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Effect of abemaciclib (LY2835219) on enhancement of chemotherapeutic agents in ABCB1 and ABCG2 overexpressing cells *in vitro* and *in vivo*

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ABSTRACT

Multidrug resistance (MDR) is the major obstacle of the success in cancer chemotherapy. The overexpression of ATP-binding cassette (ABC) transporters, particularly ABCB1 and ABCG2, play a significant role in mediating MDR by pumping anticancer drugs out of cancer cells. Abemaciclib (LY2835219) is an orally bioavailable CDK4/6 inhibitor under phase III clinical trials. Here, we found that LY2835219 remarkably enhanced the efficacy of chemotherapeutic drugs in ABCB1 or ABCG2 over-expressing cancer cells *in vitro* and *in vivo*. Furthermore, LY2835219 significantly increased the intracellular accumulation of doxorubicin (DOX) and rhodamine 123 (Rho 123) by inhibiting ABCB1 or ABCG2-mediated drug efflux in the transporters-overexpressing cells. Mechanistically, LY2835219 is likely a competitive inhibitor of ABCB1 and ABCG2 for its competition with [125I]-iodoarylazidoprazosin for photo affinity labeling of the transporters. On the other hand, at the transporters-inhibiting concentrations, LY2835219 did not alter the expression level of ABCB1 and ABCG2, and the phosphorylation status of retinoblastoma (Rb) pathway in both parental and their resistant cells. In conclusion, these findings revealed a novel role of LY2835219 in reversing ABCB1 or ABCG2-mediated MDR, which may be benefit to the patients with MDR cancer for combinational therapy.

1. Introduction

Multidrug resistance (MDR) refers to the cancer cells' ability to resistant to a broad variety of structurally and mechanistically different anticancer drugs, which is one of the major clinical obstacles in cancer chemotherapy [1]. MDR can be caused by several mechanisms, such as efflux transporters, apoptosis regulation, autophagy regulation, DNA repair, and epigenetic regulation [2]. One of the most common mechanisms of MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transporters, which mediate the efflux of anticancer drugs to limit the effective use of chemotherapeutic drugs [3]. So far, at least 48 human ABC proteins have been found to be involved in MDR as drug efflux pumps [3]. Among these ABC transporters, the ABC transporter subfamily B member 1 (ABCB1) and subfamily G member 2 (ABCG2) have been reported to play important roles in inducing MDR in several cancers, such as lung, breast, colon, ovarian cancers and melanomas [4–9]. These pumps significantly reduce the intracellular concentration of anticancer compound which are structurally and biochemically distinct to mediate MDR. A wide variety of chemotherapeutic drugs are substrates of ABCB1, such as taxanes, vinca alkaloid, anthracyclines and so on, while methotrexate and flavopiridols are substrates of ABCG2 [10]. Therefore, developing inhibitors of these transporters is a promising strategy to overcome MDR and retrieve the effectiveness of conventional anticancer drugs. Moreover, ABCB1 and ABCG2 are expressed simultaneously in many MDR cancer cells. Therefore, inhibitors which target ABCB1 as well as ABCG2 have promising clinical application prospects [11]. Recently, several small molecule kinase inhibitors which were usually originally designed for

Abbreviations: MDR, multidrug resistance; DOX, doxorubicin; Rho 123, rhodamine 123; ABC, adenosine triphosphate (ATP)-binding cassette; ABCB1, ABC transporter subfamily B member 1; ABCG2, ABC transporter subfamily G member 2; CDK, cyclin D-cyclin dependent kinase; Rb, retinoblastoma; VRP, verapamil; FTC, fumitremorgin C; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; IAAP, iodoarylazidoprazosin.

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other targets in cell-signal network have been found to interact with ABC transporters [12,13]. Most of these inhibitors were either put in clinical application or under evaluation in clinical trials. Consequently, repositioning these drugs as ABC transporters inhibitors has great advantages in terms of lower drug development costs to accelerate its application in clinic to benefit patients.

CDK4/6 inhibitors which target CDK4 (INK4)-retinoblastoma (Rb) pathway to control cell cycle progression by regulating the G1-S checkpoint have emerged as promising candidates for cancer treatment [14,15]. Abemaciclib (LY2835219) is an orally bioavailable CDK4/6 inhibitor under phase III clinical trials, which have shown its anti-proliferation activity in a series of cancers both *in vitro* and *in vivo* [14–16]. Moreover, combination of LY2835219 with other targeted therapies have been reported to overcome acquired or de novo treatment resistance to increase the effectiveness of chemotherapy [17–19]. In this study, we investigated the effect of LY2835219 on the circumvention of MDR *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco BRL (Thermo Fisher Scientific Inc., Waltham, MA, USA). Doxorubicin (DOX), paclitaxel, cisplatin, topotecan, mitoxantrone, verapamil (VRP), fumitremorgin C (FTC), rhodamine 123 (Rho123), G418, hydroxyethyl cellulose (HEC), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenylte trazoliumbromide (MTT) and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich (St. Louis, MO,USA). DOX, paclitaxel, cisplatin, topoteacan, mitoxantrone and VRP were dissolved in saline. FTC and Rho123 were dissolved in DMSO. LY2835219 and palbociclib were purchased from MedChemexpress (Monmouth Junction, NJ, USA). LY2835219 and palbociclib were dissolved in DMSO. Monoclonal antibodies against ABCB1, ABCG2 were purchased from Santa Cruz Biotechnology (Paso Robles, CA, USA), and antibodies against CDK4, CDK6, RB and p-RB (Ser780) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against tubulin and GAPDH was from Kangcheng (Shanghai, China). SYBR Green qPCR Master Mix was purchased from ExCell Bio (Shanghai, China).

2.2. Cell culture

The human oral epidermoid carcinoma cell line KB and its vincristine-selected ABCB1 overexpressing derivative KBv200 cells [20]; the human breast carcinoma cell line MCF-7 and its doxorubicin selected ABCB1 and ABCG2 overexpressing derivative MCF-7/Adr cells [21,22]; the human colon carcinoma cell line S1 and its mitoxantrone selected ABCG2-overexpressing derivative S1-M1-80 cells [23] and the human embryonic kidney cell line HEK293 and its stable pcDNA3.1, ABCB1 and ABCG2 stable gene transfect cell lines HEK293/pcDNA3.1, HEK293/ABCB1, HEK293/ABCG2-R2 were kind gift provided by Dr. Susan Bates (National Cancer Institute, NIH, Bethesda, MD, U.S.A) [24]. The transfected cells were cultured in medium containing 2 mg/mL G418. All cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. All cells were grown in drug-free culture medium for more than 2 weeks before assay. Cell lines used in this study were thawed from early passage stocks and were passaged for less than 6 months.

2.3. Cell cytotoxicity assay

The MTT assay was used to assess cytotoxicity as described previously [25]. Briefly, cells growing in logarithmic phase were seeded at a density of 2000–6000 cells per well in 96-well plates. When the cells became adherent 24 h later, a range of different concentrations of conventional chemotherapeutic drugs with or without a fixed combination of LY2835219 were added to the wells. The concentrations of agents used in this assay are: Taxel 0.1–10 μM, DOX 0.5–25 μM, Cisplatin 25 μM, Topotecan 1–100 μM, Mitoxantrone 2.5–100 μM, depending on the reaction of the cell lines on these anticancer drugs. After 68 h, MTT (5 mg/ml, 20 μL) was added into each well, and 4 h later, the medium was discarded and 150 μL DMSO was added into the wells to dissolve the formazan product from the metabolism of MTT. Finally, optical density was measured at 540 nm, with background subtraction at 670 nm by a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). The half maximal (50%) inhibitory concentration (IC_{50}) value of a substance was calculated by the Bliss method [26]. The fold reversal of MDR was calculated as previously described [27]. All experiments were repeated at least three times, and the results were shown as mean values ± standard deviation (SD).

2.4. Establishment of KBv200 cell xenograft model and reversal of MDR by LY2835219 in vivo

The KBv200 cell xenograft model was established as described previously with minor modification [28]. Athymic nude mice (female, 5–6 weeks old, 18–20 g) were purchased from the Vital River (Beijing, China). Briefly, KBv200 cells (2.0×10^6) were subcutaneously injected into the right armpit of athymic nude mice. When the tumors reached a mean diameter of 0.5 cm, the 32 mice were randomized into four groups and treated as follows: (1) control (vehicle of LY2835219, p.o., qd and saline i.p. q3d); (2) paclitaxel (20 mg/kg, i.p., q3d); (3) LY2835219 (10 mg/kg, p.o., qd); and (4) LY2835219 (10 mg/kg, p.o., qd given 1 h before injecting paclitaxel) + paclitaxel (20 mg/kg, i.p., q3d). The LY2835219 was dissolved in 1% HEC in 20 mM phosphate buffer (pH2.0) and administered orally by gavage (final volume 0.2 mL) at the indicated dose and schedule. Paclitaxel was formulated in saline and administered by intraperitoneal injection. The body weights of the animals and the two perpendicular diameters (A and B) were recorded every 2 days, and tumor volume (V) was estimated according to the following formula: $V = (\pi/6)[(A + B)/2]^3$. The curve of tumor growth was drawn according to tumor volume and time of implantation. The mice were sacrificed when the mean of tumor weights was over 1 g in the control group. Tumor tissues were excised from the mice and their weights were measured. The ratio of growth inhibition (IR) was calculated according to the following formula [28]:

$$IR\ (\%) = \frac{1 - \text{Mean tumor weight of experiment group}}{\text{Mean tumor weight of control}} \times 100$$

All of the animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University.

2.5. DOX and Rho 123 accumulation

The effect of LY2835219 on the accumulation of DOX and Rho 123 was measured by flow cytometry as previously described [24]. Briefly, the cells were incubated in six-well plates to allow attach to the well overnight. Then the cells were exposed to different concentrations of LY2835219 (0, 0.1, 0.2, 0.4 μM). After 3 h, DOX (10 μM) or Rho 123 (5 μM) was added to the medium for further incubation for another 3 h or 0.5 h, then the cells were collected, centrifuged and washed three times with ice-cold