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Inhibiting the function of ABCB1 and ABCG2 by the EGFR tyrosine kinase inhibitor AG1478

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ABSTRACT

The tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is a potent and specific EGFR tyrosine kinase inhibitor (TKI); its promising pre-clinical results have led to clinical trials. Overexpression of ATP-binding cassette (ABC) transporters such as ABCB1, ABCC1 and ABCG2 is one of the main causes of multidrug resistance (MDR) and usually results in the failure of cancer chemotherapy. However, the interaction of AG1478 with these ABC transporters is still unclear. In the present study, we have investigated this interaction and found that AG1478 has differential effects on these transporters. In ABCB1-overexpressing cells, non-toxic doses of AG1478 were found to partially inhibit resistance to ABCB1 substrate anticancer drugs as well as increase intracellular accumulation of [^{3}H]-paclitaxel. Similarly, in ABCG2-overexpressing cells, AG1478 significantly reversed resistance to ABCG2 substrate anticancer drugs and increased intracellular accumulation of [^{3}H]-mitoxantrone as well as fluorescent compound BODIPY-prazosin. AG1478 also profoundly inhibited the transport of [^{3}H]-$E_2$17βG and [^{3}H]-methotrexate by ABCG2. We also found that AG1478 slightly stimulated ABCB1 ATPase activity and significantly stimulated ABCG2 ATPase activity. Interestingly, AG1478 did not inhibit the photolabeling of ABCB1 or ABCG2 with [^{125}I]-iodoarylazidoprazosin. Additionally, AG1478 did not alter the sensitivity of parental, ABCB1- or ABCG2-overexpressing cells to non-ABCB1 and non-ABCG2 substrate drug and had no effect on the function of ABCC1. Overall, we conclude that AG1478 is able to inhibit the function of ABCB1 and ABCG2, with a more pronounced effect on ABCG2. Our findings provide valuable contributions to the development of safer and more effective EGFR TKIs for use as anticancer agents in the clinic.

1. Introduction

The tyrphostin (tyrosine phosphorylation inhibitor) 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is a low molecular weight, highly potent, reversible, selective inhibitor of the epidermal growth factor receptor (EGFR, HER1/ErbB1) [1]. It not only competes with ATP at the ATP binding site of the kinase domain of EGFR, but also induces the formation of

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inactive, unphosphorylated EGFR dimers in the presence and absence of ligand [2]. AG1478 inhibits the tyrosine kinase activity of EGFR with IC_{50} values in the nanomolar range [3], and its important chemical features for activity against EGFR include: the presence of electron-donating groups at positions 6 and 7 on the quinazoline; the presence of small lipophilic groups at position 3 of the aniline; and the orientation of the quinazoline ring nitrogens [4]. AG1478 is active in various types of cancer cell lines both *in vitro* and *in vivo*. In cell culture experiments, AG1478 demonstrates potent antiproliferative effects [5–7], and also enhances cellular sensitivity to cytotoxic drugs such as cisplatin, doxorubicin and etoposide [8,9]. In nude mouse tumor xenograft models, AG1478 directly inhibits the growth of human glioma xenografts that overexpress mutant EGFR (Δ2-7 EGFR), and sensitizes these xenografts to the cytotoxicity of cisplatin and temozolomide; it also enhances the activity of the monoclonal antibody mAb 806 [10,11]. In addition, AG1478 enhances the efficacy of radioimmunotherapy with 90Y-CHX-A''-DTPA-hu3S193 in A431 squamous carcinoma cells nude mouse xenografts [12]. In a preclinical analysis, the initial pharmacokinetics and pharmacodynamics of AG1478 were evaluated in mice with novel AG1478 formulations in β-cyclodextrin (Captisol®) [13].

In this study, we investigated the effects of AG1478 on multidrug resistance (MDR)-linked ATP-binding cassette (ABC) transporters like ABCB1 (P-glycoprotein/P-gp, MDR1), ABCC1 (multidrug resistance protein 1, MRP1) and ABCG2 (breast cancer resistance protein, BCRP/MXR) in cancer cells. Our results show that AG1478 slightly reverses ABCB1-mediated MDR whereas it significantly inhibits ABCG2-mediated MDR by direct effect on drug efflux, but had no effect on ABCC1-mediated MDR.

2. Materials and methods

2.1. Reagents

[^{3}H]-paclitaxel (37.9 Ci/mmol), [^{3}H]-mitoxantrone (4 Ci/mmol) and [^{3}H]-methotrexate (23 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA). [^{3}H]-$E_2$17βG (40.5 Ci/mmol) and [^{125}I]-Iodoarylazidoprazosin (IAAP) (2200 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). The fluorescent compound BODIPY-prazosin was purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies C-219 (against ABCB1) and BXP-34 (against ABCG2) were acquired from Signet Laboratories Inc. (Dedham, MA). Anti-actin monoclonal antibody (sc-8432) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Alexa flour 488 goat anti-mouse secondary antibody for immunocytochemistry was purchased from Molecular Probes (Eugene, OR). AG1478 was purchased from A.G. Scientific, Inc. (San Diego, CA). Fumitremorgin C (FTC) was synthesized by Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory, NCI, NIH (Bethesda, MD). Valspodar (PSC833) was obtained from Novartis Pharmaceuticals (East Hanover, NJ). A monoclonal anti-ABCB1 (MDR1) antibody (catalog no. P7965) and other chemicals were purchased from Sigma Chemical Co (St. Louis, MO).

2.2. Cell lines and cell culture

The ABCB1-overexpressing drug-resistant cell line KB-C2 was established by step-wise selection of the parental human epidermoid carcinoma cell line KB-3-1 in increasing concentrations of colchicine and was cultured in medium containing 2 μg/ml of colchicines [14]. An ABCC1-overexpressing MDR cell line, KB-CV60, was also cloned from KB-3-1 cells and was maintained in medium with 1 μg/ml of cepharanthine and 60 ng/ml of vincristine [15]. Both KB-C2 and KB-CV60 cell lines were kindly provided by Dr. Shin-ichi Akiyama (Kagoshima University, Japan). HEK293/pcDNA3.1, ABCG2-482-R5, ABCG2-482-G2, and ABCG2-482-T7 cells were established by selection with G418 after transfecting HEK293 with either empty pcDNA3.1 vector or pcDNA3.1 vector containing full length *ABCG2* coding either arginine (R), glycine (G), or threonine (T) at amino acid 482, respectively, and were cultured in medium with 2 mg/ml of G418 [16]. All the cell lines were grown as adherent monolayers in flasks with DMEM culture medium (Hyclone Co., Omaha, NE) containing 10% bovine serum at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Preparation of membrane vesicles and total cell lysates

Membrane vesicles were prepared by the nitrogen cavitation method as previously described [17]. Vesicles were stored at −80 °C until ready for use. To prepare the total cell lysates, cells were harvested and rinsed twice with PBS. Cell extracts were prepared with RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min with occasional rocking followed by centrifugation at 12,000 g at 4 °C for 15 min. The supernatant containing total cell lysate was stored at −80 °C until ready for use. The protein concentration was determined by the Bradford method. High Five insect cells (Invitrogen, Carlsbad, CA) were infected with the recombinant baculovirus carrying the human *MDR1* or *ABCG2* cDNAs with a poly-His tag at the C-terminal end [BV-MDR1(His_6)] or [BV-ABCG2(His_{10})] as described previously [18]. The membrane vesicles of High Five insect cells were prepared as previously described [19] and stored at −70 °C.

2.4. Western blot and immunocytochemistry analyses

Total cell lysate (50 μg protein) or membrane vesicles (15 μg protein) were resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. After incubating in blocking solution in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, the membranes were first incubated overnight with primary monoclonal antibodies against ABCB1(C219) or actin at 1:200 dilution or ABCG2 at 1:500 dilution at 4 °C, and were then overnight at 4 °C with HRP-conjugated secondary antibody (1:1000 dilution). The protein–antibody complex was detected by chemoluminescence. The protein expression was quantified by Scion Image software (Scion Co., Frederick, MD). For immunocytochemistry analysis, cells (2×10^3) are seeded in 24 well plates, AG1478 at 10 μM was added into the wells after overnight