Cancer Letters 409 (2017) 56–65

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

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

Chung-Pu Wu [a, b, c, d, *], Sung-Han Hsiao [a], Megumi Murakami [e], Ming-Jie Lu [a], Yan-Qing Li [a, b], Chia-Hung Hsieh [f, g], Suresh.V. Ambudkar [e], Yu-Shan Wu [h, **]

[a] Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
[b] Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
[c] Molecular Medicine Research Center, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
[d] Department of Neurosurgery, Chang Gung Memorial Hospital, Tao-Yuan, Taiwan
[e] Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, United States
[f] Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan
[g] Department of Biomedical Informatics, Asia University, Taichung, Taiwan
[h] Department of Chemistry, Tunghai University, Taichung, Taiwan

ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form
25 August 2017
Accepted 28 August 2017

Keywords:
ABC transporter
ABCG2
EGFR inhibitor
Multidrug resistance
Tyrphostin RG14620

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 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.

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

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-diphenyllapatinibrazolium bromide; Vi, sodium orthovanadate; IAAP, Iodoarylazidoprazosin.

* Corresponding author. 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan.
** Corresponding author. Department of Chemistry, Tunghai University, 181 Taichung Harbor Road Section 3, Taichung, Taiwan.
E-mail addresses: wuchung@mail.cgu.edu.tw (C.-P. Wu), yushanwu@thu.edu.tw (Y.-S. Wu).

http://dx.doi.org/10.1016/j.canlet.2017.08.035
0304-3835/

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 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.

Materials and methods

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). [^{125}I]-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.

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, 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, 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, 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 μM of mitoxantrone was added to S1-M1-80 cell culture medium, as described [68]. All cell lines were maintained at 37 °C in 5% CO_2 humidified air and placed in drug-free medium 7 days prior to assay.

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 0.25 μM calcein-AM or 1 μM 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.

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, 5000 cells were plated in each well of 96-well plates in 100 μL of culture medium and maintained at 37 °C. After 24 h, an additional 100 μL 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-8) 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].

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].

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].