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Molecular docking and pharmacogenomics of *Vinca* alkaloids and their monomeric precursors, vindoline and catharanthine

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ABSTRACT

Vinblastine and vincristine are dimeric indole alkaloids derived from *Catharanthus roseus* (formerly: *Vinca rosea*). Their monomeric precursor molecules are vindoline and catharanthine. While vinblastine and vincristine are well-known mitotic spindle poisons, not much is known about vindoline and catharanthine. Vindoline and catharanthine showed weak cytotoxicity, while vinblastine, vincristine, and the semisynthetic vindesine and vinorelbine revealed high cytotoxicity towards cancer cells. This may reflect a general biological principle of poisonous plants. Highly toxic compounds are not only active towards predators, but also towards plant tissues. Hence, plants need mechanisms to protect themselves from their own poisons. One evolutionary strategy to solve this problem is to generate less toxic precursors, which are dimerized to toxic end products when needed. As shown by *in silico* molecular docking and biochemical approaches, vinblastine, vincristine and vinorelbine bound with high affinity to α/β-tubulin and inhibited tubulin polymerization, whereas the effects of vindoline and catharanthine were weak. Similarly, vinblastine produced high fractions of mono- and multipolar mitotic spindles, while vindoline and catharanthine did only weakly affect bipolar mitotic spindle formation. Here, we show that vinblastine contributes to cell death by interference with spindle polarity. P-glycoprotein-overexpressing multidrug-resistant CEM/VCR1000 cells were highly resistant towards vincristine and cross-resistant to vinblastine, vindesine, and vinorelbine, but not or only weakly cross-resistant to vindoline and catharanthine. In addition to tubulin as primary target, microarray-based mRNA signatures of responsiveness of these compounds have been identified by COMPARE and signaling pathway profiling.

1. Introduction

Plants produce secondary metabolites as defense weapons against microbial infections by viruses, bacteria, or protozoa and parasites such as insects or worms as well as against herbivores. Many plants are poisonous, while others can serve as medicinal plants with pharmacological activity. As shown in a previous survey conducted by the National Cancer Institute (NCI), USA, more than two thirds of all anticancer drugs established in anticancer therapy are natural products, derivatives of natural products or mimic bioactive principles of natural products [1].

Among the clinically established natural products with anticancer activity are the *Vinca* alkaloids vinblastine and vincristine and more recently, the semi-synthetic derivatives vindesine and vinorelbine, which are highly useful drugs for the treatment of certain malignancies.

Vinca alkaloids arrest tumor cells during mitosis by binding to tubulin and depolymerization of microtubules [2]. This leads to cell cycle arrest in mitosis [3]. Besides interaction of *Vinca* alkaloids with tubulins, other mechanisms upstream (*e.g.* membrane-bound drug efflux transporters) and downstream (*e.g.* signal transduction pathways, programmed cell death) also account to the drugs' efficacy towards cancer cells.

Abbreviations: ABC transporter, ATP-binding cassette transporter; HNSCC, head and neck squamous cell carcinoma; IC_{50}, 50% inhibition concentration; RMSD, root mean square deviations; mRNA, messenger RNA; NCI, national cancer institute.

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Vinblastine and vincristine are dimeric indole alkaloids derived from *Catharanthus roseus* (formerly: *Vinca rosea*). Their monomeric precursor molecules are vindoline and catharanthine. While there is clear evidence for the action of vinblastine and vinblastine as mitotic spindle poisons, not much is known about the monomers vindoline and catharanthine.

Both precursor molecules are less cytotoxic than their dimeric drugs, vinblastine and vincristine. The question arises, whether this reflects a biological principle of poisonous plants. Poisonous natural products such as vinblastine and vincristine are effective defence mechanisms against herbivores and other predators. However, these compounds may also reveal toxicity to the plants themselves. Hence, they may generate and store large amounts of less toxic precursor molecules for self-protection, whereas the final synthesis of highly toxic end products occurs only upon appropriate external stimulation.

In the present investigation, we hypothesized that different cytotoxicities of monomeric precursors and dimeric end products should affect binding to the primary target of *Vinca* alkaloids, the microtubules. In addition, dimeric second-generation drugs, the semisynthetic vindesine and vinorelbine have been included in the study. A comparative analysis of functional effects of the above mentioned compounds on microtubule formation (effect on α/β-tubulin polymerisation) has been carried out *in vitro*. The relative binding affinities of vindoline and catharanthine were estimated from Dixon plots assuming that all compounds either directly or indirectly (allosteric modulation) interfere with the [^{3}H]-vinblastine binding sites in α/β-tubulin. The experimental data have been compared to molecular modelling studies. The binding of *Vinca* alkaloids to tubulin may not only cause inhibition of microtubule elongation, but may also affect mitotic spindle formation. The formation of multipolar mitotic spindles by inhibition of centrosomal coalescence has been anticipated as novel treatment strategy [4,5]. Therefore, we have analyzed the capacity of *Vinca* alkaoids to induce multipolar mitotic spindles. Finally, we have analyzed the role of drug resistance mechanisms for monomeric and dimeric *Vinca* alkaloids. We first analyzed cross-resistance of vincristine-resistant CEM/VCR1000 leukemia cells towards vindoline and catharanthine in comparison to vinblastine, vindesine, and vinorelbine. Then, we have analyzed other determinants of responsiveness towards *Vinca* alkaloids in the cell line panel of the NCI by means of COMPARE-analyses of microarray-based transcriptome-wide mRNA expression.

2. Material and methods

2.1. Compounds

Vindoline and cantharanthine were isolated from *Cataranthus roseus* as described [6]. Vinblastine sulphate, vincristine sulphate, vindesine sulphate salt, and vinorelbine ditartrate salt vindesine were obtained from Sigma–Aldrich (Taufkirchen, Germany). Vindoline and catharanthine are precursor molecules in the biosynthesis route, while vinblastine and vincristine are end products (Fig. 1). The entire biosynthesis pathway has previously been elucidated [7]. Vindoline and catharanthine were isolated from *Cataranthus roseus* by two of the authors (YF and YZ). Vindesine and vinorelbine are semi-synthetic derivatives and were obtained from Sigma–Aldrich (Taufkirchen, Germany).

2.2. Cell lines

Human CCRF-CEM leukemia cells were maintained in RPMI medium (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum in a humidified 7% CO_2 atmosphere at 37 °C. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase. The multidrug resistance gene 1 (*ABCB1, MDR1*)-expressing CEM/VCR1000 subline was maintained in 1000 ng/mL vincristine. The establishment of the resistant subline has been described [8]. Sensitive and resistant cells were kindly provided by Dr. A. Sauerbrey (Dept. of Pediatrics, University of Jena, Jena, Germany).

The HNSCC cell line SCC114 (oral squamous cell carcinoma) cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Biochrom AG, Berlin, Germany). When indicated, vindoline, catharanthine, or vinblastine were added to the cell culture medium for 24 h. In all experiments, the final DMSO concentration was <1%.

The panel of 60 human tumor cell lines of the Developmental Therapeutics Program of the NCI consisted of leukemia, melanoma, non-small cell lung cancer, colon cancer, renal cancer, and ovarian cancer cells, cells of tumors of the central nervous system, prostate carcinoma, and breast cancer. Their origin and processing have been previously described [9].

2.3. Sulforhodamine B assay

The determination of drug sensitivity in the NCI cell lines by the sulforhodamine B assay has been reported [10]. The 50% inhibition concentration (IC_{50}) values for vinblastine, vincristine, catharanthine, and vindoline have been deposited in the database of the database of the Developmental Therapeutics Program of the NCI (http://www.dtp.nci.nih.gov).

2.4. Growth inhibition assay

The *in vitro* response to drugs was evaluated by means of a growth inhibition assay as described [10]. Aliquots of 5×10^4 cells/mL were seeded in 24-well plates and compounds were immediately added at different drug concentrations to allow calculation of 50% inhibition concentration (IC_{50}) values. Cells were counted seven days after drug treatment. The resulting growth data represent the net outcome of cell proliferation and cell death.

2.5. Preparation of pure α/β-tubulin (>95%)

Tubulin was isolated as pure α/β-tubulin from fresh pig brain according to a previously described method [11]. Fresh brains were obtained from the local slaughterhouse and processed immediately without prior cooling. In brief, 150–200 g cleaned pig brain was put into ice-cooled depolymerization buffer (50 mM MES, 1 mM $CaCl_2$, adjusted to pH 6.9 with KOH) and homogenized in a Polytron mixer. The homogenate was centrifuged in a Sorvall SLA-1500 rotor at 14,500 rpm for 60 min. The supernatant was transferred into an Erlenmeyer flask in high-molar PIPES-buffer (1 M PIPES, 10 mM $MgCl_2$, 20 mM EGTA adjusted to pH 6.9 with KOH) plus ATP (1.5 mM final concentration) and glycerol (98%) ad 300 mL. The resulting suspension was mixed and incubated at 37 °C for 1 h. Aliquots were transferred into ultracentrifuge tubes and centrifuged in a Beckman Ti50.2 rotor at 32,500 rpm (96,000 × *g*) for 75 min at 30 °C. The microtubule protein pellets were suspended in depolymerization buffer and put on ice prior to ultracentrifugation at 4 °C. The procedure was repeated for two polymerization cycles (total of three cycles) and the final α/β-tubulin pellets were suspended in ice-cold Brinkley Buffer (BRB80; 80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA adjusted to pH 6.8 with KOH) prior to shock-freezing in liquid nitrogen and subsequent storage at −80 °C. Purity and concentration of α/β-tubulin were determined by SDS-PAGE gel electrophoresis and spectrophotometrically ($A = \varepsilon \cdot c \cdot d$ with a given extinction coefficient of 115,000 $M^{-1}\,cm^{-1}$) at 280 nm. This procedure typically yielded 60–100 mg of α/β-tubulin per 100 g of brain.