biochemical assays demonstrating that tyrphostin RG14620 stimulates ATP hydrolysis and inhibits photoaffinity labeling of ABCG2 with IAAP, and by a

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Conflict of interest

The authors declare no conflict of interest.

docking analysis of tyrphostin RG14620 in the drug-binding pocket of this transporter. Taken together, our findings indicate that tyrphostin RG14620 is a potent and selective modulator of ABCG2 that may be useful to overcome chemoresistance in patients with drug-resistant tumors.

Keywords

ABC transporter; ABCG2; EGFR inhibitor; Multidrug resistance; Tyrphostin RG14650

1. Introduction

During the course of cancer chemotherapy, many patients develop multidrug resistance (MDR) and are no longer responsive to most conventional anticancer agents, including those that are functionally and structurally unrelated. Unfavorable clinical outcomes caused by the MDR phenotype in cancer patients often lead to cancer relapse and the eventual death of these patients. In spite of the presence of many mechanisms that are known to be involved, operating independently or in unison, the overexpression of ATP-Binding Cassette (ABC) transporters remains a leading cause of acquired cancer drug resistance [19]. MDR-linked ABC transporters such as ABCB1 (P-glycoprotein, P-gp), ABCC1 (multidrug-resistance protein 1, MRP1) and ABCG2 (breast cancer resistance protein, BCRP) are membrane proteins from the ABC protein superfamily that can generate energy from ATP hydrolysis to actively transport anticancer agents across cell membranes [19, 67]. Collectively, this energy-dependent drug transport system is capable of effluxing a large proportion of clinically active anticancer agents, preventing drugs from reaching their targets within a cancer cell [8, 11, 37, 53]. In addition, the transport function of these transporters has an important physiological role in the protection of vital organs such as the brain and testes from xenobiotics. Considering the tissue localization of these transporters, it is not surprising that they have a great impact on the absorption, distribution, metabolism and elimination (ADME) of almost all classes of drugs [7, 20, 53].

It was discovered over 30 years ago that verapamil, a calcium channel blocker that was intended to be used for hypertension and angina, was able to reverse MDR caused by ABCB1 [59]. For many years, direct inhibition of the drug efflux function of a drug transporter has been considered by researchers to be the most effective way to reverse MDR in cancer cells overexpressing ABC drug transporters [65]. Unfortunately, there is currently no modulator that is clinically applicable to reverse MDR in cancer patients, and the search for a selective and potent modulator of MDR-linked ABC drug transporter is still ongoing [65]. One of the major obstacles in developing an effective inhibitor is associated with the lack of selectivity caused by the substantial overlapping of substrate specificity of major ABC drug transporters. Problems associated with high toxicity and unforeseen drug-drug interactions are also common with synthetic inhibitors. Recently, rather than developing novel inhibitors, drug repurposing (also referred to as drug repositioning) of existing therapeutic agents has become a more practical solution to overcome MDR mediated by ABC drug transporters [63]. Therefore, we chose to investigate whether small molecule targeted drugs, such as epidermal growth factor receptor (EGFR) inhibitors, could reverse MDR mediated by ABC drug transporters.

The transmembrane epidermal growth factor receptor (EGFR) belongs to the ErbB/HER family of receptor tyrosine kinases that plays a key role in regulating the proliferation of normal epithelial cells. However, EGFR is often overexpressed, dysregulated, or mutated in tumors, leading to constitutive activation of many intracellular protein kinase signaling pathways that are involved in the development and growth of various epithelial malignancies [35]. Consequently, EGFR is one of the most important molecular targets for cancer treatment. Protein tyrosine kinase inhibitors of the tyrphostin family are compounds small in molecular weight that were designed to selectively target the substrate site of the EGFR kinase domain [18, 31]. Recently, additional biological and pharmacological activities of tyrphostins have been reported, including potent anti-arthritic effects [1], antiviral effects on encephalomyocarditis virus (EMCV) and hepatitis C virus (HCV) [17], as well as inhibition of oxidative stress-induced activation of several members of the transient receptor potential cation (TRP) channels [57]. Tyrphostin RG14620 is one of the most potent tyrphostins, known to inhibit the proliferation of various types of cancer, both *in vitro* and *in vivo* [38, 49, 70]. In addition, co-administration of tyrphostin RG14620 and other therapeutic agents has been reported to be an effective combination regimen. One study demonstrated that tyrphostin RG14620 and retinoids act cooperatively in inhibiting the growth of ovarian cancer cells [22]. Another showed that combination therapy of paclitaxel, tyrphostin RG14620 and the mammalian target of rapamycin (mTOR) inhibitor acts synergistically to promote cell death in endometrial cancer cells [30, 69].

In the present study, we investigated the effect of tyrphostin RG14620 on MDR mediated by the three major ABC drug transporters ABCB1, ABCC1 and ABCG2 in cancer cells. Our data show that tyrphostin RG14620 is a potent and selective modulator of ABCG2. Tyrphostin RG14620 enhances drug-induced apoptosis and reverses MDR in ABCG2-overexpressing cancer cells through direct inhibition of the transport function of ABCG2 protein.

2. Materials and methods

2.1. Chemicals

Phosphate-buffered saline (PBS), RPMI medium, fetal calf serum (FCS), Dulbecco's Modified Eagle's medium (DMEM), trypsin-EDTA, penicillin, and streptomycin were purchased from Gibco, Invitrogen (CA, USA). [¹²⁵I]-Iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA). Annexin V: FITC Apoptosis Detection Kit was purchased from BD Pharmingen (San Diego, CA, USA). Tyrphostin RG14620 and all other chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise.

2.2. Cell culture conditions

The human epidermal carcinoma cell line KB-3-1 and its ABCB1-overexpressing sublines KB-C-1, KB-8-5-11, KB-V-1, human ovarian carcinoma cell line OVCAR-8 and its ABCB1-overexpressing subline NCI-ADR-RES, human non-small cell lung carcinoma cell line H460 and its ABCG2-overexpressing subline H460-MX20, pcDNA3.1-HEK293, ABCB1-transfected MDR19-HEK293, ABCC1-transfected MRP1-HEK293 and ABCG2-

transfected R482-HEK293, were cultured in DMEM. The human large-cell lung carcinoma cell line COR-L23/P and its ABCC1-overexpressing subline COR-L23/R, human colon carcinoma cell line S1 and its ABCG2-overexpressing subline S1-M1-80, human lung adenocarcinoma epithelial cell line A549 and its ABCG2-overexpressing subline A549-Bec150, were cultured in RPMI-1640. All cell lines were cultured in medium supplemented with 10% FCS, 2 mM L-glutamine and 100 units of penicillin/streptomycin/mL. HEK293 and HEK293 transfected lines were maintained in 2 mg/mL G418 [68], whereas 1 mg/mL vinblastine was added to KB-V-1 cell culture medium [43], and 80 μ M of mitoxantrone was added to S1-M1-80 cell culture medium, as described [68]. All cell lines were maintained at 37 °C in 5% CO₂ humidified air and placed in drug-free medium 7 days prior to assay.

2.3. Fluorescent drug accumulation assay

Intracellular accumulation of fluorescent substrates was determined using a FACScan flow cytometer (BD Biosciences) and subsequently analyzed using Cell Quest software (Becton-Dickinson), as described previously [21, 46]. Briefly, after harvesting cells by trypsinization and centrifugation, 3×10^5 cells were resuspended in 4 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% FCS before 0.5 μ M calcein-AM or 1 μ M pheophorbide A (PhA) was added. Calcein-AM is transported by both ABCB1 and ABCC1, whereas PhA is transported by only ABCG2. The fluorescent drug efflux mediated by ABCB1, ABCC1 or ABCG2 was carried out in the presence or absence of tyrphostin RG14620, tariquidar (an inhibitor of ABCB1), MK-571 (an inhibitor of ABCC1), or Ko143 (an inhibitor of ABCG2), as described previously [41]. Calcein fluorescence was detected with excitation and emission wavelengths of 485 and 535 nm, whereas PhA fluorescence was detected with excitation and emission wavelengths of 395 and 670 nm.

2.4. Cytotoxicity assay

In order to determine the sensitivities of cells to tested drugs, cytotoxicity assays were carried out according to the method described by Ishiyama *et al* [26]. Briefly, 5,000 cells were plated in each well of 96-well plates in 100 μ L of culture medium and maintained at 37 °C. After 24 h, an additional 100 μ L of tested drug at various concentrations was added to each well and incubated for an additional 72 h before developing with either Cell Counting Kit-8 (CCK) or MTT reagent. For the MDR reversal assays, additional tyrphostin RG14620 or reference inhibitors of ABCB1, ABCC1 or ABCG2, at nontoxic concentrations, were added to the cytotoxicity assays. Finally, the extent of reversal was determined based on the calculated fold-reversal (FR) values, as described previously [13].

2.5. Immunoblotting

The antibodies BXP-21 (1:500) and α -tubulin (1:2000) were used in Western blot immunoassays to detect ABCG2 and tubulin, respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000) was used as the secondary antibody. Signals were detected as described previously [68].

2.6. Apoptosis assay

The percentage of apoptotic cells induced by the indicated regimens was determined using the annexin V–FITC and propidium iodide (PI) staining method, as described previously [25]. Cells were treated with tyrphostin RG14620, topotecan or a combination of topotecan and tyrphostin RG14620 as indicated for 48 h. Cells were harvested, centrifuged and resuspended in FACS buffer containing 1.25 µg/mL annexin V–FITC (PharMingen) and 0.1 mg/mL PI and incubated for 15 min at room temperature. The labeled cells (10,000 per sample) were analyzed by FACScan using CellQuest software. Phosphatidylserine PS-positive and PI-negative cells were counted as early apoptotic cells with intact plasma membranes, whereas PS-positive and PI-positive cells are considered as either necrotic or late apoptotic with leaky membranes [6].

2.7. ATPase assay and photoaffinity labeling of ABCG2 with [¹²⁵I]IAAP

The vanadate (Vi)-sensitive ATPase activity of ABCG2 in membrane vesicles of High-Five cells expressing ABCG2 was measured by endpoint P_i assay as described previously [3]. The [¹²⁵I]IAAP photoaffinity labeling assay was carried out as described previously [46, 68]. Briefly, membranes of ABCG2-overexpressing MCF7-FLV1000 cells (50 – 75 µg protein/100 µL) were incubated with tyrphostin RG14620 for 10 min at room temperature in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 before 3–6 nM [¹²⁵I]IAAP (2200 Ci/mmol) was added. The samples were then exposed to 366 nm UV light and processed as described previously [46, 68].

2.8. Docking of tyrphostin RG14620 in the drug-binding pocket of human ABCG2

The energy was minimized for both the ABCG2 protein structure (PDB:5NJG) [54] and tyrphostin RG14620 using Accelrys Discovery Studio 4.0. Ligand preparation and docking was performed by the CDOCKER module of the same software and the ligand poses were ranked using the CDOCKER interaction energy parameters (kcal/mol). Ligand protein binding energy in kcal/mol was calculated using the analyze tool in DS where energy of binding (E_{binding}) = energy of complex ($E_{\text{ligand-enzyme}}$) – energy of ligand (E_{ligand}) – energy of receptor (E_{enzyme}) using the implicit solvent model GBSW.

2.9. Statistical analysis

Values are shown as mean ± standard error of the mean (SEM), and the IC₅₀ values are mean ± standard deviation (SD) calculated from at least three independent experiments. KaleidaGraph (Reading, PA, USA) and GraphPad Prism (La Jolla, CA, USA) software was used to plot the curves and perform statistical analysis. The improvement in fit was analyzed by two-sided Student's t-test and labeled “statistically significant” if the probability, *p*, was less than 0.05.

3. Results

3.1. Tyrphostin RG14620 inhibits the transport activity of ABCG2

To determine the potency and selectivity of tyrphostin RG14620 for the transport function of major MDR-linked ABC drug transporters, we first examined the effect of tyrphostin

RG14620 on ABCB1-, ABCC1- or ABCG2-mediated fluorescent substrate transport. The accumulation of fluorescent calcein in ABCB1-transfected HEK293 (MDR19-HEK293) and ABCC1-transfected HEK293 (MRP1-HEK293) cells, or the accumulation of fluorescent pheophorbide A (PhA) in ABCG2-transfected HEK293 (R482-HEK293) cells in the presence (*shaded, solid lines*) or absence (*solid lines*) of 10 μ M tyrphostin RG14620 was measured and analyzed as described in *Materials and methods*. In the presence of tyrphostin RG14620, we found a significant increase in PhA accumulation in R482-HEK293 cells, but considerably less accumulation of calcein in MDR19-HEK293 and MRP1-HEK293 cells (Fig. 1A–1C). Since tyrphostin RG14620 appears to be selective for ABCG2 relative to ABCB1 and ABCC1, we further examined the effect of tyrphostin RG14620 on the transport function of ABCG2 in ABCG2-overexpressing human cancer cell lines. To eliminate the possible cell type specific response to tyrphostin RG14620, ABCG2-overexpressing human S1-M1-80 colon cancer and human H460-MX20 lung cancer cell lines were used in the study. Similar to what we observed in R482-HEK293 cells, the presence of tyrphostin RG14620 significantly increased the accumulation of PhA in both the S1-M1-80 (Fig. 1D) and H460-MX20 (Fig. 1E) cancer cell lines. Of note, tyrphostin RG14620 had no significant effect on the accumulation of calcein or PhA in drug-sensitive parental cells (Fig. 1A – 1E, left panels). The reference inhibitors tariquidar, MK-571 and Ko143 were used in the experiments as positive controls (Fig. 1, *dotted lines*) for complete inhibition of ABCB1, ABCC1 and ABCG2 transport, respectively.

3.2. Tyrphostin RG14620 reverses ABCG2-mediated drug resistance

Given that tyrphostin RG14620 increases the accumulation of ABCG2 drug substrate in ABCG2-overexpressing cells, we next examined the reversal effect of tyrphostin RG14620, at nontoxic concentrations, on drug resistance mediated by ABCG2. We found that tyrphostin RG14620 was unable to reverse ABCB1-mediated resistance to colchicine in MDR19-HEK293 cells (Fig. 2A, *right panel*) or ABCC1-mediated resistance to etoposide in MRP1-HEK293 cells (Fig. 2B, *right panel*). In contrast, without affecting the proliferation of parental cells (Fig. 2, *left panels*), tyrphostin RG14620 reversed ABCG2-mediated resistance to mitoxantrone in R482-HEK293 cells in a concentration-dependent manner (Fig. 2C, *right panel*). Similarly, tyrphostin RG14620 restored the sensitivity of ABCG2-overexpressing S1-M1-80 and H460-MX20 cancer cells to mitoxantrone (Fig. 2D and 2E) and topotecan (Table 1), which are well-known anticancer drug substrates of ABCG2 [40]. The calculated IC₅₀ and fold-reversal (FR) values in these multidrug-resistant cell lines are summarized in Table 1. The FR value corresponds to the extent of reversal of multidrug-resistant cells to a particular chemotherapeutic agent by a modulator, which was calculated as described previously [13]. At 2 μ M, tyrphostin RG14620 significantly restored the sensitivity of S1-M1-80 colon cancer cells to topotecan and mitoxantrone by approximately 41-fold and 47-fold, respectively. Similarly, at the same concentration, RG14620 resensitized H460-MX20 lung cancer cells to topotecan and mitoxantrone by approximately 6-fold and 7-fold, respectively (Table 1). As positive controls, tariquidar, MK-571 and Ko143 were used here to demonstrate the reversal of drug resistance mediated by ABCB1, ABCC1 and ABCG2 [66].

3.3. Tyrphostin RG14620 does not affect the protein expression of ABCG2

Next, the effect of tyrphostin RG14620 on ABCG2 protein expression was determined in ABCG2-overexpressing H460-MX20 cancer cells, as it has been shown that drug-induced transient down-regulation of ABCG2 can also lead to the re-sensitization of ABCG2-overexpressing multidrug-resistant cancer cells to chemotherapeutic agents [12, 33, 62]. Cells were treated with increasing concentrations (0 – 2 μ M) of tyrphostin RG14620 for 72 h and processed by immunoblotting as described in *Materials and methods*. We found that tyrphostin RG14620 has no significant effect on ABCG2 protein expression in H460-MX20 cancer cells (Fig. 3). Our data indicate that the re-sensitization of ABCG2-overexpressing cells to chemotherapeutic drugs was a result of direct inhibition of ABCG2-mediated drug transport by tyrphostin RG14620, and not related to the down-regulation of ABCG2 at the protein level.

3.4. Tyrphostin RG14620 enhances drug-induced apoptosis in ABCG2-overexpressing cancer cells

The effect of tyrphostin RG14620 on drug-induced apoptosis was examined in drug-sensitive S1 and ABCG2-overexpressing S1-M1-80 cancer cells. Cells were treated with topotecan in the presence or absence of tyrphostin RG14620 for 48 h, processed and analyzed as described in *Materials and methods*. As shown in Fig. 4, 5 M of topotecan significantly increased apoptosis in S1 cells, but not in S1-M1-80 cells (approximately 53% of early and late apoptosis in S1 cells, in contrast to 8% in S1-M1-80 cells). However, in the presence of 5 μ M of tyrphostin RG14620, the cell death population induced by topotecan in S1-M1-80 cells increased substantially (from approximately 8% to 44%), comparable to the level of apoptosis induced by topotecan in S1 cells. Of note, the level of apoptosis in S1 and S1-M1-80 cell lines was not affected by tyrphostin RG14620 alone. Our results indicate that tyrphostin RG14620 enhances the sensitivity of ABCG2-overexpressing cells to anticancer drugs.

3.5. Tyrphostin RG14620 stimulates ATPase activity of ABCG2

The effect of tyrphostin RG14620 on ABCG2 ATPase activity was examined to gain insight into the interaction between tyrphostin RG14620 and ABCG2. As shown in Fig. 5, tyrphostin RG14620 stimulated the vanadate-sensitive ATPase activity of ABCG2 in a concentration-dependent manner, with approximately 2.5-fold maximum stimulation and a calculated EC₅₀ value of 224 ± 24 nM. Since substrate transport mediated by ABC transporters is coupled to ATP hydrolysis [4, 5], our results suggest that most likely tyrphostin RG14620 behaves as a substrate of ABCG2.

3.6. Tyrphostin RG14620 inhibits [¹²⁵I]IAAP photoaffinity labeling of ABCG2

[¹²⁵I]IAAP is a photoaffinity analogue of prazosin that binds directly to the substrate-binding site of ABCG2, and has been used to characterize the substrate-binding site of that transporter [47]. Knowing that tyrphostin RG14620 behaves as a substrate of ABCG2, we next used the [¹²⁵I]IAAP photoaffinity labeling assay to determine whether tyrphostin RG14620 competes with the binding of another drug substrate at the substrate-binding site of ABCG2, and thus restores chemosensitivity in ABCG2-overexpressing cells. We found

that tyrphostin RG14620 at 10 μ M inhibited the photolabeling of [125 I]IAAP to ABCG2 by approximately 55% (Fig. 6A), indicating that tyrphostin RG14620 does bind to the substrate-binding site of ABCG2.

3.7. Tyrphostin RG14620 docking analysis using the cryo-EM structure of human ABCG2

For identification of the potential site(s) where tyrphostin RG14620 interacts with the substrate-binding pocket of ABCG2, docking studies of tyrphostin RG14620 were performed on 7 possible binding cavities within the transmembrane domain (TMD) of the recently published cryo-EM structure of a homodimer of the human ABCG2 transporter [54]. The best ligand binding pose (CDOCKER interaction energy = -26.6 kcal/mol) lies in a site lined by Val⁴⁰¹, Thr⁴⁰², Val⁴⁰⁴ from transmembrane helix 1b (TM1b of monomer 2), Phe⁴³², Asn⁴³⁶, Thr⁴³⁵, Phe⁴³⁹ from TM2 and Met⁵⁴⁹ from TM5 of monomer 1 of the dimer (Fig. 6B), with calculated binding energy of -35.9 kcal/mol. This analysis demonstrates that residues from both monomers of ABCG2 are required for interaction with RG14620.

3.8. Overexpression of ABCG2 does not reduce the susceptibility of cancer cells to tyrphostin RG14620

We examined the cytotoxicity of tyrphostin RG14620 in drug-sensitive and MDR cancer cell lines to determine whether cancer cells overexpressing ABCB1, ABCC1 or ABCG2 are resistant to treatment with tyrphostin RG14620. The IC₅₀ values of tyrphostin RG14620 are summarized in Table 2. The extent of cellular resistance to tyrphostin RG14620 caused by the overexpression of ABCB1, ABCC1 or ABCG2 is represented by the resistance factor (RF), which was calculated by dividing the IC₅₀ value of MDR subline by the IC₅₀ value of the respective parental line. We found that ABCB1-, ABCC1- and ABCG2-overexpressing multidrug-resistant cancer cell lines were equally sensitive to tyrphostin RG14620 as their drug-sensitive parental cancer cell lines.

4. Discussion

ABCG2 is one of the more recently identified ABC drug efflux pumps [2, 7, 34, 39] that is capable of transporting and conferring resistance to a wide range of chemotherapeutic agents, including but not limited to anthracyclines, mitoxantrone, etoposide, topotecan, CPT-11, SN-38, methotrexate and many tyrosine kinase inhibitors in cancer cells [8, 11, 16, 34, 37]. ABCG2 is highly expressed in tissues such as the gastrointestinal tract, liver, kidney, blood-brain barrier (BBB) and blood-placental barrier (BPB), thus affecting the overall oral ADME of substrate drugs [9, 11]. The physiological importance of ABCG2 can be demonstrated by the transport of its physiological substrates such as porphyrins [27], sterols [55], and urate [64]. The Q141K variant of ABCG2 linked to gout disease, is quite prevalent in certain ethnic populations [64]. In addition, ABCG2 was identified as a marker for cancer stem cells (CSCs) [24, 72], which are believed to have a critical role in tumorigenesis and metastasis [61]. One study demonstrated that the overexpression of ABCG2 caused by drug selection regimens contributes to “stem cell-like” properties in multidrug-resistant breast cancer cell lines [10], further suggesting that ABCG2 may play a protective role in the survival of CSCs [29, 36, 71]. More importantly, high ABCG2 expression has been linked to

poor clinical outcomes especially in patients with lung carcinoma [29], esophageal squamous cell carcinoma [23, 58] and acute myeloid leukemia (AML) [14, 15, 51, 60].

In recent years, a tremendous effort has been made to develop novel inhibitors or repurpose existing drugs to restore the sensitivity of drug-resistant cancer cells to anticancer agents [46, 48]. Numerous EGFR inhibitors have previously been shown to reverse ABCG2-mediated MDR by directly inhibiting the function of ABCG2 [13, 32, 44, 56]. However, due to a large overlap in the substrate specificity of ABCB1 and ABCG2, most of them were not specific. For instance, EGFR inhibitors such as erlotinib, lapatinib and pelitinib reverse ABCG2-mediated MDR in cancer cells through direct inhibition of the drug efflux function of ABCG2. However, these EGFR inhibitors are not transporter-specific and are known to interact strongly with another multidrug transporter (ABCB1) [13, 44, 56]. This prompted us to search for an EGFR inhibitor that could selectively reverse MDR-mediated by ABCG2 at nontoxic concentrations.

In this study, we discovered that tyrphostin RG14620 increased the accumulation of PhA by inhibiting the efflux function of ABCG2 in the same manner as other EGFR inhibitors that interact with the ABC drug transporters ABCB1 and ABCG2 [44, 45, 50]. However, tyrphostin RG14620 is selective for ABCG2 relative to ABCB1 and ABCC1. More importantly, tyrphostin RG14620 enhances topotecan-induced apoptosis and reverses ABCG2-mediated resistance to topotecan and mitoxantrone at low, nontoxic concentrations and in a dose-dependent manner. Any compound that binds to the transporter in a drug-binding pocket will inhibit the photolabeling of ABCG2 with [¹²⁵I]IAAP [42, 68], a photo affinity substrate of ABCG2 that is known to be transported by ABCG2 [47]. Therefore, the results of the IAAP photoaffinity labeling and docking analysis using the recently published structure of a homodimer of human ABCG2 further supports the notion that tyrphostin RG14620 binds strongly to the substrate-binding site of ABCG2 and competes with the binding of another drug substrate at the same site. Consistent with these data, we found that tyrphostin RG14620 stimulates the ATP hydrolysis of ABCG2 and that ABCG2-overexpressing cells are not resistant to tyrphostin RG14620, indicating that it is a high-affinity substrate of ABCG2 that competes with the transport of other anticancer drugs by this transporter. Of note, the IC₅₀ values of tyrphostin RG14620 determined in our study were comparable to those reported previously [30, 49, 70]. As with any other combination therapy, it is possible that concomitant administration of tyrphostin RG14620 with various anticancer agents may cause undesirable clinical responses due to unforeseen drug-drug interactions. Tyrphostin RG14620 may alter the pharmacokinetics of other anticancer substrate drugs by inhibiting the function of ABCG2 in the GI tract and at the blood-brain barrier similar to the way gefitinib, an EGFR inhibitor, affects the pharmacokinetics of irinotecan [52] and topotecan [28] in mice.

In summary, considering that the function of the ABCG2 drug transporter has a significant impact on the therapeutic efficacy of many chemotherapeutic agents, combination treatment with anticancer drugs and selective modulators such as tyrphostin RG14620 should be further tested in preclinical animal model studies to overcome ABCG2-mediated MDR in cancer patients in the future.

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Abbreviations

MDR	multidrug resistance
ABC	ATP-binding cassette
EGFR	Epidermal growth factor receptor
FCS	fetal calf serum
CCK-8	Cell Counting Kit-8
IMDM	Iscove's Modified Dulbecco's Medium
FR	fold-reversal
MTT	3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide
Vi	sodium orthovanadate
IAAP	Iodoarylazidoprazosin

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Highlights

- Tyrphostin RG14620, an EGFR inhibitor of the tyrphostin family, directly inhibits the transport function of ABCG2.
- Tyrphostin is ABCG2 specific.
- Tyrphostin RG14620 enhances drug-induced apoptosis and restores chemosensitivity of ABCG2-overexpressing multidrug-resistant cancer cells.
- Docking analysis indicates tyrphostin RG14620 binds to the drug-binding pocket of ABCG2.
- Tyrphostin RG14620 is a potent and selective modulator of ABCG2 that is useful to overcome chemoresistance in patients with drug-resistant tumors.

Fig. 1.

Tyrphostin RG14620 inhibits ABCG2-mediated fluorescent drug efflux. The accumulation of fluorescent calcein (0.5 μ M calcein-AM) in HEK293 cells (A and B, left panels), ABCB1-transfected MDR19-HEK293 cells (A, right panel) and ABCC1-transfected MRP1-HEK293 cells (B, right panel), or 1 μ M pheophorbide A (PhA) in HEK293 cells (C, left panel) and in ABCG2-transfected R482-HEK293 cells (C, right panel), or in human colon S1 cells (D, left panel) and their ABCG2-overexpressing variant S1-M1-80 cancer cells (D, right panel), or in human lung H460 cells (E, left panel) and their ABCG2-overexpressing

variant H460-MX20 cancer cells (E, right panel), was measured in the absence (solid lines) or presence of 20 μ M tyrphostin RG14620 (shaded, solid lines) or a reference inhibitor (dotted lines) for ABCB1 (3 μ M tariquidar), ABCC1 (25 μ M MK-571) or ABCG2 (3 μ M Ko143), and analyzed immediately by flow cytometry as described previously [68]. Representative histograms of three independent experiments are shown.

Fig. 2.

Effect of tyrphostin RG14620 on drug resistance mediated by ABCB1, ABCC1 or ABCG2. Drug-sensitive parental HEK293 cells (A-C, left panels) and HEK293 cells transfected with human ABCB1 (A, right panel), ABCC1 (B, right panel) or ABCG2 (C, right panel), as well as (D) drug-sensitive S1 colon cells (left panel) and their ABCG2-overexpressing variant S1-M1-80 cells (right panel) and (E) drug-sensitive H460 cells (left panel) and their ABCG2-overexpressing variant H460-MX20 (right panel) lung cancer cells were treated with doxorubicin, etoposide or mitoxantrone in the absence (open circles) or presence of

tyrphostin RG14620 at 0.1 μM (filled circles), 0.2 μM (open squares), 0.5 μM (filled squares) 1.0 μM (open triangles) or 2.0 μM (filled triangles), respectively, as described in *Materials and methods*. Points, mean values from at least three independent experiments; bars; SEM.

Fig. 3.

Effect of tyrphostin RG14620 on ABCG2 protein expression in human cancer cell lines. Immunoblot detection of human ABCG2 protein expression in (A) ABCG2-overexpressing H460-MX20 lung cancer cells and (B) quantification of ABCG2 in total cell lysate protein (10 μ g/lane) from cells treated with DMSO (vehicle control) or increasing concentrations of tyrphostin RG14620 (RG) for 72 h as described previously [68]. α -tubulin was used as an internal control for equal loading. Values are presented as mean $\pm$ SEM calculated from three independent experiments.

Fig. 4. Tyrphostin RG14620 enhances topotecan-induced apoptosis in ABCG2-overexpressing S1-M1-80 cancer cells. (A) Drug-sensitive human colon S1 cancer cells (top panels) and ABCG2-overexpressing drug-resistant variant S1-M1-80 cancer cells (lower panels) were treated with either DMSO (*control*), 5 μ M tyrphostin RG14620 (+ *RG14620*), 5 μ M topotecan (+ *topotecan*) or a combination of 5 μ M topotecan and 5 μ M of tyrphostin RG14620 (*topotecan + RG14620*) for 48 h. Cells were isolated and analyzed by flow cytometry as described previously [25]. Representative dot plots and the mean values of

three independent experiments are shown. (B) Quantification of topotecan-induced apoptosis in human colon S1 and S1-M1-80 cells. Values are presented as mean $\pm$ S.D. calculated from three independent experiments. ** $p < 0.01$; *** $p < 0.001$, versus the same treatment without tyrphostin RG14620.

Fig. 5.

Tyrphostin RG14620 stimulates vanadate-sensitive ABCG2 ATPase activity. The effect of 0 – 5 μM of tyrphostin RG14620 on vanadate-sensitive ABCG2 ATP hydrolysis was determined by endpoint P_i assay, as described previously [3]. Points indicate the mean of at least three independent experiments; bars indicate the SEM. The inset shows the ATPase activity in the presence of lower concentrations from 0 to 0.5 μM of RG compound.

Fig. 6. Binding of tyrphostin RG14620 to ABCG2 protein. (A) Tyrphostin RG14620 (RG14620) inhibits photoaffinity labeling of ABCG2 with [125 I]IAAP. Membrane protein (50–75 μ g protein) from MCF-7-FLV1000 cells was incubated in the absence or presence of 10 μ M of RG14620 before 3–6 nM [125 I]IAAP (2,200 Ci/mmol) was added to the samples, as detailed in *Materials and methods*. Photo-labeled ABCG2 was processed and visualized as described previously [46, 68]. Representative autoradiogram and values represent mean $\pm$ S.D. calculated from three independent experiments. (B) Binding mode of RG14620 with human

ABCG2 structure (PDB: 5NJG) as predicted by Accelrys Discovery Studio 4.0 software as described in *Materials and methods*. RG14620 is shown as a molecular model with the atoms colored as carbon- gray, hydrogen-light gray, chlorine-green, sulfur-yellow, nitrogen-blue and oxygen-red. The same color scheme is used for interacting amino acid residues.

Fig. 7.

Comparison of the levels of anticancer drug accumulation in ABCG2-overexpressing MDR cancer cells in the absence or presence of tyrphostin RG14620. In the absence of tyrphostin RG14620 (left panel), anticancer drugs (red circles) are transported out of cancer cells by ABCG2, thereby reducing their intracellular concentration and rendering chemotherapy ineffective. In the presence of tyrphostin RG14620 (green triangle, right panel), anticancer drugs are no longer pumped out by ABCG2, resulting in effective killing of cancer cells.

Table 1

Reversal effect of tyrphostin RG14620 on drug resistance mediated by ABCB1, ABCC1 or ABCG2.

Treatment	Concentration (μM)	Mean IC ₅₀ [†] ± SD and (FR [‡])	
		pcDNA-HEK293 [nM]	MDR19-HEKS293 [nM]
Colchicine	–	8.23 ± 1.43 (1.0)	252.77 ± 49.96 (1.0)
+ RG14620	0.5	7.50 ± 1.77 (1.1)	269.62 ± 57.46 (0.9)
+ RG14620	1	6.56 ± 1.45 (1.3)	285.15 ± 58.38 (0.9)
+ RG14620	2	6.97 ± 1.76 (1.2)	281.30 ± 53.91 (0.9)
+ Tariquidar	1	4.84 ± 1.34 [*] (1.7)	10.60 ± 2.48 ^{**} (23.8)
		pcDNA-HEK293 [μM]	MRP1-HEKS293 [μM]
Etoposide	–	0.28 ± 0.06 (1.0)	48.58 ± 6.71 (1.0)
+ RG14620	0.5	0.29 ± 0.06 (1.0)	44.05 ± 9.70 (1.1)
+ RG14620	1	0.32 ± 0.07 (0.9)	32.82 ± 8.38 (1.5)
+ RG14620	2	0.27 ± 0.06 (1.0)	35.04 ± 8.62 (1.4)
+ MK-571	25	0.20 ± 0.05 (1.4)	8.17 ± 1.10 ^{***} (5.9)
		pcDNA-HEK293 [nM]	R482-HEKS293 [nM]
Mitoxantrone	–	2.15 ± 0.45 (1.0)	111.41 ± 16.47 (1.0)
+ RG14620	0.5	1.77 ± 0.28 (1.2)	32.49 ± 4.24 ^{**} (3.4)
+ RG14620	1	1.85 ± 0.34 (1.2)	18.94 ± 3.06 ^{***} (5.9)
+ RG14620	2	2.22 ± 0.44 (1.0)	14.21 ± 2.54 ^{***} (7.8)
+ Ko143	1	1.82 ± 0.27 (1.2)	5.86 ± 1.00 ^{***} (19.0)
		H460 [nM]	H460-MX20 [μM]
Topotecan	–	44.08 ± 6.19 (1.0)	1.66 ± 0.44 (1.0)
+ RG14620	0.1	40.56 ± 5.59 (1.1)	1.23 ± 0.35 (1.3)
+ RG14620	0.2	46.73 ± 5.18 (1.0)	0.67 ± 0.18 [*] (2.5)
+ RG14620	0.5	44.14 ± 6.06 (1.0)	0.54 ± 0.15 [*] (3.1)
+ RG14620	1	37.83 ± 5.48 (1.2)	0.40 ± 0.11 ^{**} (4.2)
+ RG14620	2	35.84 ± 5.58 (1.2)	0.27 ± 0.07 ^{**} (6.1)
+ Ko143	1	31.03 ± 4.71 [*] (1.4)	0.26 ± 0.07 ^{**} (6.4)
		H460 [nM]	H460-MX20 [μM]
Mitoxantrone	–	27.66 ± 10.47 (1.0)	1.61 ± 0.34 (1.0)
+ RG14620	0.1	24.34 ± 8.45 (1.1)	1.52 ± 0.41 (1.1)
+ RG14620	0.2	23.62 ± 7.88 (1.2)	1.19 ± 0.36 (1.4)
+ RG14620	0.5	21.17 ± 7.19 (1.3)	0.73 ± 0.25 [*] (2.2)
+ RG14620	1	17.69 ± 5.72 (1.6)	0.38 ± 0.07 ^{**} (4.2)
+ RG14620	2	19.95 ± 6.65 (1.4)	0.23 ± 0.06 ^{**} (7.0)
+ Ko143	1	21.06 ± 8.39 (1.3)	0.10 ± 0.02 ^{**} (16.1)

Treatment	Concentration (μM)	Mean IC ₅₀ [†] ± SD and (FR) [‡]	
		pcDNA-HEK293 [nM]	MDR19-HEKS293 [nM]
		S1 [nM]	S1-M1-80 [μM]
Topotecan		27.36 ± 4.47 (1.0)	13.56 ± 3.51 (1.0)
+ RG14620	0.1	25.51 ± 3.87 (1.1)	5.83 ± 1.72 * (2.3)
+ RG14620	0.2	25.35 ± 3.99 (1.1)	4.10 ± 1.29 * (3.3)
+ RG14620	0.5	23.55 ± 3.08 (1.2)	2.71 ± 0.85 ** (5.0)
+ RG14620	1	23.50 ± 2.88 (1.2)	1.44 ± 0.36 ** (9.4)
+ RG14620	2	23.83 ± 3.00 (1.1)	0.33 ± 0.11 ** (41.1)
+ Ko143	1	26.75 ± 4.22 (1.0)	0.43 ± 0.13 ** (31.5)
Mitoxantrone	–	3.75 ± 0.43 (1.0)	46.93 ± 9.36 (1.0)
+ RG14620	0.1	4.01 ± 0.44 (0.9)	33.91 ± 5.69 (1.4)
+ RG14620	0.2	3.84 ± 0.41 (1.0)	16.32 ± 2.62 ** (2.9)
+ RG14620	0.5	4.41 ± 0.44 (0.9)	5.35 ± 0.92 ** (8.8)
+ RG14620	1	3.92 ± 0.40 (1.0)	2.63 ± 0.60 ** (17.8)
+ RG14620	2	4.86 ± 0.73 (0.8)	1.01 ± 0.21 ** (46.5)
+ Ko143	1	2.66 ± 0.41 (1.4)	0.77 ± 0.14 ** (60.9)

Abbreviation: FR, fold-reversal.

[†]IC₅₀ values are mean ± SD calculated from dose-response curves obtained from three independent experiments using cytotoxicity assay as described in *Materials and methods*.

[‡]FR values were obtained by dividing IC₅₀ values of cells treated with the indicated anticancer drug in the absence of a modulator by IC₅₀ values of cells treated with the same anticancer drug in the presence of a modulator.

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$

Table 2

Cytotoxicity of tyrphostin RG14620 in human cancer cell lines overexpressing ABCB1, ABCC1 or ABCG2.

Cell line	Type	Transporter expressed	IC ₅₀ (μM) [†]	RF [‡]
KB-3-1	epidermal	–	8.98 ± 3.50	1.0
KB-C-1	epidermal	ABCB1	9.88 ± 2.73	1.1
KB-8-5-11	epidermal	ABCB1	9.59 ± 2.80	1.1
KB-V-1	epidermal	ABCB1	12.56 ± 3.97	1.4
OVCAR-8	ovarian	–	35.80 ± 2.52	1.0
NCI-ADR-RES	ovarian	ABCB1	28.28 ± 3.19	0.8
COR-L23/P	lung	ABCC1	11.68 ± 3.24	1.0
COR-L23/R	lung	ABCC1	10.46 ± 2.93	0.9
H460	lung	–	17.68 ± 3.84	1.0
H460-MX20	lung	ABCG2	17.33 ± 2.39	1.0
S1	colon	–	25.26 ± 4.58	1.0
S1-M1-80	colon	ABCG2	23.06 ± 4.53	0.9
A549	lung	–	21.02 ± 5.19	1.0
A549-Bec150	lung	ABCG2	17.95 ± 1.52	0.9

Abbreviation: RF, resistance factor.

[†]IC₅₀ values are mean ± SD calculated from dose-response curves obtained from three independent experiments using cytotoxicity assay as described in *Materials and methods*.

[‡]RF values were calculated by dividing IC₅₀ values of ABC transporter overexpressing cells by IC₅₀ values of respective parental cells.

**P* < 0.05;

***P* < 0.01;

****P* < 0.001