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Original research article

L-1416, a novel MDR reversing agent with possible reduced calcium antagonism

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ABSTRACT

Background: Multidrug efflux transporter P-glycoprotein (P-gp) is highly expressed on membrane of tumor cells and supposed to be implicated in the resistance to tumor chemotherapy. However, currently none of P-gp inhibitors has been approved by Food and Drug Administration not only due to toxicity but also lack of efficacy in clinical trials.
Methods: To solve the problem, our lab synthesized a novel compound named 1416 [1-(2,6-dimethylphenoxy)-3,4-dimethoxyphenylethylamino) propane hydrochloride] with the hope of high P-gp inhibition and low side effects. Caco-2 cell monolayer and tumor bearing mice were used to evaluate the P-gp inhibition of 1416 *in vitro* and *in vivo*, respectively. One of its potential side effects, calcium antagonism was also evaluated.
Results: Results showed that 1416 showed a similar P-gp inhibition as verapamil in Caco-2 cell monolayer. No significant difference was observed in antitumor enhancement when the optical isomers of 1416 (D-1416 and L-1416) were co-administered with vinblastine. In calcium antagonism, L-1416 showed less calcium inhibition than both D-1416 and verapamil.
Conclusion: The novel compound 1416 could significantly increase the antitumor effects of cytotoxic drugs and one of its optical isomers, L-1416, might be more promising due to its potential low calcium antagonism.

Introduction

Currently, the successful chemotherapy of solid and hematological tumors has been affected by intrinsic or acquired drug resistance, called as multidrug resistance (MDR). One reason causing MDR in cancer cells is the over-expression of P-glycoprotein (P-gp) on the surface of resistant cells, which is an ATP-dependent drug transport protein consisting of 1480 amino acids [1]. P-gp acts as a non-specific drug efflux pump, which reduces the intracellular concentration of cytotoxic drugs [2]. Therefore, inhibiting the function of P-gp is thought to be one of the most useful methods to reverse the acquired MDR [3].

Currently, many potential P-gp inhibitors have been reported and extensively evaluated [4,5]. The first generation of P-gp inhibitors were found by accident in the 1980s, such as VER, cyclosporin, tamoxifen, and several calmodulin antagonists [6]. These compounds were designed for other pharmacological uses, but were occasionally found to show the ability of P-gp inhibition. Many of these first generation P-gp inhibitors are P-gp substrates and can compete with cytotoxic compounds at the binding site of P-gp [7]. However, the first generation P-gp inhibitors often show low binding affinity to P-gp. Generally, these P-gp inhibitors are not so effective in human trials. Usually, to get effective drug efficacy, a significantly higher dosage is needed, usually resulting in unacceptable toxicities [8].

Most of the second generation P-gp inhibitors were designed based on the optimized chemical structure of the first generation, such as dexverapamil, valspodar (PSC 833), and biricodar (VX-710)

Abbreviations: MDR, multidrug resistance; VER, verapamil; VBL, vinblastine; DOX, doxorubicin; VCR, vincristine.

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[9]. These compounds are more potent than the first generation for less toxic, better pharmacologic profile and reduction of necessitated chemotherapy dose [8]. However, the second generation P-gp inhibitors showed unpredictable pharmacokinetic interactions with co-administered chemotherapy agents [6]. These are due to the fact that a competition exists between chemotherapy agents and P-gp inhibitors for cytochrome P450 3A4 activity [10]. Furthermore, due to the P-gp inhibition, the second generation P-gp inhibitors also elicit pharmacodynamic interactions through the competitive inhibition of all P-gp-medicated transport [11,12]. Therefore, it is difficult to establish a safe and effective dose of the co-administered chemotherapy agent and these second generation inhibitors, which limit their clinical usage [8,10]. The third generation P-gp inhibitor was always found by combinatorial chemistry screening, such as XR9576. XR9576 is novel and shows high P-gp affinity [13]. In clinical trials, XR9576 could reverse the resistance of paclitaxel and vinorelbine in ovarian cancer [14,15]. However, phase III clinical studies of XR9576 co-treating with vinorelbine in non-small cell lung cancer were terminated early for adverse events and mortality rates that would have made it impossible to demonstrate any survival [16,17]. Among the side effects of P-gp inhibitors, calcium antagonism is the most serious one. Calcium antagonism can block the calcium channels on myocardial cells and vascular smooth muscle cells, resulting in significant cardiovascular side effects like decreased myocardial contractility and self-discipline of sinus node [18]. Many potent P-gp inhibitors have been abandoned for calcium antagonism like VER, quinine and cyclosporin A [19–21]. Therefore, finding new efficient P-gp inhibitors with less or no side effects, especially with less calcium antagonism is thought to be the main direction of P-gp inhibitor discovery.

To develop a new P-gp inhibitor, our lab synthesized a novel compound called 1416 [1-(2,6-dimethylphenoxy)-3,4-dimethoxyphenylethylamino) propane hydrochloride] (dark red dotted line box, Fig. 1). 1416 consists of two parts: one (blue dotted line box, Fig. 1) is derived from VER (green dotted line box, Fig. 1), which removed the structure of D-620 (N-dealkylnorverapamil), and a non P-gp inhibition part [22]. The other is derived from Mexiletine (red dotted line box, Fig. 1), an anti-arrhythmic drug, to minimize the possibility of arrhythmia arising from calcium antagonism [23]. To evaluate its P-gp inhibition ability, Caco-2 cell line was chosen to evaluate the P-gp inhibition of 1416. The antitumor enhancement of optical isomers including D-1416 and L-1416 were evaluated both *in vivo* and *in vitro*. Calcium antagonism of D-1416 and L-1416 were also evaluated and compared with VER.

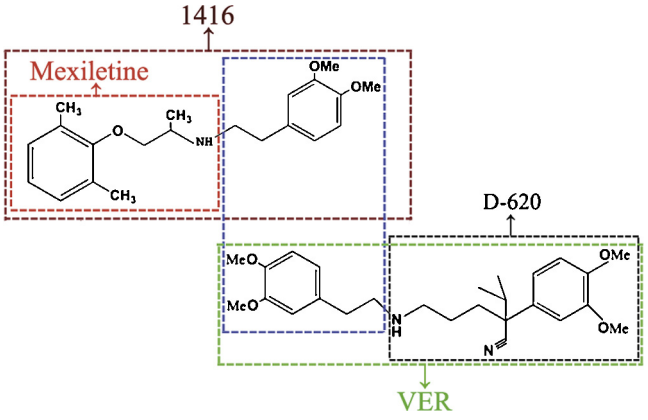

Fig. 1. The chemical structures of VER (green dotted line box), Mexiletine (red dotted line box), 1416 (dark red dotted line box), D-620 (black dotted line box) and the common group of VER and 1416 (blue dotted line box). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Materials and methods

Chemicals

VER, VBL and DOX were bought from Sigma Aldrich (St. Louis, MO, USA). 1416, a novel compound, were synthesized by our lab. D-1416 and L-1416 were also split by our lab.

Cell lines and cell culture

K562 (human chronic myeloid leukemia cell), K562/ADR (resistant to DOX), S180 (mouse fibro sarcoma cell) were kindly gift from China Pharmaceutical University (Nanjing, China). Caco-2 cell (human colon carcinoma cell) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 (human breast cancer cell), MCF-7/ADR (resistant to DOX), KB (oral cancer cell) and KB/VCR (resistant to VCR) were purchased from Nanjing KeyGen Biotech Co. Ltd (Nanjing, China).

Caco-2 monolayer and bidirectional permeability assay

Caco-2 cell culturing and bidirectional permeability assay were performed. Briefly, Caco-2 cells were seeded onto 24-well polycarbonate filter membranes (Millipore, USA) at a density of 8×10^4 cells/cm^2. The cells grew in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G and100 μg/ml streptomycin. The culture medium was changed every other day at first and then replaced every day after culturing for a week. About 21 days later, the monolayer could be used for permeability assay when the trans-epithelial electrical resistance (TEER) was more than 600 Ω/m^2.

The transport medium in this experiment was Hank's balanced salt solution with 10 mM Hepes. The medium was washed twice and soaked with transport medium. Permeability studies were initiated on the monolayers by adding an appropriate volume (apical = 0.4 ml, basal = 0.6 ml) of buffer containing VBL (100 μM), VBL plus 100 μM VER, or VBL plus 10 μM and 20 μM 1416, in triplicate, to the apical compartment (for apical to basal transport) or to the basal compartment (for basal to apical transport) of the transwell, respectively. Samples (100 μL) were taken from both the apical and basal side of the membrane during the test at 20 min, 40 min, 60 min, 90 min, 120 min and 150 min. After the transport experiment, sodium fluorescein (100 μM) was added to the apical side at the end of experiment, and the leakage of sodium fluorescein was examined from the receiving chamber after 60 min incubation. Typical sodium fluorescein flux values across Caco-2 monolayers were below 0.5% h^{-1}. The permeated amount of VBL was measured by high-performance liquid chromatography through LC-10AT (Shimadzu, Japan) at a wavelength of 262 nm.

Rhodamine123 (Rho123) accumulation and retention

MCF-7 and MCF-7/ADR cells (about 10^5 cells/ml) were cultured for 1 h at 37 °C in a 5% CO_2 incubator after adding different compounds: 10 μM VER, 10 μM D-1416, and 10 μM L-1416. Then 3.5 μg/ml Rhodamine123 was added into each well and cultured for 2 h. Cells were washed three times using cold PBS buffer and fluorescent microscopy (TE2000-U, Nikon, Japan) was used to observe the fluorescence of cells.

Cytotoxicity assay and MDR assay

KB and KB/VCR cell lines were used in this experiment. About 2×10^4 cells were seeded into 96-well plates and cultured