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Alkaloids derived from genus *Veratrum* and *Peganum* of Mongolian origin as multidrug resistance inhibitors of cancer cells

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ABSTRACT

Alkaloids comprise one of the largest groups of plant secondary metabolites including vinca alkaloids. The ability of six alkaloids from *Veratrum lobelianum*, one from *Veratrum nigrum* and three from *Peganum nigellastrum* to modify transport activity of *MDR1* was studied. Flow-cytometry in a multidrug-resistant human *MDR1*-gene-transfected mouse lymphoma cells (L5178Y) was applied. The inhibition of multidrug resistance was investigated by measuring the accumulation of rhodamine-123 in cancer cells.

Veralosinine and veranigrine were the most effective resistance modifiers. In a checkerboard method veralosinine and veranigrine enhanced the antiproliferative effects of doxorubicin on MDR cells in combination. The structure–activity relationships were discussed.

1. Introduction

In the last years great efforts have been made to discover effective and nontoxic drugs that are able to inhibit multidrug resistance (MDR). The transporters of various drugs are responsible for multidrug resistance. The main 5 transporter families are the ATP binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), resistance, nodulation and cell division (RND) and multidrug-toxic compounds extrusion (MATE) family. These drug export mechanism leads to a decreased response to the cytotoxic drugs. Furthermore, once cancer cells become resistant to one drug they often show resistance to a large variety of other cytotoxic drugs [1].

MDR is defined as resistance of tumor cells to the cytotoxic action of multiple structurally dissimilar and functionally divergent chemotherapeutic agents including vinca alkaloids [2]. Such resistance is considered to be one of the major reasons of failure of chemotherapy in the treatment of cancer patients. MDR is manifested by the reduced intracellular drug accumulation resulting from increased drug efflux by an adenosine triphosphate ATPase membrane transporter [3]. This ATP-dependent efflux mechanism due to special membrane transporters encoded by the members of the ABC-transporter superfamily consists of 7 families from A to G [4].

One of the most important ABC-efflux pumps responsible for MDR is encoded by the *MDR1* (or *ABCB1*) gene and termed as *MDR1*, ABCB1, P-gp or P-170 [5]. *MDR1* is constitutively expressed in organs such as liver, kidney and adrenal cortex, organs responsible for the elimination of toxicants from the body suggesting its role in detoxification [6]. But *MDR1* is found to be overexpressed in various human cancers and cancerous cell lines expressing MDR phenotype and the amount of *MDR1* is correlated with the degree of resistance particularly at low to moderate levels of resistance [7].

Plant cells produce a large variety of alkaloids, which have diverse chemical structures and biological activities [8]. Some of them are used as medicines (*Vinca* alkaloids, podophyllotoxin, taxanes) and play an important role in plants as an endogenous biological barrier to protect against pathogens or herbivores because of their strong antimicrobial activities and cytotoxicity.

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Vinca alkaloids are substrates of ABC-type drug exporters but also inhibitors [9]. Based on these observations we focused our recent research on the MDR inhibitory effects of various plant alkaloids.

Plants from genus *Veratrum* (Liliaceae) and genus *Peganum* (Zygophyllaceae) were traditionally used as medicinal substances by Mongolia for a long history. The main chemical ingredients in *Veratrum lobelianum* Bernh., *V. nigrum* L. and *Peganum nigellastrum* Bunge. species are steroidal and indole-type alkaloids. The alkaloids have extensive pharmacological actions including anticancer, anti-Alzheimer's disease, anti-diabetes, antimicrobial, anti-inflammatory activities [10,11].

The genus *Veratrum* (Liliaceae) comprises about 40 species which can be found in many areas of the temperate northern hemisphere. The *Veratrum* species have been found to be a rich source of new and bioactive steroidal alkaloids, some of which are well known for their pharmacological potential [12]. The wild-growing plants from genus *Peganum* L. include five species with medicinally important properties [13].

Based on our earlier studies the aim of the present study was to investigate the anticancer properties of a set of alkaloids isolated from *V. lobelianum* Bernh., *V. nigrum* L. and *P. nigellastrum* Bunge. species, including their *MDR1* inhibitory effect. The alkaloids neogermitrine, (**1**), verabenzoamine, (**2**) and 15-O-(2-methylbutyroyl)germine, (**4**) (all of germine-type alkaloids), veratroilzigadenine, (**3**, zygadenine-type alkaloid) and veralosinine (**5**, steroidal alkaloid) are isolated from *V. lobelianum*. Veranigrine (**6**, steroidal alkaloid) is isolated from *V. nigrum*. From *Peganum nigellastrum* are isolated deoxypeganine, (**7**) and peganine, (**9**) (both quinasoline-type alkaloids) and harmine, (**8**, indole-type alkaloid). Several classes of compounds that inhibit efflux by *MDR1* and enhance the accumulation and efficacy of anticancer compounds have been identified, like steroidal alkaloids [14] and indole-type alkaloids [15].

2. Materials and methods

2.1. Tested compounds

The alkaloids neogermitrine, (**1**), verabenzoamine, (**2**), veratroilzigadenine, (**3**), 15-O-(2-Methylbutyroyl)germine, (**4**), veralosinine, (**5**), veranigrine, (**6**), deoxypeganine, (**7**), harmine, (**8**) and peganine, (**9**) were isolated from the roots and rhizomes of three Mongolian species: *Veratrum lobelianum* compounds **1–5**, *V. nigrum* **6,** and *Peganum nigellastrum* **7–9.** They were identified on the base of physical constants and spectral data described in our previous studies in details [16–18].

2.2. Cell cultures

L5178 mouse T-cell lymphoma cells (ECACC cat. no. 87111908, U.S. FDA, Silver Spring, MD, USA) were transfected with pHa *MDR1/A* retrovirus, as described previously [19,20]. The human *MDR1* gene-expressing cell line was selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype. L5178Y (parental, PAR) mouse T-cell lymphoma cells and the human *MDR1*-transfected subline (MDR) were cultured at 37 °C in McCoy's 5A medium (Sigma-Aldrich Kft, Budapest, Hungary) supplemented with 10% heat-inactivated horse serum (Csertex Kft., Budapest, Hungary), L-glutamine (Invitrogen Corp., Carlsbad, CA, USA) and penicillin-streptomycin mixture (Sigma) in 100 U/L and 100 mg/L concentration, respectively. The mouse lymphoma cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

2.3. Assay for antiproliferative effect

The cell growth inhibitory effects of the compounds alone were tested in 96-well flat-bottomed microtitre plates. The compounds were diluted in two-steps from a starting concentration of 50 μg/ml in a final volume of 150 μl medium, and dimethyl sulphoxide (DMSO) was used as a control. A total of 6×10^3 cells in 50 μl of medium were added to each well, with the exception of the medium control wells. The culture plates were further incubated at 37 °C for 72 h, at the end of which 15 μl of MTT solution (thiazolyl blue tetrazolium bromide solved in PBS to a final concentration of 5 mg/ml, Sigma) were added to each well. After further incubation at 37 °C for 4 h, 100 μl of sodium dodecyl sulphate (SDS, Sigma) solution (10%) were measured into each well and the plates were further incubated at 37 °C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Inhibition of cell growth was determined as a percentage according to the formula:

$$IC_{50} = 100 - \left[\frac{OD\ treated\ cells - OD\ medium\ control}{OD\ cell\ control - OD\ medium\ control} \times 100\right]$$

where IC_{50} is defined as the inhibitory concentration that reduces the growth of the compound-exposed cells by 50%.

2.4. Assay for reversal of MDR in tumor cells

The mouse lymphoma cells were adjusted to a density of 2×10^6/mL, resuspended in serum-free McCoy's 5A medium and distributed in 0.5-mL aliquots into Eppendorf centrifuge tubes, PAR cells were used as negative control. The tested compounds were added at various concentrations in different volumes (2.0–20.0 μL) of the 1.0–10.0 mg/mL stock solutions, and the samples were incubated for 10 min at room temperature. Next, the indicator rhodamine 123 (R123) was added to the samples at a final concentration of 10 μg/ml and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured with a FACS Star Plus flow cytometer (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Verapamil (EGIS Pharmaceuticals PLC, Budapest, Hungary) was used as a positive control in the rhodamine 123 exclusion experiments at a final concentration of 22 μM. The percentage mean fluorescence intensity was calculated for the treated MDR cell lines as compared with the untreated MDR cells. Fluorescent activity