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AST1306, a potent EGFR inhibitor, antagonizes ATP-binding cassette subfamily G member 2-mediated multidrug resistance

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ABSTRACT

AST1306, an inhibitor of EGFR and ErbB2, is currently in phase I of clinical trials. We evaluated the effect of AST306 on the reversal of multidrug resistance (MDR) induced by ATP-binding cassette (ABC) transporters. We found that AST1306 significantly sensitized the ABC subfamily G member 2 (ABCG2)-overexpressing cells to ABCG2 substrate chemotherapeutics. AST1306 significantly increased intracellular accumulation of [^{3}H]-mitoxantrone in ABCG2-overexpressing cells by blocking ABCG2 efflux function. Moreover, AST1306 stimulated the ATPase activity of ABCG2. Homology modeling predicted the binding conformation of AST1306 to be within the transmembrane region of ABCG2. In conclusion, AST1306 could notably reverse ABCG2-mediated MDR.

Introduction

Intrinsic or acquired multidrug resistance (MDR) often leads to failure of cancer chemotherapy [1]. MDR is characterized by resistance to structurally and mechanistically distinct classes of antineoplastic drugs. One of the major mechanisms in cancer cells that give rise to MDR is the overexpression of ATP-binding cassette (ABC) transporters [2]. Currently, 49 human ABC transporters have been identified and classified into 7 subfamilies (A-G) based on sequence and structure. Among the currently identified ABC transporters, 14 are involved in the development of MDR [3,4]. ABCB1, ABCC1 and ABCG2 are the most influential in MDR. ABCB1 (also known as MDR1 or P-glycoprotein), a 170 kDa membrane glycoprotein, can transport a wide range of amphipathic and hydrophobic compounds, such as anthracyclines, taxanes, epipodophyllotoxin derivatives and vinca alkaloids [5]. ABCCs are composed of 13 members (ABCC1-ABCC13), of which ABCC13 is a non-functional gene-encoding transporter [6,7]. ABCC1 (also known as MRP1), a 190 kDa transmembrane protein, confers resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins and methotrexate, but not to taxanes [8–10]. ABCC10 (also known as MRP7), a 171 kDa membrane protein, confers resistance to various anticancer drugs such as taxanes and vinca alkaloids [11–13]. ABCG2 (also known as BCRP or MXR), a 72 kDa protein, is a half-transporter because it contains only one transmembrane domain (TMD) and one nucleotide binding domain (NBD). The substrates of ABCG2 include sulfated hormone metabolites, glucuronidated methotrexate, mitoxantrone (MX), topotecan and irinotecan [14,15]. These ABC transporters act as the cancer cells' defense against substrate chemotherapeutic drugs; therefore, blocking these transporters would provide an effective strategy towards increasing the intracellular accumulation of chemotheraputic agents [16–18].

Tyrosine kinase inhibitors (TKIs) are a class of anticancer agents that act by competing with the binding site of the catalytic domain of several oncogenic tyrosine kinases. This inhibitory effect is key towards blocking the cellular replication, survival, metastasis and angiogenesis of cancer cells [19]. Substantial studies have reported that certain TKIs modulate the MDR phenotype mediated by ABC transporters, and have encouraged ongoing clinical trials to evaluate the potential of these TKIs to circumvent the anticancer drug resistance [20,21].

Elevated levels of the epidermal growth factor receptor (EGFR) and its cognate ligands have been identified as a common component of multiple cancer types, and appear to promote solid tumor growth [22]. AST1306 is a novel oral multitargeted TKI that inhibits wild type EGFR, ErbB2, as well as mutant EGFR T790 M/L858R [23]. AST1306 was designed and synthesized based on the chemical structure of lapatinib, including the key chemical group

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of irreversible EGFR inhibitors. Moreover, both *in vitro* and *in vivo* studies showed a prominent effect of AST1306 on the ERbB2-overexpressing tumors [23]. In our study, we focus on the modulation of ABC transporters by AST1306.

Materials and methods

Chemicals and reagents

MX, SN-38, paclitaxel, vincristine, verapamil, cisplatin, penicillin/streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). AST1306 was obtained as a gift from Selleck Chemicals (Houston, TX). PAK-104P was a gift of Prof. Shin-Ichi Akiyama (Kagoshima University, Kagoshima, Japan) from Nissan Chemical Ind. Co., Ltd. (Chiba, Japan). Cepharanthine was generously provided by Kakenshoyaku Co. (Tokyo, Japan). Fumitremorgin C (FTC) was synthesized by Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory (NCI, NIH, Bethesda, MD). [^{3}H]-MX (4 Ci/mM) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Human ABCG2 (Arg482) vesicles and control vesicles for ABC transporters were purchased from BD Bioscience (San Jose, CA). Monoclonal antibodies BXP-21 against ABCG2 were purchased from Signet Laboratories, Inc. (Dedham, MA). Anti-β-actin monoclonal antibody (sc-8432) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell lines and cell culture

Human non-small cell lung carcinoma cells H460 and ABCG2-overexpressing H460/MX20 drug selected cells were kindly provided by Dr. Susan E. Bates (NCI, NIH, Bethesda, MD). H460/MX20 cells were maintained in DMEM with addition of 20 nM MX [24]. Wild-type ABCG2-482-R2, mutant ABCG2-482-G2 and mutant ABCG2-482-T7 cells were established by selection with G418 after transfecting HEK293 with either the empty pcDNA3.1 vector or the pcDNA3.1 vector containing the full-length ABCG2, coding arginine (Arg), glycine (Gly) or threonine (Thr) at amino acid position 482, respectively [25]. The ABCB1-overexpressing KB-C2 cell line was established by a stepwise exposure of a human epidermoid carcinoma cell line KB-3-1 to increasing concentrations of colchicine up to 2 μg/ml [26]. HEK293/ABCC1 and HEK293/ABCC10 cells were generated by transfecting the HEK293 cells with ABCC1 expression vector and ABCC10 expression vector, respectively [27,28]. HEK293/ABCC1 was generated in Dr. Ambudkar's laboratory (NCI, NIH, Bethesda, MD) and the plasmid containing *ABCC10* was generously provided by the late Dr. Gary Kruh (University of Illinois at Chicago, Chicago, IL). All cells were grown as adherent monolayers in DMEM culture medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum at 37 °C, in a humidified incubator containing 5% CO_2.

Cytotoxicity assay

Cytotoxicity assay was analyzed using a slightly modified MTT colorimetric assay [29]. After the cells were evenly distributed into 96-well multi-plate, different concentrations of chemotherapeutic drugs were added into designated wells after pre-incubation with reversal agents for 2 h. Finally, the absorbance was read at 570 nm using Opsys microplate reader (Dynex Technologies, Chan-tilly, VA). The resistance-fold was calculated by dividing the IC_{50} for the MDR cells with or without inhibitor by that of the parental cells without inhibitor.

Immunoblotting analysis

The effect of AST1306 on the expression level of ABCG2 was tested after the cells were treated with AST1306 at 1 μM for 0, 24, 48 and 72 h. Protein lysates were isolated and prepared for Western blot analysis as previously described [30,31].

Immunofluorescence staining

H460/MX20 cells were cultured into 96-well plates coated with poly-l-lysine and treated with 1 μM AST1306 for 72 h. After treatment, cells were stained as previously described [32]. Images were taken by using an IX70 microscope with IX-FLA fluorescence and CCD camera.

2.6. [^{3}H]-MX accumulation assay

The cells were collected and four aliquots (approximately 5×10^6 cells) were suspended in the medium and incubated with or without AST1306 (0.25 μM or 1 μM), at 37 °C for 2 h. The cells were then treated with 0.2 μM [^{3}H]-MX and incubated for another 2 h at 37 °C. The medium was then removed and the cells were rinsed three times with cold PBS. The cells were then lysed by adding 200 μl lysis buffer and transferred to scintillation vials. Each sample was placed in scintillation fluid and radioactivity was measured using a Packard TRI-CARB 1900CA liquid scintillation analyzer from Packard Instrument Company Inc. (Downers Grove, Illinois, USA) [33,34].

[^{3}H]-MX efflux assay

To test the drug efflux, the cells were pretreated in the same manner as in the drug accumulation experiment. [^{3}H]-MX was then added to the cells and further incubated for 2 h, after which the cells were washed in ice-cold PBS and supplemented with fresh medium with or without AST1306 at 1 μM at 37 °C. After various time points, the aliquots of cells were removed and immediately washed three times with ice-cold PBS. The cells were collected and lysed for the detection of radioactivity as described previously [33,35].

ATPase assay of ABCG2

The vanadate (Vi)-sensitive ATPase activity of ABCG2 was measured in the membrane vesicles obtained from BD Biosciences (San Jose, CA). The membrane vesicles (20 μg protein/reaction) were incubated in ATPase assay buffer at 37 °C for 5 min with or without 400 μM vanadate. We then incubated the membrane vesicles with different concentrations of AST1306, ranging from 0 to 40 μM, at 37 °C for 5 min, followed by an addition of 10 μl of 25 mM Mg-ATP solution. The reaction was allowed to continue for another 10 min at 37 °C, and then terminated by an addition of 30 μl 10% SDS solution to the reaction mix. The amount of P_i released was detected and quantified by adding 200 μl of detection reagent. The reaction mix was then further incubated for 20 min at 37 °C. The absorption was detected between 630 and 850 nm using a spectrophotometer [35].

Docking simulation

AST1306 structure was built and docked against various grids on human ABCG2 homology model as per previous protocols [15,36]. All computations were carried out on a Dell Precision 490n dual processor with the Linux OS (Ubuntu 12.04 LTS).

Statistical analysis

All experiments were repeated at least three times. Differences between runs were determined using the two-tailed student's *t*-test and statistical significance was determined at $p < 0.05$.

Results

Cytotoxicity of AST1306 on sensitive and resistant cells

We tested the cytotoxicity of AST1306 on different cell lines by MTT assay prior to investigating the reversal effect. The results showed that more than 85% of the cells survived at the concentration of 1 μM AST1306 in these cell lines (Fig. 1B–F). Based on these results, 1 μM was chosen as a maximum concentration for combination treatment with known ABCB1, ABCG2, ABCC1 or ABCC10 substrate antineoplastic drugs.

Reversal effect of AST1306 on MDR cells overexpressing ABCG2

The IC_{50} values of the substrate antineoplastic drugs in the ABCG2-overexpressing cells and parental cells when combined with or without AST1306 are summarized in Table 1. The H460/MX20 cells showed high resistance towards MX and SN-38 as compared to H460 cells. However, the resistance of MX and SN-38 in H460/MX20 cells was overcome by treatment with AST1306 (Table 1). AST1306 at 1 μM only produced approximately two-fold sensitization to MX and SN-38 in the parental H460 cells with low levels of intrinsic ABCG2. This effect was modest as compared to that of H460/MX20 cells. It has been reported that mutations at amino acid 482 in ABCG2 could alter the substrate and antagonist specificity of ABCG2 [25,37]. Therefore, we investigated whether AST1306 could reverse ABCG2-mediated resistance to its substrates in cells transfected with either the wild-type (Arg482) or mutant (Arg482Gly and Arg482Thr) forms of ABCG2. As shown in Table 2, AST1306 could significantly increase sensitivity to MX and SN-38 in ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells, respectively, and this effect was similar to that obtained with 1 μM FTC. These results

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