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## **The effect of conjugation on antitumor activity of vindoline derivatives with octaarginine, a cell-penetrating peptide**

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## "Dedicated to the memory of Professor Csaba Szántay"

### Abstract

Some *Vinca* alkaloids (e.g. vinblastine, vincristine) have been widely used as antitumor drugs for a long time. Unfortunately, vindoline, a main alkaloid component of *Catharanthus roseus* (L.) G. Don, itself, has no antitumor activity. In our novel research program we have prepared and identified new vindoline derivatives with moderate cytostatic activity.

Here we describe the effect of conjugation of vindoline derivative with oligoarginine (tetra-, hexa- or octapeptides) cell-penetrating peptides on the cytostatic activity *in vitro* and *in vivo*. Br-Vindoline-(L)-Trp-OH attached to the *N*-terminus of octaarginine was the most effective compound *in vitro* on HL-60 cell line. Analysis of the *in vitro* activity of two isomer conjugates (Br-vindoline-(L)-Trp-Arg<sub>8</sub> and Br-vindoline-(D)-Trp-Arg<sub>8</sub>) suggest the covalent attachment of the vindoline derivatives to octaarginine increased the antitumor activity significantly against P388 and C26 tumour cells *in vitro*. The cytostatic effect was dependent on the presence and configuration of Trp in the conjugate as well as on the cell line studied. The configuration of Trp notably influenced the activity on C26 and P388 cells: conjugate with (L)-Trp was more active than conjugate with the (D)-isomer. In contrast conjugates had very similar effect on both the HL-60 and MDA-MB-231 cells. In preliminary experiments conjugate Br-vindoline-(L)-Trp-Arg<sub>8</sub> exhibited some inhibitory effect on the tumor growth in P388 mouse leukemia tumor-bearing mice. Our results indicate that the conjugation of modified vindoline could result in an effective compound even with *in vivo* antitumor activity.

Key words: *Vinca* alkaloids, vindoline, cell-penetrating peptide, peptide-conjugates, antitumor activity

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## Introduction

*Vinca* alkaloids were first isolated from the leaves of *Catharanthus roseus* (L.) G. Don. The major alkaloid in this plant is vindoline<sup>1,2</sup>. Some of the minor alkaloids (e.g. vincristine, vinblastine) of this plant have been widely used as antitumor drugs for a long time. These bisindole alkaloids, which contain a rearranged catharanthine and vindoline moiety, disturb the dynamics of microtubular system causing microtubule instability<sup>3,4</sup>. Vindoline, the most complex part of bisindole alkaloids, is considered as their bio- as well as synthetic precursor. Unfortunately, early studies showed that vindoline does not exhibit any antitumor activity but it might be functioning as the anchor in the binding of bisindole alkaloids to tubulin<sup>5</sup>. Because small structural changes may cause drastic differences in the antitumor efficacies, potencies, and side effects, much synthetic effort has been directed toward modifying the structure of *Vinca* alkaloids to obtain efficient drug candidates. One of the first such attempts was to prepare 17-desacetylvinblastine derivatives by the replacement of the methyl ester group at position C-16 in the vindoline part with an amide group (vindesine)<sup>6</sup> and then to modify this group by incorporating different alkyl groups or amino acids<sup>7,8</sup>. Among the amino acid derivatives, the presence of hydrophobic amino acid (Leu, Ile, Val, or Trp) exhibited pronounced activity. The Trp-OEt derivative was found to be very active on P388 and L1210 leukemia cells implanted intravenously in DBA/2 mice. For further improvement, by modification of the vindoline ring system a new set of compounds was prepared<sup>9</sup> and attached to (L)- or (D)-Trp-OMe<sup>10</sup>. These conjugates showed higher *in vitro* cytostatic activity on HL-60 human leukemia cells, while the unmodified vindoline was inefficient even at 100  $\mu$ M concentration. It should be noted that the (L)- and (D)-Trp containing derivatives showed lower activity as compared to vinblastine.

Cell-penetrating peptides (CPP), mainly positively charged short amino acid sequences, are able to pass through the cell membrane<sup>11</sup>. Based on the experimental data, the family of cell-penetrating peptides can be divided into three main classes; (a) primary amphipathic, (b) secondary amphipathic and (c) non-amphipathic cell-penetrating peptides<sup>12</sup>. Oligoarginines were *de novo* designed in accord with early-discovered CPPs containing several arginine residues in their sequences<sup>13</sup>. Among these compounds octaarginine (Arg<sub>8</sub>) was found to be a CPP with optimal length for efficient internalization into mouse macrophage RAW264.7 cells<sup>14</sup>. Based on these observations octaarginine was used frequently to transport different molecular cargos into cells. We also used successfully this peptide to deliver calpain enzyme

substrate peptide<sup>15</sup> and antitumor drug (daunomycin, vinblastine, pemetrexed or methotrexate)<sup>16-19</sup>.

Previously, we prepared and studied the (L)-Trp modified derivatives of 17-desacetylvinblastine<sup>16</sup> with essentially the same activity on human leukemia cells (HL-60) as that of vinblastine sulfate, a drug used in cancer treatment. Conjugation with octaarginine preserved the activity of the free isomers on both sensitive (HL-60) and drug-resistant leukemia cells (HL-60-MRP1 and HL-60-MDR1).

The biological data obtained with these Trp derivatives of vinblastine inspired us to prepare vindoline derivative isomers and isomer conjugates. Thus, the (L)- and (D)-Trp derivatives of Br-vindoline and conjugates with oligoarginines were prepared by solution-phase conjugation and the *in vitro* cytostatic effect was studied on several tumor cell lines. In addition, the *in vivo* antitumor effect of conjugates was analyzed in two mouse tumor models.

## Materials and Methods

### General Procedures

Analytical RP-HPLC was performed a) on an Exformma (Exformma Technology (ASIA) Co., Ltd, Hong Kong, China) HPLC system using an Agilent Zorbax SB-C18 column (4.6mm x 150mm, I.D., 100Å pore size, 5 µm silica), linear gradient elution (0 min 0% B, 2 min 0% B, 22 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80:20, V/V)) was used at a flow rate of 1 mL/min at ambient temperature, or b) a Knauer (H. Knauer GmbH, Berlin, Germany) HPLC system using Phenomenex SYNERGY MAX-RP (4.6mm x 250mm, I.D., 80 Å pore size, 4 µm silica), linear gradient elution (0 min 0% B, 5 min 0% B, 50 min 90% B) with eluent A and eluent B was used at a flow rate of 1 mL/min at ambient temperature. The UV detection was carried in both cases at  $\lambda = 220$  nm.

The peptides were purified on a semi-preparative Phenomenex Jupiter C18 column (250x10mm I.D. 300 Å pore size, 10 µm silica (Torrance, CA, USA)). The flow rate was 4 mL/min. Linear gradient elution (0 min 0% B; 5 min 0 % B, 50 min 50 % B) and UV detection ( $\lambda = 220$  nm) was applied. The conjugates were purified by using the same system and condition, but the linear gradient was 0 min 25% B; 5 min 25 % B, 50 min 70 % B.

TLC was carried out using Kieselgel 60 F<sub>254</sub> (Merck) glass plates.

The molecular mass of peptides and conjugates was determined by ESI-MS was performed on a Bruker Daltonics Esquire 3000 plus (Germany) ion trap mass spectrometer. The samples were dissolved in acetonitrile–water (50:50, V/V), containing 0.1% acetic acid.

Melting points were measured on VEB Analytik Dresden PHMK-77/1328 apparatus and are uncorrected. IR spectra were recorded on Zeiss IR 75 and 80 instruments. NMR measurements were performed on a Varian 800 MHz NMR spectrometer equipped with a  $^1\text{H}\{^{13}\text{C}/^{15}\text{N}\}$  Triple Resonance  $^{13}\text{C}$  Enhanced Salt Tolerant Cold Probe operating at 800 MHz for  $^1\text{H}$  and 201 MHz for  $^{13}\text{C}$ , and a Varian 500 MHz NMR spectrometer equipped with a  $^1\text{H}\{^{13}\text{C}/^{15}\text{N}\}$  5 mm PFG Triple Resonance  $^{13}\text{C}$  Enhanced Cold Probe operating at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ . Chemical shifts are given on the delta scale as parts per million (ppm) with tetramethylsilane (TMS) ( $^1\text{H}$ ) or dimethylsulfoxide- $d_6$  ( $^{13}\text{C}$ ) as the internal standard (0.00 ppm and 39.5 ppm, respectively).  $^1\text{H}$ - $^1\text{H}$ , direct  $^1\text{H}$ - $^{13}\text{C}$ , and long-range  $^1\text{H}$ - $^{13}\text{C}$  scalar spin-spin connectivities were established from 2D gDQFCOSY, zTOCSY, gHSQCAD, and gHMBCAD experiments, respectively. All pulse sequences were applied by using the standard spectrometer software package. All experiments were performed at 298 K. HRMS analyses were performed on an LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) system. The ionization method was ESI operated in positive ion mode. For the CID experiment helium was used as the collision gas, and normalized collision energy (expressed in percentage), which is a measure of the amplitude of the resonance excitation RF voltage applied to the endcaps of the linear ion trap, was used to induce fragmentation. The protonated molecular ion peaks were fragmented by CID at a normalized collision energy of 35–55%. The samples were dissolved in methanol. Data acquisition and analysis were accomplished with Xcalibur software version 2.0 (Thermo Fisher Scientific).

### Synthesis of vindoline derivatives

The synthesis of the methyl esters of Br-vindoline-Trp-OMe and Br-vindoline-D-Trp-OMe conjugate was described earlier<sup>10</sup>. The esters were then hydrolysed using LiOH x H<sub>2</sub>O in MeOH. The methyl ester derivatives (**1a** and **1b**) were dissolved in a mixture of MeOH and distilled water (3:1 V/V ratio at c= 0.04 M), and lithium hydroxide monohydrate (8 eqv.) was added. The reaction mixture was kept in a refrigerator at 4 °C for 24 h. 10 mL of distilled water was then added to the solution and the methanol was evaporated in vacuum. The solution was acidified to pH 7 with 5% HCl and evaporated to dryness in vacuum. The crude product was purified by preparative TLC.

*N*-[10-bromo-17-*O*-desacetyl-16-des(methoxycarbonyl)vindoline-16-carbonyl]-*L*-tryptophan  
(**2a**)

167 mg (0.246 mmol) methyl ester (**1a**) was hydrolysed and the crude product was purified by preparative TLC (CHCl<sub>3</sub>-MeOH 7:3, V/V) and 70 mg (29 %) of **2a** was isolated as pale yellow crystals. Mp 251-253 °C (decomp.). TLC (CHCl<sub>3</sub>-MeOH 7:3), *R<sub>f</sub>*=0.44.

IR (KBr) 3406, 1655, 1605, 1498, 1458, 1229, 746 cm<sup>-1</sup>.

<sup>1</sup>H NMR (799.7 MHz, DMSO-*d*<sub>6</sub>) δ 0.62 (3H, t, *J* 7.5 Hz, H<sub>3</sub>-18), 0.88 (1H, dq, *J* 13.5, 7.5 Hz, H<sub>γ</sub>-19), 1.33 (1H, dq, *J* 13.5, 7.5 Hz, H<sub>α</sub>-19), 2.04–2.11 (2H, m, H<sub>α</sub>-6 and H<sub>γ</sub>-6), 2.53–2.58 (1H, m, H<sub>γ</sub>-5), 2.69 (4H, s, N(1)-CH<sub>3</sub>, H-21), 2.80 (1H, br d, *J* 16.3 Hz, H<sub>γ</sub>-3), 3.13–3.18 (2H, m, H<sub>α</sub>-1'' and H<sub>γ</sub>-1''), 3.19–3.23 (1H, m, H<sub>α</sub>-5), 3.32–3.36 (1H, buried m, H<sub>α</sub>-3), 3.37 (1H, s, H-2), 3.79 (3H, s, C(11)-OCH<sub>3</sub>), 3.80–3.90\* (2H, overlapping singlet and broad resonance, H-17 and C(17)-OH), 4.20–4.32 (1H, m, H-2''), 5.54 (1H, br d, *J* 10.2 Hz, H-15), 5.76 (1H, dd, *J* 10.2, 5.1 Hz, H-14), 6.24 (1H, s, H-12), 6.90 (1H, dd, *J* 7.6, 7.4 Hz, H-5'), 7.00 (1H, dd, *J* 8.1, 7.4 Hz, H-6'), 7.15 (1H, br, H-2'), 7.23 (1H, s, H-9), 7.27 (1H, d, *J* 8.1 Hz, H-7'), 7.58 (1H, d, *J* 7.6 Hz, H-4'), 7.75 (1H, d, *J* 7.6 Hz, H-4''), 8.68\* (1H, br, C(16)-OH), 10.71 (1H, br, H-1'). <sup>13</sup>C NMR (201.1 MHz, DMSO-*d*<sub>6</sub>) δ 7.5 (C-18), 27.4 (C-1''), 31.9 (C-19), 37.9 (N(1)-CH<sub>3</sub>), 42.1 (C-20), 44.4 (C-6), 50.26 (C-5), 50.35 (C-3), 52.0 (C-7), 53.7\* (br, C-2''), 56.0 (C(11)-OCH<sub>3</sub>), 66.9 (C-21), 72.9 (C-17), 79.3 (C-16), 83.8 (C-2), 93.4 (C-12), 96.9 (C-10), 110.5\* (br, C-3'), 110.8 (C-7'), 117.7 (C-5'), 118.8 (C-4'), 120.3 (C-6'), 122.6 (C-14), 123.5 (C-2'), 126.0 (C-9), 126.5 (C-8), 127.8 (C-3a'), 131.4 (C-15), 135.7 (C-7a'), 153.0 (C-13), 155.7 (C-11), 170.9\* (br, C-3''), 170.9 (C(16)-CONH), \*: assignment of exchanging resonances may be uncertain.

HRMS: M+H=665.19653 (C<sub>33</sub>H<sub>38</sub>O<sub>6</sub>N<sub>4</sub>Br; delta=-0.6 ppm). HR-ESI-MS-MS (CID=35 %) rel. int. %): 647(100); 619(2); 479(8); 443(23); 433(23); 373(3).

*N*-[10-bromo-17-*O*-desacetyl-16-des(methoxycarbonyl)vindoline-16-carbonyl]-*D*-tryptophan (**2b**)

249 mg (0.366 mmol) methyl ester (**1b**) was hydrolysed and the crude product was purified by preparative TLC (CHCl<sub>3</sub>-MeOH 7:3, V/V) and 126 mg (52 %) of **2b** was isolated as pale yellow crystals. Mp 215-217 °C (decomp.). TLC (CHCl<sub>3</sub>-MeOH 7:3), *R<sub>f</sub>*=0.28.

IR (KBr) 3406, 1649, 1605, 1499, 1040, 746 cm<sup>-1</sup>.

<sup>1</sup>H NMR (499.9 MHz, DMSO-*d*<sub>6</sub>) δ 0.58 (3H, t, *J* 7.3 Hz, H<sub>3</sub>-18), 0.82 (1H, dq, *J* 13.3, 7.3 Hz, H<sub>γ</sub>-19), 1.24–1.31 (1H, buried m, H<sub>α</sub>-19), 1.95–2.10 (2H, m, H<sub>α</sub>-6 and H<sub>γ</sub>-6), 2.23 (3H, s, N(1)-CH<sub>3</sub>), 2.51–2.58 (1H, m, H<sub>γ</sub>-5), 2.60 (1H, s, H-21), 2.79 (1H, br d, *J* 15.6 Hz, H<sub>γ</sub>-3), 3.05–3.13 (2H, overlapping broad resonances, H<sub>γ</sub>-1'', H-2), 3.15–3.38 (3H, buried m, H<sub>α</sub>-3, H<sub>α</sub>-5, H<sub>α</sub>-1''), 3.74 (3H, s, C(11)-OCH<sub>3</sub>), 3.81–4.01\* (2H, overlapping singlet and broad

resonance, H-17 and C(17)-OH), 4.17–4.30 (1H, br, H-2''), 5.52 (1H, br d, *J* 10.7 Hz, H-15), 5.73 (1H, dd, *J* 10.7, 5.0 Hz, H-14), 6.13 (1H, s, H-12), 6.94 (1H, dd, *J* 7.6, 7.4 Hz, H-5'), 7.01 (1H, dd, *J* 8.1, 7.4 Hz, H-6'), 7.17–7.23 (2H, overlapping singlet and broad resonance, H-2' and H-9), 7.28 (1H, d, *J* 8.1 Hz, H-7'), 7.57 (1H, d, *J* 7.6 Hz, H-4'), 8.00 (1H, br d, *J* 5.0 Hz, H-4''), 8.88\* (1H, br, C(16)-OH), 10.71 (1H, br, H-1'), <sup>13</sup>C NMR (125.7 MHz, DMSO-*d*<sub>6</sub>) δ 7.5 (C-18), 27.7 (C-1''), 31.9 (C-19), 37.9 (N(1)-CH<sub>3</sub>), 42.1 (C-20), 44.5 (C-6), 50.2 (C-3), 50.5 (C-5), 52.0 (C-7), 54.3\* (br, C-2''), 56.0 (C(11)-OCH<sub>3</sub>), 67.3 (C-21), 72.8 (C-17), 79.4 (C-16), 83.6 (C-2), 93.4 (C-12), 97.0 (C-10), 110.9 (C-7'), 111.2 (C-3'), 117.7 (C-5'), 118.4 (C-4'), 120.4 (C-6'), 122.4 (C-14), 123.2 (C-2'), 125.9 (C-9), 126.6 (C-8), 127.9 (C-3a'), 131.4 (C-15), 135.8 (C-7a'), 153.0 (C-13), 155.7 (C-11), 171.6\* (br, C-3''), 171.6 (C(16)-CONH) \*: assignment of exchanging resonances may be uncertain.

HRMS: M+H=665.19645 (C<sub>33</sub>H<sub>38</sub>O<sub>6</sub>N<sub>4</sub>Br; delta=-0.7 ppm). HR-ESI-MS-MS (CID=35 %) rel. int. %): 647(100); 619(3); 479(5); 443(18); 433(19); 373(3).

### Synthesis of the conjugates of vindoline derivatives with oligoarginine

Oligoarginines (tetra-, hexa- and octaarginine) was synthesised on Rink Amide MBHA resin using standard Fmoc/<sup>t</sup>Bu strategy as described before<sup>19</sup>. After cleavage from the resin the crude product was purified by RP-HPLC and was allowed to react with vindoline derivatives as reported earlier<sup>16</sup>. Briefly, the vindoline derivatives (**2a** and **2b**) were conjugated with oligoarginine in DMF using 1.2 eqv. HOBt and DIC as the coupling reagents in the presence of 5 eqv. DIEA as the base. The conjugation reaction proceeded for 6 h at RT. DMF was then evaporated in vacuum, the crude product was dissolved in eluent A and was purified by RP-HPLC as described above. The Br-vindoline-Arg<sub>8</sub> conjugate was prepared by the reaction of 10-bromo-17-dezacylvindoline-16-carboxylic acid hydrazide and *H*-Arg<sub>8</sub>-NH<sub>2</sub> as described earlier<sup>10</sup> with one modification. The octaarginine component was dissolved in DMF instead of DCM. The solvents were then evaporated and the residue was dissolved in the mixture of eluent A and B (3:1 V/V) and purified by RP-HPLC as described above.

### Cell cultures for *in vitro* studies

For the analysis of *in vitro* cytostatic effect MCF-7 (ATCC: HTB-22)<sup>20</sup>, MDA-MB-231 (ATCC: HTB-26)<sup>21</sup> and HL-60 (ATCC: CCL-240)<sup>22</sup> cells were used. MCF-7, human breast adenocarcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS), non-essential amino acids (NEAA), pyruvate (1 mM), L-glutamine (2 mM) and gentamicin (160 µg/ml). HL-60 human promyelocytic leukemia cells were grown in



RPMI-1640 supplemented with 10% FCS, (L)-glutamine (2 mM) and gentamicin (160 µg/ml). MDA-MB-231 human triple negative breast adenocarcinoma cells were cultured in RPMI-1640 supplemented with 10% FCS, (L)-glutamine (2 mM) and gentamicin (16 µg/ml). Cells were maintained in plastic tissue culture dishes at 37°C with a humidified atmosphere containing 5% CO<sub>2</sub> / 95% air.

P388 mouse leukemia and C26 murine colon carcinoma cell lines were obtained from ATCC. Cells were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing penicillin-streptomycin (Sigma) and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### **Analysis of the *in vitro* cytostatic activity of the conjugates**

The *in vitro* cytostatic effect of the compounds was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay (MTT-assay) on HL-60, MDA-MB-231, MCF-7 C26 and P388 cells. The cells were grown to confluency in a cell culture flask and were then plated into a 96-well plate with an initial cell number of  $5.0 \times 10^3$ , and in the case of P388, a  $2.0 \times 10^4$  per well. After 24 h of incubation at 37°C, cells were treated with the compounds at a  $1.28 \times 10^{-3}$ -100 µM concentration range for 3 h in a 200 µL final volume. Control cells were treated with serum-free medium only at 37°C for 3 h. After incubation the cells were washed twice with serum-free medium and further cultured up to 72 h in serum-containing medium. In order to evaluate cell viability, an MTT assay<sup>23,24</sup> was carried out and the IC<sub>50</sub> value was determined. Briefly, 45 µL of MTT solution (2 mg/mL, in serum-free medium) was added to each well following 4 h of incubation, plates were centrifuged at 900 g for 5 min, and the supernatant was removed. The precipitated purple crystals were dissolved in 100 µL of DMSO and the absorbance was determined at  $\lambda = 540$  nm and  $\lambda = 620$  nm using ELISA plate reader (iEMS Reader, Labsystems, Finland). The percentage of cytostasis was calculated using the following equation: Cytostatic effect (%) =  $[1 - (OD_{\text{treated}}/OD_{\text{control}})] \times 100$ ; where OD<sub>treated</sub> and OD<sub>control</sub> correspond to the optical densities of the treated and untreated cells, respectively. In each case three independent experiments were carried out with 4 parallel measurements. The 50% inhibitory concentration (IC<sub>50</sub>) values were determined from the dose-response curves. The curves were defined by using Microcal™ Origin1 (version 9.2) software: cytostasis was plotted as a function of concentration, fitted to a sigmoidal curve, and the IC<sub>50</sub> value was determined. IC<sub>50</sub> represents the concentration of a compound that is required for 50% inhibition of *in vitro* proliferation and expressed as micromolar units.

## Experimental Animals

First generation of hybrid BDF1 (C57BL/6 female and DBA/2 male) adult female mice, and BALB/c female mice, weighing 22-24 g from our specified pathogen-free (SPF) animal colonies (Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary) were used. The animals were kept in makrolon cages at 22-24°C (40-50% humidity), with a lighting regimen of 12/12 h light and dark. The animals had free access to tap water and were fed with a sterilized standard diet (Charles River VRF1, autoclavable, Germany) ad libitum. The animals used in these studies were cared for according to the „Guiding Principles for the Care and Use of Animals” based upon the Helsinki declaration and they were approved by the local ethical committee (permission number: PEI/001/2574-6/2015).

## Analysis of the *in vivo* antitumor activity of the conjugates

The antitumor activity of the conjugates was studied on P388 (mouse leukemia) and C26 cells (murine colon carcinoma) in BDF1 or BALB/c mice.

*Solid tumor models:* P388 and C26 cells ( $1-2 \times 10^6$  in 200  $\mu$ l serum-free medium per mouse) were injected subcutaneously into BDF1 or BALB/c mice (females, 7-10 animals per group), respectively. The animals were treated at Day1 and Day6 after tumor cell inoculation with the tested compounds at doses of 10, 20, and 40 mg/kg. Two dimensions (length and width) of the growing tumor were measured by caliper three times per week and tumor volumes were calculated using the formula:  $\text{length} \times \text{width}^2 \times \pi/6$ . The treatments were terminated between Day22-33, respectively.

## Results

*Vinca* alkaloids are clinically used drugs against several kinds of tumors. Unfortunately, only the minor alkaloid components of *Catharanthus roseus* (L.) G. Don were found to be effective. The major alkaloid of the plant– vindoline – exhibited no antitumor activity<sup>25</sup>. In our ongoing project with *Vinca* alkaloids effective vinblastine conjugates with octaarginine<sup>16</sup> and various vindoline derivatives with modest activity<sup>10</sup> were synthesized. We described earlier that derivatives of Br-vindoline containing (L)- or (D)-Trp-OMe moiety could exhibit moderate cytostatic activity<sup>10</sup>. Here we report on studies in which the effect of conjugation with cell-penetrating peptides on this activity was investigated.

### **Synthesis of Br-vindoline derivatives with/without (L)- or (D)-Trp-OMe**

Vindolines substituted at position 10 (**1a, b**) were coupled with amino acid esters as described before<sup>10</sup>. The esters (**1a, b**) were treated in a methanol-water mixture with lithium hydroxide (Scheme 1) at 4 °C to produce compounds with free carboxylic acid function for peptide conjugation (**2a, b**). The synthesis of 10-bromo-17-dezacylvindoline-16-carboxylic acid hydrazide (**3**) was presented by us earlier<sup>10</sup>.

### **Synthesis of vindoline derivative-oligoarginine conjugates**

Oligoarginines are well-known cell-penetrating peptides which can deliver a wide range of compounds into cells efficiently. Our earlier results indicated also that octaarginine was the most effective compound in the delivery of antitumor drugs (e.g. vinblastine, daunomycin) into cell<sup>16,17</sup>. This activity was dependent on the number of Arg residues present in the conjugate. Therefore here we have also studied the effect of the chain length of oligoarginine on the activity of vindoline conjugates and conjugates of Br-vindoline-(L)-Trp-OH (Scheme 2 a) with tetra-, hexa- or octaarginine were synthesized. Earlier in the case of the vinblastine-Trp-OH conjugates<sup>16</sup> we observed that the configuration of Trp had high impact on the biological activity, the octaarginine conjugates of Br-vindoline with (L)- and (D)-Trp-OH were prepared and comparatively studied. In order to evaluate the importance of the Trp moiety, the 10-bromo-17-dezacylvindoline-16-carboxylic acid hydrazide was also directly coupled to the most effective oligoarginine (octaarginine, Scheme 2 b).

The oligoarginines (tetra-, hexa- and octaarginines) were built up by Fmoc/<sup>t</sup>Bu strategy with a free *N*-terminal amino group and carboxamid *C*-terminus. The purified peptides were then conjugated with the vindoline derivatives in solution by the *in situ* active ester strategy in DMF using DIC and HOBt as the coupling reagents. The conjugates were purified by RP-HPLC and analyzed by analytical RP-HPLC and ESI-MS mass spectrometry (**Table 1**). The conjugation of Br-vindoline and octaarginine was carried out as described earlier<sup>10</sup> with some modification.

### ***In vitro* cytostatic effect of conjugates**

First, we studied the effect of the length of oligoarginine on antitumor activity by comparing *in vitro* cytotoxicity of tetra-, hexa- and octaarginine conjugates on HL-60 cells (**Table 2**). While oligoarginine and Br-vindoline had no effect, the Trp derivative of Br-vindoline (**2a**) exhibited some moderate activity [ $IC_{50} = 83.1 \pm 10.7 \mu M$ ]. This could be increased by conjugation with oligoarginine. The presence of tetraarginine already enhanced the biological

activity of (**2a**), but the presence of eight arginine residue had the most pronounced influence [ $IC_{50} = 12.9 \pm 4.4 \mu M$ ].

In the next set of experiments the analysis of *in vitro* cytostatic effect of compounds and octaarginine conjugates was extended to various tumor cell lines – HL-60, MCF-7, MDA-MB-231, C26 and P388 (**Table 3**). Considering our previous observation on the marked effect of the configuration on activity with vinblastine-Trp-OH isomer conjugates (with L- or D-Trp enantiomer)<sup>16</sup>, the analysis of the D-Trp containing compounds were also incorporated (**Table 3**).

As expected vindoline and Br-vindoline expressed no activity on any cell lines studied (HL-60, MDA-MB-231, MCF-7, C26 and P388 cell lines). The presence of Trp-OMe moiety almost regardless to the configuration of the Trp residue, increased the antitumor effect of Br-vindoline and the studied derivatives (**1a, b**) had moderate biological activity in a range of  $IC_{50} = 73.8 \pm 10.4 \mu M - 5.3 \pm 1.5 \mu M$ . It is interesting to note that in case of P388 cells, compounds **1a** and **1b** had not only the most marked effect, but also slightly different activity depending on the Trp configuration ( $IC_{50} = 5.3 \pm 1.5 \mu M$  for L-Trp, and  $IC_{50} = 15.9 \pm 2.8 \mu M$  for D-Trp, respectively).

The corresponding  $IC_{50}$  values derived from the treatment of cells with octaarginine conjugates indicated the same tendency: the (L)-Trp containing conjugate (Br-Vind-(L)-Trp-Arg<sub>8</sub>) was more effective ( $IC_{50} = 3.1 \pm 0.5 \mu M$ ) as compared with the isomer conjugate (Br-Vind-(D)-Trp-Arg<sub>8</sub>) ( $IC_{50} = 18.0 \pm 1.2 \mu M$ ).

The conjugates with Br-vindoline-(L)-Trp-OH or Br-vindoline-(D)-Trp-OH had cytostatic activity on other cell lines as well and this effect was generally higher or essentially the same (e.g. P388) in comparison with the free, unconjugated vindoline derivatives (**1a, b**). It is important to mention that conjugates of Br-vindoline without the Trp moiety (Br-Vind-Arg<sub>8</sub>) were slightly less effective on MCF7, C26 and P388 cells. The *in vitro* cytostatic activity was also dependent on the cell line: very similar effect of octaarginine conjugates was observed on HL-60 and MDA-MB-231 cells with  $IC_{50}$  values in the range of  $IC_{50} = 11.5-15.1 \mu M$  and  $IC_{50} = 10.0-15.1 \mu M$ , respectively. On the other hand, the configuration of Trp notably determined the influence of the conjugate on the activity on C26 and P388 cells: conjugate with (L)-Trp was more active than conjugate with the (D)-isomer.

### ***In vivo* antitumor activity of conjugates**

The effect of Trp containing conjugates on the tumor growth was studied *in vivo* by using two tumor models. P388 (mouse leukemia) and C26 cells (murine colon carcinoma) were injected subcutaneously into mice. Then the animals were treated with the conjugate on Day 1 and 6 at 10 mg/kg body weight concentrations. Vinblastine as a clinically used drug was used as positive control (1 mg/kg body weight). In the case of P388 tumor we observed differences: the treatment with conjugate containing (L)-tryptophan [Br-vind-(L)-Trp-Arg<sub>8</sub>] conjugate exhibited a tendency to inhibit the tumor growth (**Figure 1**), while conjugates with (D)-tryptophan (Br-vind-(D)-Trp-Arg<sub>8</sub>) had essentially no inhibitory effect.. It is interesting to mention that the Br-vind-(L)-Trp-Arg<sub>8</sub> conjugate – under these conditions –was slightly, but not significantly more influential than vinblastine, a known cytostatic agent.

In contrast, the treatment of C26 colon carcinoma with isomer conjugates (Br-vind-(L)-Trp-Arg<sub>8</sub> or Br-vind-(D)-Trp-Arg<sub>8</sub> conjugate) essentially no change in the tumor volume was observed. Interestingly the vinblastine treatment had no marked influence on tumor growth (**Figure 2**).

## Discussion

*Vinca* alkaloids – vinblastine, vincristine – are effective and clinically used antitumor drugs. Unfortunately, one of the major alkaloids – vindoline – has not antitumor activity<sup>7,8</sup>. In our research program some vindoline derivatives were synthesized and their *in vitro* cytostatic activity was characterized<sup>10</sup>. While vindoline has no antitumor activity<sup>25</sup>, some of its derivatives (e.g. Br-vindoline-(L/D)-Trp-OMe) could have same modest activity.

Thus these compounds might provide new avenues for tumor treatment with a new group of *vinca* alkaloid derivatives. The mechanism of action of this novel vindoline derivatives could be different from the clinically used compounds (vinblastine, vincristine) and could result in more efficient treatment.

We observed earlier that conjugation of vinblastine to octaarginine retained the activity and the conjugates showed selectivity for inhibiting the mitotic spindle formation. Thus Br-vindoline-(L/D)-Trp-OMe derivatives were selected for studying the effect of conjugation with oligoarginine as a cell-penetrating peptide<sup>16</sup>. For analyzing the effect of tryptophan on the biological activity 10-Br-vindoline (**3**), 10-Br-vindoline-(L)-Trp-OH (**1a**) and 10-Br-vindoline-(D)-Trp-OH (**1b**) were conjugated with oligoarginine with different length. The effect of the chirality of tryptophan on the biological activity was investigated. For conjugation Br-vindoline derivatives were coupled to the *N*-terminal amino group of oligoarginine in solution (Scheme 2).

Considering that *vinca* alkaloids are typically used in the treatment of cancerous diseases of the blood (non-Hodgkin's lymphoma, Hodgkin's disease)<sup>26</sup> the effect of conjugation was studied on HL-60 cells. The conjugation with tetra-, hexa- and octaarginine increased drastically the cytostatic effect in comparison to the effect of methyl esters (**1a**, **1b**) or a carboxylic acid derivative (**2a**). Although all conjugates had a cytostatic effect, the conjugate with octaarginine showed the highest activity which is in good correlation with earlier data. In further experiments only the effect of conjugates with octaarginine was investigated.

In the next step the antitumor effect of octaarginine conjugates of Br-vindoline derivatives with/without Trp residue was studied *in vitro* on several cell lines. The compounds expressed an antitumor effect *in vitro* on human leukemia (HL-60) and breast cancer (MDA-MB-231 and MCF-7) cells too. Although the Trp-OMe residue increased the efficacy of Br-vindoline, it had no influence in the conjugates on HL-60 and MDA-MB-231 cells, all conjugates had essentially the same activity. In contrast, conjugates with Trp were more active on the MCF-7 cells, but the cytostatic activity was independent of the configuration of tryptophan moiety.

The effect was also studied on mouse leukemia (P388) and murine colon carcinoma cells (C26). On these cells all conjugates have cytostatic effect, but the Trp increased this activity, and its configuration also has influence on the activity. Conjugate with (L)-tryptophan was twice or six times active on P388 and C26 cells, respectively. These data showed that the conjugation with octaarginine, the well-known cell-penetrating peptide, increased the activity of the moderately active vindoline derivatives. Both the presence of Trp<sup>27</sup> and octaarginine could increase the cellular-uptake and thus the activity of Br-vindoline derivatives.

Based on the *in vitro* results the Br-vind-(L)-Trp-Arg<sub>8</sub> and Br-vind-(D)-Trp-Arg<sub>8</sub> conjugates were selected for preliminary *in vivo* study using mouse tumor models. We observed that the configuration of Trp had some impact on the activity; the conjugate with (L)-isomer was more active than with (D)-isomer. Although both conjugates were active *in vitro* on these cells, in the case of mouse P388 leukemia cells, only the Br-vind-(L)-Trp-Arg<sub>8</sub> conjugate could inhibit the tumor growth at 10 mg/kg concentration. Since no effect was documented on C26 murine colon carcinoma cells, one can conclude that the isomer-related effect is also dependent on the properties of the tumor investigated. For further studies are planned to optimize the conditions and also inclusion of other tumor models are considered.

This study demonstrated that vindoline, the main alkaloid component of *Catharanthus roseus* (L.) G. Don., has a potency to be chemically transformed to an active anticancer agent. To the best of our knowledge, the conjugates reported here are the first vindoline derivatives with significant *in vitro* antitumor activity. Conjugates reported in this paper were effective against certain types of tumor cells *in vitro* at micromolar range. Our results indicate that conjugation with oligoarginine as cell-penetrating peptide could increase the *in vitro* antitumor effect of the free vindoline derivative. Preliminary *in vivo* results presented might also indicate the antitumor potency of conjugate. It is important to emphasize that the role of cell-penetrating peptide in this effect needs to be clarified. It is worth mentioning that the octaarginine part of conjugates might increase the tumor accumulation of conjugates.<sup>28</sup> It is attractive to speculate that the vindoline based derivatives/conjugates could be considered as a potential new member of the *vinca* alkaloid family (e.g. vincristine, vinblastine) with different tumor cell targeting mechanism.

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### Conflict of interest

The authors declare no financial or non-financial conflict of interest.

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**Table 1** Chemical characteristics of conjugates

Compounds	R <sub>t</sub> (min)	ESI-MS <sup>c</sup>	
		M <sub>cal.</sub>	M <sub>meas.</sub>
Br-Vind-Arg <sub>8</sub> *	10.2 <sup>a</sup>	1727.9	1728.6
Br-Vind-(L)-Trp-Arg <sub>4</sub>	28.7 <sup>b</sup>	1289.3	1289.0
Br-Vind-(L)-Trp-Arg <sub>6</sub>	27.9 <sup>b</sup>	1601.7	1601.4
Br-Vind-(L)-Trp-Arg <sub>8</sub>	13.5 <sup>a</sup> or 27.2 <sup>b</sup>	1914.1	1914.9
Br-Vind-(D)-Trp-Arg <sub>8</sub>	13.5 <sup>a</sup>	1914.1	1914.9

\* Arg<sub>n</sub>: H-(Arg)<sub>n</sub>-NH<sub>2</sub>

<sup>a</sup> Column: Agilent Zorbax SB-C18 4.6x150mm, 100Å; Gradient: 0 min 0% B, 2 min 0% B, 22 min 90% B; Flow rate: 1mL/min; RT, λ=220 nm.

<sup>b</sup> Column: Agilent Zorbax SB-C18 4.6x150mm, 100Å; Gradient: 0 min 0% B, 2 min 0% B, 22 min 90% B; Flow rate: 1mL/min; RT, λ=220 nm.

<sup>c</sup> ESI-MS analysis was carried out on a Bruker Esquire 3000 plus equipment (Germany). The sample was dissolved in acetonitrile-water (50:50, V/V), 0.1% acetic acid.

**Table 2** *In vitro* cytostatic activity of vindoline-derivative and -conjugates on sensitive HL-60 tumour cells: the effect of the length of oligoarginine

Compound	IC <sub>50</sub> (sd) (μM)
	HL-60
Arg <sub>8</sub>	> 800
Br-Vindoline	> 100
Br-Vind-(L)-Trp-OH ( <b>2a</b> )	83.1 (10.7)
Br-Vind-(L)-Trp-Arg <sub>4</sub>	29.9 (18.1)
Br-Vind-(L)-Trp-Arg <sub>6</sub>	21.4 (1.1)
Br-Vind-(L)-Trp-Arg <sub>8</sub>	12.9 (4.4)

<sup>a</sup> The cells were incubated with the compound for 3 h, after cultured in serum-containing medium for 3 days. The IC<sub>50</sub> values were determined by MTT assay as described in the text. Standard deviation values (sd) are also presented.

**Table 3** *In vitro* cytostatic activity of vindoline-derivatives and -conjugates on tumor cell lines

Compound	HL-60	IC <sub>50</sub> (sd) (μM)			P388
		MDA-MB-231	MCF-7	C26	
Vindoline	> 100 <sup>b</sup>	> 100	> 100	> 100	> 100
Br-Vindoline	> 100	> 100	> 100	> 100	> 100
Br-Vind-(L)-Trp-OMe ( <b>1a</b> )	56.8 (9.8) <sup>b</sup>	n.d.	n.d.	18.4 (2.1)	5.3 (1.5)
Br-Vind-(D)-Trp-OMe ( <b>1b</b> )	73.8 (10.4) <sup>b</sup>	37.3 (13.7)	28.5 (5.7)	21.6 (4.3)	15.9 (2.8)
Br-Vind-Arg <sub>8</sub>	11.6 (5.3)	10.3 (3.3)	12.0 (3.3)	25.9 (2.7)	26.6 (5.0)
Br-Vind-(L)-Trp-Arg <sub>8</sub>	11.5 (4.6)	13.2 (0.4)	6.4 (3.6)	4.3 (0.41)	3.1 (0.5)
Br-Vind-(D)-Trp-Arg <sub>8</sub>	15.1 (0.5)	10.0 (3.7)	5.1 (1.1)	10.8 (0.7)	18.0 (1.2)

<sup>a</sup> The cells were incubated with compound for 3 h, after cultured in serum-containing medium for 3 days. The IC<sub>50</sub> values were determined by MTT assay as described in the text. Standard deviation values (sd) are also presented.

<sup>b</sup> From the literature (10)

n.d. not determined