Synthesis and in Vitro Antitumor Effect of Vinblastine Derivative−Oligoarginine Conjugates

Zoltán Bánóczi, Álmos Gorka-Kereskényi, Judit Reményi, Erika Orbán, László Hazai, Natália Tőkési, Judit Oláh, Judit Ovádi, Zoltán Béni, Viktor Háda, Csaba Szántay, Jr., Ferenc Hudecz,* György Kalaus, and Csaba Szántay

Research Group of Peptide Chemistry, Eötvös Loránd University (ELTE), Hungarian Academy of Sciences, Budapest 112, P.O. Box 32, H-1518, Hungary, Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, Budapest, Gellért tér 4, H-1521 Hungary, Research Group for Alkaloid Chemistry, Hungarian Academy of Sciences, University of Technology and Economics, Budapest, Gellért tér 4, H-1521 Hungary, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Karolina u 29, H-1113 Hungary, Spectroscopic Research, Gedeon Richter Plc., Budapest 10, P.O. Box 27, H-1475 Hungary, and Department of Organic Chemistry, Eötvös Loránd University (ELTE), Budapest, Pázmány P. s. 1./a, H-1117 Hungary. Received January 15, 2010; Revised Manuscript Received July 12, 2010

Vinblastine is a widely used anticancer drug with undesired side effects. Its conjugation with carrier molecules could be an efficient strategy to reduce these side effects. Besides this, the conjugate could exhibit increased efficiency against resistant cells, e.g., due to the altered internalization pathway. Oligoarginines, as cell-penetrating peptides, can transport covalently attached compounds into different kinds of cells and enhance the efficiency of those compounds. We report here the coupling of vinblastine through its carboxyl group at position 16 with the *N*-terminal amino function of L-Trp methyl ester. After hydrolysis of the ester group, 17-desacetylvinblastineTrp was conjugated to the *N*-terminal amino group of oligoarginine via the *C*-terminal carboxyl group of the Trp moiety in solution. The antitumor effect of conjugates was studied on sensitive and resistant human leukemia (HL-60) cells in vitro. Our data suggest that all conjugates investigated possess an antiproliferative effect against the studied cells. However, the effect was dependent on the number of Arg residues in the conjugates: $Arg_8 > Arg_6 \gg Arg_4$. The conjugate with Arg_8 exhibited similar efficicacy as compared with free 17-desacetylvinblastineTrp. The in vitro studies also showed that the tubulin binding ability of vinblastine was essentially preserved even in the octaarginine conjugate. We also observed that two isomers were formed during conjugation. These isomers showed different levels of activity against tubulin polymerization in vitro and in vivo. The 17-desacetylvinblastineTrp-Arg_8-1 isomer conjugate possessed high selectivity against the mitotic spindles. HRMS and NMR data suggest that 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 are epimers at the tryptophan α carbon atom.

INTRODUCTION

Vinca-alkaloids (e.g., vincristine, vinblastine) are widely used antitumor drugs isolated first from the leaves of *Catharanthus roseus* (L.) G. Don. They cause a cytotoxic effect by disturbing the dynamics of the microtubular system (*1, 2*). In vivo, these compounds interact with tubulin at the ends of microtubules in mitotic spindles and promote microtubule instability (*3*). They are bisindole alkaloids containing a rearranged catharanthine and a vindoline moiety. Vindoline, a major alkaloid of *Catharanthus roseus*, constitutes the most complex half of vinblastine, and it is both a biosynthetic and synthetic precursor of the natural product. Upon studying the tubulin binding ability of the two parts separately, it turned out that only the catharanthine part could bind to tubulin, but this interaction is weaker than that for the bisindole alkaloids (*4*). It was concluded that the indole part of catharanthine could be responsible for the binding of vinblastine/vincristine to tubulin and the vindoline moiety could function as an anchoring residue.

The structure of vinblastine and vincristine differs only in the nature of the substituent, a methyl or a formyl group, respectively, on the vindoline *N*-atom, but this "minor" difference leads to different antitumor efficacies, potencies, and toxicities. Vinblastine is in clinical use with success in the treatment of testicular cancer, non-Hodgkin's lymphoma, and breast cancer. However, vincristine is more effective against Hodgkin's disease and pediatric solid tumors. Vinblastine treatment can result in leucopenia, while vincristine might be neurotoxic. These differences indicate that there is a marked relationship between the chemical structure and the biological effect of these alkaloids. To eliminate or reduce the side effects of these potent vinca-alkaloids, much effort has been directed toward the synthesis of new derivatives.

In the first experiments, Barnett et al. synthesized 17-desacetylvinblastine-amide (vindesine) by modifying the methyl ester group at position C-16 of the vindoline part. Vindesine and vincristine exhibited very similar biological activity spectra in animal experiments, but the neurotoxicity of the former was lower (*5*). Further compounds were prepared by modification of the amide group by incorporating different alkyl groups (*6*) or amino acids (*7*). Among the amino acid derivatives, the hydrophobic amino acid (Leu, Ile, Val, and Trp) containing derivatives showed pronounced activity. The Trp-OEt derivative had an outstanding effect on P388 and L1210 leukemias implanted intravenously in DBA_2 mice (*7*).

Various bioconjugates of vinblastine derivatives were also prepared for targeting. Conjugates with tumor-specific antibodies (*8*), for example, KS1/4 (*9*), carcinoembryonic antigen (CEA) (*10*), epidermal growth factor receptor (*11*), or PF1/D, a squamous carcinoma-reactive monoclonal antibody (*12*), were also produced and tested extensively. In other experiments, 17-desacetylvinblastine monohydrazide was coupled with folic acid

* Address correspondence to Ferenc Hudecz, Tel.: +36-1-2090-555/6428, Fax: +36-1-372-2620; E-mail: fhudecz@caesar.elte.hu.

10.1021/bc100028z
Published on Web 10/25/2010

as a targeting unit via a peptide spacer (*13, 14*). The conjugate could bind to the folate receptor positive cells and produced specific, dose-responsive activity in vitro. In vivo tests confirmed the activity of the conjugate in both syngeneic and xenograft models with minimal to moderate toxicity.

Oligoarginines are members of the cell-penetrating oligopeptide family. These synthetic or natural peptides permeate the cell membrane and transport the covalently attached cargoes into the cytosole. Among oligoarginines, hexa-, hepta-, and octaarginine showed the most effective internalization (*15, 16*). Different oligoarginines were used efficiently to transport genetically or chemically attached protein, peptide, and noncovalently bound plasmid DNA into cells (*17*). Oligoarginines can also be used to transport covalently coupled small molecules into cells, like cyclosporin A (*18*), ferrocene (*19*), and daunomycin (*20*) derivatives or bisubstrate-analogue inhibitors of basophilic protein kinases (adenosine, 5′-adenosine-carboxic acid) (*21*).

In this work, we report on the synthesis of oligoarginine (Arg_n, where $n = 4$, 6, or 8) conjugates with the Trp-derivative of vinblastine via amide bond formation in solution. We observed and isolated two isomers after conjugation. The effect of these compounds on sensitive and resistant HL-60 (human leukemia) cells was studied. Furthermore, we described our findings on the influence of conjugates on the in vitro and in vivo tubulin polymerization. Data suggest that the cytotoxicity of the conjugates was dependent on the chain length of the peptides. Moreover, only the 17-desacetylvinblastineTrp-Arg_8-1 conjugate exhibited the capability to destroy only the mitotic spindle and cause the apoptosis of the cells that are in mitosis. On the basis of HRMS and NMR data, we also report on the structure identification of the isomers proposing that 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 are epimers at the α-carbon atom of the Trp residue.

EXPERIMENTAL PROCEDURES

Fmoc-Arg(Pbf)-OH was purchased from Novabiochem (Läufelfingen, Switzerland), while thioanisole, 1,2-ethanedithiol (EDT), *N*-diisopropyl-ethylamine (DIEA), 1-hidroxybenztriazole (HOBt), *N,N′*-diisopropylcarbodiimide (DIC), trifluoracetic acid (TFA), phenol, ninhydrine, piperidine, 1,8-diazabicyclo[*5.4.0*]undec-7-ene (DBU) were Fluka (Buchs, Switzerland) products. The Rink-amid MBHA resin was from Novabiochem (Läufelfingen, Switzerland). Reagents and solvents used in the biological assays were purchased from Sigma-Aldrich Ltc (Budapest, Hungary). Solvents (dimethyl formaide (DMF), dichloromethane (DCM), diethyl ether, acetonitrile (AcN)) for syntheses and purification were obtained from Reanal (Budapest, Hungary).

General Procedures. Analytical RP-HPLC was performed on a Knauer (Herbert Knauer GmbH, Berlin, Germany) system using a Phenomenex SYNERGI MAX-RP column (250 × 4.6 mm I.D., 4 μm silica, 80 Å pore size) (Torrance, CA, USA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) was generated using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile−water (80:20, V/V) as eluent B. Flow rate of 1 mL/min was applied at ambient temperature. The samples were dissolved in eluent B. The crude products were purified on a semipreparative Phenomenex Jupiter C18 column (250 × 10 mm I.D., 10 μm silica, 300 Å pore size) (Torrance, CA, USA). The flow rate was 4 mL/min. Peaks were detected at λ=220 nm. Positive ion electrospray ionization mass spectrometric analysis was performed on a Bruker Esquire 3000 plus (Germany). The samples were dissolved in acetonitrile−water (50:50, V/V), containing 0.1% acetic acid. High-resolution MS measurements were carried out on a Thermo LTQ FT Ultra mass spectrometer using ESI (source voltage, 4.2 kV; capillary voltage, 43 V; capillary temperature, 200 °C; sheath gas flow rate, 10 arb; aux gas flow rate, 2 arb; sweep gas flow rate, 2 arb; solvent, MeOH/H_2O 1:1 + 1 v/v % cc. AcOH) ionization technique.

All NMR measurements were performed on a Varian 800 MHz NMR spectrometer equipped with a ^{13}C enhanced HCN cold probe. $^1H-^1H$, direct $^1H-^{13}C$, long-range $^1H-^{13}C$ scalar, and dipolar spin−spin connectivities were established from ^1H, 2D-GHSQCAD, GCOSY, 2D-GHMBCAD, and 2D-ROESY NMR experiments, respectively. All pulse sequences were applied by using the standard spectrometer software package.

Synthesis of Oligoarginines. Oligoarginines (tetra-, hexa-, and octaarginine) were synthesized manually by solid-phase methodology on Rink-amide resin (0.3 g, 0.69 mmol/g) using Fmoc/^{t}Bu strategy. The side chain of Arg was protected with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group. N^{α}-Fmoc group was removed with 2% piperidine plus 2% DBU in DMF (2 + 2+5 + 10 min) followed by washing with DMF (8 × 0.5 min). For coupling, the Arg derivative and DIC and HOBt dissolved in DMF were used in 3-fold molar excess for the resin capacity. The reaction proceeded at RT for 60 min. Then, the resin was washed (DMF 2 × 0.5 min, DCM 3 × 0.5 min). The efficiency of coupling was checked by ninhydrin assay (*22*). The amount of resin was divided into three parts during the synthesis. After assembly, the peptide was cleaved from the resin by 10 mL of TFA using 0.75 g of phenol, 0.5 mL of distilled water, 0.5 mL of thioanisole, and 0.25 mL of 1,2-ethandithiol as scavengers. The crude product was precipitated by dry diethyl ether, dissolved in 10% acetic acid, and freeze−dried. Purification was done by RP-HPLC as described above. The following gradients were applied for purification: 0 min 0% B; 5 min 0% B; 50 min 50% B.

Synthesis of Conjugates. First, 17-desacetylvinblastineTrp was prepared by coupling the unprotected 17-desacetylvinblastine to Trp as described earlier (*7*) with some modifications. Briefly, vinblastine was refluxed in an ethanol−chloroform mixture with 98% hydrazine monohydrate instead of anhydrous hydrazine used in the published paper mentioned above. The corresponding acid azide was prepared with sodium nitrite in the presence of hydrochloric acid. The latter compound, without isolation, was allowed to react with L-Trp methyl ester in dichloromethane at 4 °C to give the Trp derivative of 17-desacetylvinblastine (*23*). After hydrolysis of the carboxylic ester, the corresponding 17-desacetylvinblastineTrp carboxylic acid (3 mg, 3.15 μmol) was coupled to oligoarginines (Arg_n, where $n = 4$, 6, or 8) (6.3 μmol) by DCC (1.3 mg, 6.3 μmol); HOBt (1 mg, 6.3 μmol) in the presence of DIEA (1.1 μL, 6.3 μmol) in 0.5 mL DMF. The reaction proceeded at RT for 4 h. The DMF was evaporated and conjugates were purified by RP-HPLC using the following gradient: 0 min 5% B; 5 min 5% B; 50 min 80% B as described above.

Cell Cultures. HeLa (ATCC, CCL-2) cells were grown in DME/F-12 medium supplemented with 10% FCS, 1 mM sodium pyruvate, 100 U/mL streptomycin, and 100 μg/mL penicillin (all from Sigma) (complete medium) in a humidified 37 °C incubator with 5% CO_2. The sensitive and resistant HL-60 human leukemia cells (ATCC: CCL-240) were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 160 μg/mL gentamycin. The cell culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2.

MTT Assay. For cytostatic studies, sensitive and resistant HL-60 cells were placed in a 96-well plate with each well containing 5×10^3 cells. After incubation at 37 °C for 24 h, the cultured cells were treated with the solution of conjugates in serum-free RPMI-1640 medium for 3 h. Compounds were used at the $c = 2 \times 10^{-3}$ to 8×10^2 μM range. In control experiments, cells were treated with serum-free medium at 37 °C for 3 h. After incubation, cells were washed with serum-

free medium three times, and serum-containing medium was added to the cells. After 3 days at 37 °C, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out. The yellow solution of MTT at a concentration of 0.2 mg/mL was added to each well. The obtained purple crystal was dissolved in DMSO, and the optical density (OD) of the samples was measured at $\lambda = 540$ nm using ELISA Reader (Labsystems MS Reader, Finland). The percentage of the antitumor effect was calculated using the following equation:

$$\text{antitumor effect } \% = (1 - \text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100$$

where $\text{OD}_{\text{treated}}$ and $\text{OD}_{\text{control}}$ correspond to the optical densities of the treated cells and the control cells, respectively, at $\lambda = 540$ nm. Results obtained from in vitro cytotoxicity measurements were analyzed using sigmoidal curve fitting.

Tubulin Preparation. Microtubule-associated protein (MAP)-depleted tubulin was purified from bovine brain by the method of Na and Timasheff (*24*) and stored in 10 mM phosphate buffer, pH 7.0, containing 1 M of sucrose, 0.5 mM of $MgCl_2$, and 1 mM of GTP at −80 °C. Purified tubulin showed no contamination with MAPs on overloaded SDS-PAGE. Before use, stored tubulin was dialyzed against 50 mM of 4-morpholine ethanesulfonic acid (MES) buffer, pH 6.8, at 4 °C for 3 h and then centrifuged at 100 000 *g* at 4 °C for 20 min.

Polymerization Assay. Ten micromoles of tubulin was assembled to microtubules at 37 °C in polymerization buffer (50 mM of MES buffer, pH 6.6, containing 1 mM of dithioerythritol, 1 mM of $MgCl_2$, 1 mM of EGTA (ethylene glycol tetraacetic acid), and 100 mM of KCl. The polymerization was initiated with 20 μM of paclitaxel at 37 °C. Vinblastine, 17-desacetylvinblastineTrp, and 17-desacetylvinblastineTrp−octaarginine conjugates in the 0.05−2 μM concentration range were incubated with tubulin at 37 °C for 5 min before starting the polymerization. For turbidity measurements, the absorbance was monitored at $\lambda = 350$ nm with a Cary 100 spectrophotometer (Varian, Mulgrave, Victoria, Australia). The error margin of the turbidity measurements was ±5% within a series of experiments.

Immunocytochemistry. For microscopic studies, HeLa cells were grown on 12 mm diameter coverslips. The vinblastine, 17-desacetylvinblastineTrp, and 17-desacetylvinblastineTrp−octaarginine conjugates were added to the complete medium of the cells in the 0.25−5 μM concentration range for 24 h and then were fixed with ice-cold methanol for 15 min and immunostained for α-tubulin as described by Lehotzky et al. (*25*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) after coverslips were mounted on an Aqua Poly/Mount medium (Sigma). Images were taken on a Leica DMLS microscope (Leica Microsystems, Wetzlar, Germany) equipped with a cooled CCD camera (Spot, Digital Instruments, Sterling Heights, MI, USA). Statistical analysis was performed manually by investigating random microscopic fields. Data were analyzed with Microsoft *Excel*. Images were processed with *ImageJ* software.

RESULTS AND DISCUSSION

To avoid the undesired effect of antitumor drugs and to increase their efficiency against resistant cells is a difficult task to achieve. The conjugation of small drug molecules to carriers could be considered as a potential strategy for utilization. In this paper, we describe the synthesis and in vitro antitumor effect of oligoarginine conjugates of the Trp derivative of 17-desacetylvinblastine. The influence of these compounds on the tubulin polymerization was also studied in vitro and in vivo.

Synthesis of Conjugates. Among the amino acid ester derivatives of viblastine, the tryptophan derivative showed the highest antitumor effect in mice (*7*). Accordingly, for the present studies 17-desacetylvinblastineTrp (*7, 23, 26*) was prepared with a modified procedure using 17-desacetylvinblastine (*26*), as the major metabolite of vinblastine in humans considering its pharmacological equivalency with vinblastine (*27*) (Scheme 1).

Scheme 1. Synthesis of 17-DesacetylvinblastineTrp

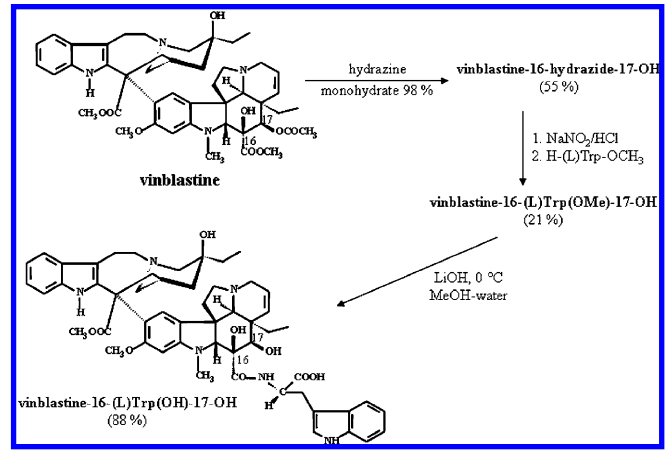

Table 1. Characteristics of 17-DesacetylvinblastineTrp−Oligoarginine Conjugates

conjugate	R_t[a] (min)	$[M+H]^+$,[b] cal. [meas.]
17-desacetylvinblastineTrp-Arg_4-1	27.9	1564.9 (1564.3)
17-desacetylvinblastineTrp-Arg_4-2	26.3	1564.9 (1564.3)
17-desacetylvinblastineTrp-Arg_6-1	26.0	1877.3 (1876.7)
17-desacetylvinblastineTrp-Arg_6-2	26.3	1877.3 (1876.7)
17-desacetylvinblastineTrp-Arg_8-1	26.3	2189.6 (2189.1)
17-dezacetylvinblasztinTrp-Arg_8-2	26.6	2189.6 (2189.1)

[a] RP-HPLC retention time, column: Phenomenex SYNERGI MAX-RP (250 × 4.6 mm I.D., 4 μm silica, 80 Å pore size). Linear gradient elution 0 min 0% B; 5 min 0% B; 50 min 90% B using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile−water (80:20, V/V) as eluent B. [b] ESI-MS.

17-DesacetylvinblastineTrp was conjugated with tetra-, hexa-, or octaarginine in solution. First, the oligoarginines were built up on Rink-amide resin by Fmoc/^{t}Bu strategy, and after cleavage from the resin by TFA, the unprotected and purified *C*-terminal peptide amides were used for the conjugation. 17-DesacetylvinblastineTrp was coupled to the peptides by the active ester method using DCC/HOBt coupling reagents in DMF in the presence of DIEA as base. The conjugates were characterized by analytical RP-HPLC and ESI-MS (Table 1 and Figure 1 in Supporting Information).

The conjugation reaction resulted in two isomers with different retention times, but with the same molecular mass in all three cases. The isomers were separated, isolated, and characterized, and their biological effects were examined. To identify the structure of the two isomer conjugates, these compounds were studied by high-resolution MS and NMR.

Identification of the Structure of the Two Isomers of the Octaarginine Conjugates. The two compounds (17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2) obtained during the preparation of the 17-desacetylvinblastineTrp octaarginine conjugate were investigated after HPLC separation.

High-Resolution Mass Spectrometry. In the direct ESI mass spectra of the samples 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 multiply charged ions of $[M+4H]^{4+}$, $[M+5H]^{5+}$ and $[M+6H]^{6+}$ can be detected. The monoisotopic mass values were obtained by the deconvolution of the mass spectra (using Xtract in *Xcalibur 2.0.7*): 17-DesacetylvinblastineTrp-Arg_8-1: *m/z* 2188.29693, calculated value for $C_{102}H_{161}O_{16}N_{39}$: 2188.29780 ($\delta$ −0.4 ppm). 17-

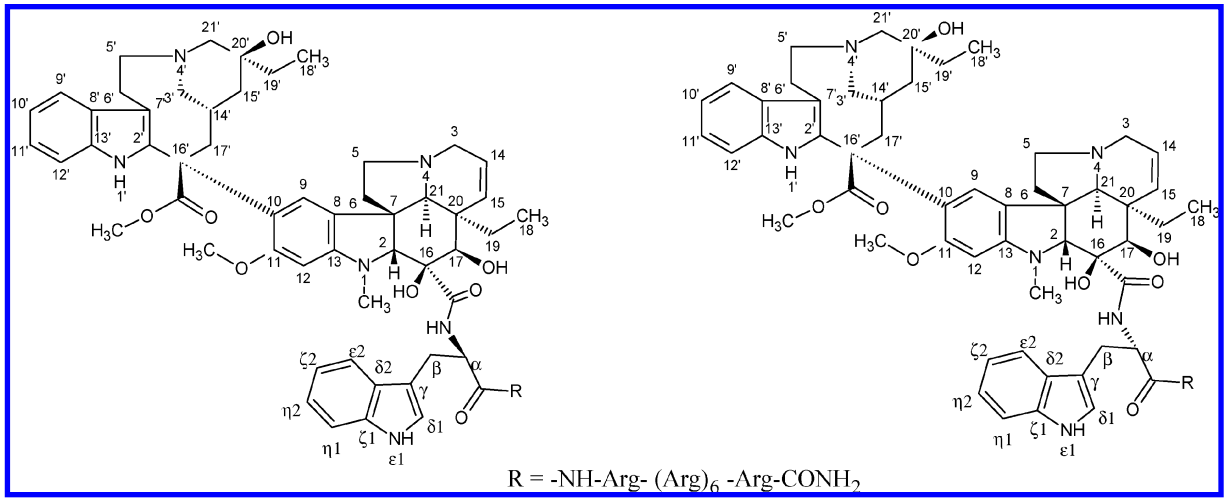

Figure 1. Structures proposed for the two isomeric Arg8 conjugates of 17-desacetylvinblastineTrp: 17-desacetylvinblastineTrp-Arg_8-1 (left) and 17-desacetylvinblastineTrp-Arg_8-2 (right).

DesacetylvinblastineTrp-Arg_8-2: *m/z* 2188.29728, calculated value for $C_{102}H_{161}O_{16}N_{39}$: 2188.29780 ($\delta$ −0.2 ppm).

The most intense $[M+5H]^{5+}$ ion peaks were fragmented by CID (collision induced dissociation) at a normalized collision energy of 25%. The monoisotopic mass values of the main fragment ions in the deconvoluted MS-MS spectra are the following: 17-DesacetylvinblastineTrp-Arg_8-1: *m/z* 2129.25013, 2015.17064, 1876.09619, 1702.96880, 1660.94691, 1537.91516, 1501.89406, 1348.74426, 1234.66543, 1192.64356, 1174.63301, 1078.56404, 648.36752, 523.28346. 17-DesacetylvinblastineTrp-Arg_8-2: *m/z* 2129.25155, 2015.17057, 1876.09677, 1234.66544, 1192.64463, 1174.63280, 953.63348, 894.46857, 708.38901, 648.36785.

On the basis of the accurate mass values of the fragment ions in the MS−MS spectra, elemental compositions were calculated using the *Xcalibur 2.0.7* software. In the range of 0.2−1.5 ppm mass accuracy, plausible elemental compositions could be obtained for all of the fragment ions. All of these elemental compositions are in agreement with the expected 17-desacetylvinblastineTrpArg_8 chemical structure. However, when comparing the MS-MS spectra of the two samples some differences can be noticed regarding the obtained fragment ions and their relative abundance values. Most of the fragment ions were obtained by the cleavage of the Arg_8 part of the molecule for both samples. In the case of 17-desacetylvinblastineTrp-Arg_8-1, more fragment ions were produced by the cleavage of the vindoline C ring (cleavage of the C_2-C_{16} and $C_{17}-C_{20}$ bonds), while exclusively for 17-desacetylvinblastineTrp-Arg_8-2, a single minor fragment ion was observed in the deconvoluted mass spectrum, which can originate from a C−C cleavage beside the carbonyl group of the Tryptophan. According to these MS data, compounds 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 are two stereoisomers of 17-desacetyvinblastineTrpArg_8.

NMR. Since both compounds 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 were available only in a limited amount for NMR measurements, only limited spectroscopic information could be obtained on an affordable time scale even on our ultrasensitive 800 MHz NMR spectrometer. Furthermore, due to their conformational dynamics both compounds gave broad ^{1}H NMR resonances in all common NMR solvents (such as DMSO-d_6, $CDCl_3$, ACN-d_3, MeOD-d_4, or D_2O), which prohibited easy access to the desired spectroscopic information (see Supporting Information Figure 2 as an example) in this concentration. Finally, a solvent mixture containing ACN-d_3 and D_2O in a one-to-one ratio was chosen as the most suitable medium. Although most of the resonances (mainly those belonging to the vindoline half of the two molecules) were still significantly broadened in this solvent mixture, structurally informative spectral information could nevertheless be obtained. In accordance with the HRMS results, these data suggest that 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 are epimers at the tryptophan α-carbon atom (Figure 1).

Partial ^{1}H and ^{13}C NMR assignments (determined from ^{1}H, 2D-GHSQCAD, GCOSY, 2D-GHMBCAD, and 2D-ROESY NMR spectra) that are indicative of the proposed epimeric structures are listed in Table 1 in Supporting Information. These structures are supported via the following argument. The chemical shifts of the catharantine parts of 17-desacetylvinblastineTrp-Arg_8-1 and 17-desacetylvinblastineTrp-Arg_8-2 (note that an almost complete assignment could be achieved for the catharantine moieties) are practically the same, telling us that the structural difference is located elsewhere in the two molecules.

Due to the low sample concentrations and to the significant broadening of some of the resonances belonging to the vindoline half of the molecules, assignments for this part of the compounds could not be given completely. Nevertheless, the similarity of the signals due to the detectable and assignable functional groups [the C(14)═C(15) double bond, the C(20)-Et, C(11)-OMe, and N(1)-Me functions], as well as those belonging to the aromatic resonances (*8, 9*) of the indole part, indicate that the vindoline parts also remained intact during the formation of the conjugates.

The most significant differences were detected in signals belonging to the tryptophan moieties, especially those belonging to the Trp α and β protons of these residues. Not only do the chemical shifts of the Trp α (5.09 and 4.84 ppm in 17-desacetylvinblastineTrp-Arg_8-2 and 17-desacetylvinblastineTrp-Arg_8-1, respectively) and β protons (3.73 ppm in 17-desacetylvinblastineTrp-Arg_8-2 and 3.58 and 3.72 ppm in 17-desacetylvinblastineTrp-Arg_8-1) differ significantly, but the coupling patterns differ as well (Figure 3 in Supporting Information). In 17-desacetylvinblastineTrp-Arg_8-2, the Trp α proton resonance appears as a triplet with a coupling constant of 5.9 Hz, while that in 17-desacetylvinblastineTrp-Arg_8-1 is a doublet of doublet with coupling constants of 10.4 and 5.6 Hz. These findings indicate that during the formation of the Arg conjugates inversion of the Trp α carbon occurred. The Trp moieties are expected to be in different conformational states in the two epimers, which accounts for the observed difference in the coupling patterns of the Trp α proton resonances.

In order to identify the two epimers, we recorded the ^{1}H NMR spectra of two smaller model systems containing only the

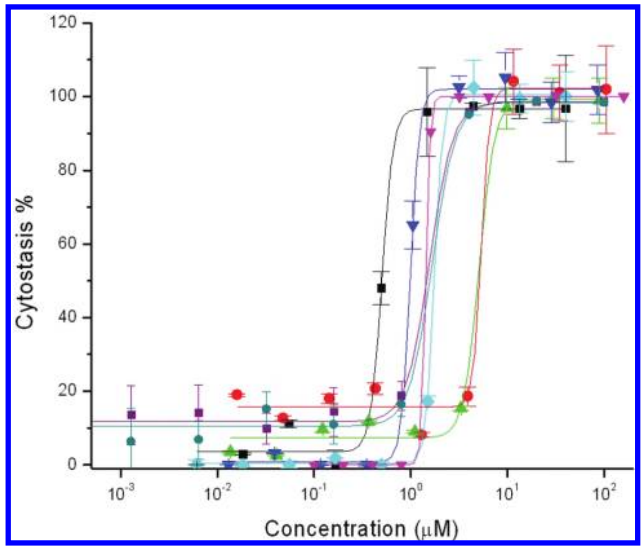

Figure 2. Cytostatic activity of vinblastine sulfate, 17-desacetylvinblastine, and its conjugates on sensitive HL-60 cells. The cells were incubated with the compound for 3 h, after growth in serum-containing medium for 3 days. The IC_{50} values were determined by MTT assay as described in the text: black -■-, 17-desacetylvinblastineTrp, $IC_{50} = 0.49$ μM; magenta -▼-, vinblastine sulfate, $IC_{50} = 1.26$ μM; red -●-, 17-desacetylvinblastineTrp-Arg_4-1, $IC_{50} = 5.25$ μM; green -▲-, 17-desacetylvinblastineTrp-Arg_4-2, $IC_{50} = 4.97$ μM; cyan -◆-, 17-desacetylvinblastineTrp-Arg_6-1, $IC_{50} = 0.97$ μM; blue -▼-, 17-desacetylvinblastineTrp-Arg_6-2, $IC_{50} = 1.75$ μM; purple -■-, 17-desacetylvinblastineTrp-Arg_8-1, $IC_{50} = 1.61$ μM; teal -●-, 17-desacetylvinblastineTrp-Arg_8-2, $IC_{50} = 1.59$ μM.

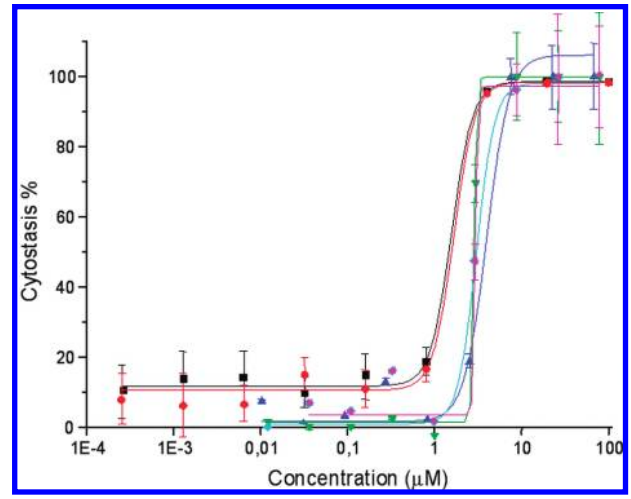

Figure 3. Cytostatic activity of 17-desacetylvinblastineTrp-Arg_8 conjugates on sensitive and resistant HL-60 cells. The cells were incubated with the compound for 3 h, after grown in serum-containing medium for 3 days. The IC_{50} values were determined by MTT assay as described in the text: black -■-, 17-desacetylvinblastineTrp-Arg_8-1/HL-60, $IC_{50} = 1.61$ μM; red -●-, 17-desacetylvinblastineTrp-Arg_8-2/HL-60, $IC_{50} = 1.59$ μM; blue -▲-, 17-desacetylvinblastineTrp-Arg_8-1/HL-60-MDR1, $IC_{50} = 2.62$ μM; green -▼-, 17-desacetylvinblastineTrp-Arg_8-2/HL-60-MDR1, $IC_{50} = 2.94$ μM; cyan -◆-, 17-desacetylvinblastineTrp-Arg_8-1/HL-60-MRP1, $IC_{50} = 2.94$ μM; magenta -▼-, 17-desacetylvinblastineTrp-Arg_8-2/HL-60-MRP1, $IC_{50} = 3.78$ μM.

"vindoline" half bound to D- and L-tryptophan (unpublished synthetic results), having the structures shown in Figure 4 in Supporting Information. In the vindoline−L-tryptophan model, the α proton appears as a triplet (5.8 Hz) similarly to epimer 17-desacetylvinblastineTrp-Arg_8-2, while in the vindoline−D-tryptophan model, it appears as a doublet of doublets (8.9 and 4.8 Hz) similarly to epimer 17-desacetylvinblastineTrp-Arg_8-1 (Figure 5 in the Supporting Information). These similarities indicate that 17-desacetylvinblastineTrp-Arg_8-2 and 17-desacetylvinblastineTrp-Arg_8-1 contain the L- and D-tryptophan moieties, respectively.

Additional synthetic and spectroscopic investigations are in progress to further verify the proposed structures as well as to explore the nature of and reasons behind the observed epimerization of Trp octaarginine during its coupling to 17-desacetylvinblastine.

Cytostatic Effect of Conjugates on HL-60 Human Leukemia Cells. The cytostatic effect of conjugates as well as of the control compounds was studied on sensitive and resistant HL-60 cells using MTT assay. The cells were treated with vinblastine sulfate, 17-desacetylvinblastineTrp, and its conjugates at different concentrations for 3 h, and then, the number of living cells was determined after 72 h. Data are summarized in Figure 2. In contrast to vinblastine sulfate ($IC_{50} = 1.26$ μM), the 17-desacetylvinblastine has more marked activity on sensitive HL-60 cells ($IC_{50} = 0.0043$ μM). The presence of Trp residue in the amino acid conjugate decreased the activity by 2 orders of magnitude ($IC_{50} = 0.49$ μM), but it is still more effective than vinblastine sulfate. On sensitive HL-60 cells, the increasing number of Arg residues enhanced the cytostatic effect of the conjugate. The octa- and hexaargininеTrp conjugate isomers have slightly higher activity ($IC_{50} = 1.61$ μM and 1.59 μM, for octaargininеTrp conjugate and $IC_{50} = 0.97$ μM and 1.75 μM for hexaargininеTrp conjugates) as compared to the tetraargininеTrp conjugates ($IC_{50} = 5.25$ μM and 4.97 μM). It is interesting to note that the octa- and hexaargininеTrp conjugate isomers almost preserve the activity of 17-desacetylvinblastineTrp, and these values are comparable with those of vinblastine sulfate ($IC_{50} = 0.97-1.75$ μM for octa- and hexaargininеTrp conjugates and $IC_{50} = 1.26$ μM for vinblastine sulfate).

The activity of compounds was studied on HL-60/MDR1 and HL-60/MRP1 resistant cells, too. The effect of vinblastine sulfate on resistant HL-60/MRP1 cells ($IC_{50} = 1.31$ μM) is similar to that of sensitive HL-60 cells ($IC_{50} = 1.26$ μM), but its efficacy was somewhat lower in the case of resistant HL-60/MDR1 cells ($IC_{50} = 2.96$ μM).

The effect of 17-desacetylvinblastine decreased markedly ($IC_{50} = 1.78$ for MDR1 and 0.24 μM for MRP1), showing that it is a good substrate of the MDR1 and MRP1 transporter proteins, somewhat better than vinblastine sulfate. The efficiency of the Trp derivative diminished dramatically in the presence of the MDR1 protein ($IC_{50} = 59.6$ μM), but the effect of the MRP1 protein was less marked ($IC_{50} = 3.98$ μM).

The antitumor effect of the conjugates increased with the number of Arg residues present in the conjugates on both resistant cell lines, but the IC_{50} values are somewhat higher than in the case of the sensitive cells. The two isomer conjugates (except the compounds with four Arg residues) show similar effects on the resistant cells. The IC_{50} values of octaarginine conjugates are shown in Figure 3.

Our results show that 17-desacetylvinblastine significantly inhibits the division of sensitive human leukemia cells. This effect, which is higher than that of vinblastine sulfate, decreases if the molecule is modified by a Trp. The conjugation of 17-desacetylvinblastineTrp to oligoarginine resulted in an antitumor effect which is dependent on the number of Arg residues. The presence of hexa- and octaarginine in the conjugates has almost no effect—these compounds exhibit essentially the same level of antitumor activity. However, tetraarginine reduces the activity significantly.

17-Desacetylvinblastine has significant cytotoxicity, higher than that of vinblastine. While the attachment of Trp dramatically decreases the effect of 17-desacetylvinblastine, this derivative still has a similar activity to vinblastine. The IC_{50} values of octa- and hexaargininеTrp conjugates suggest that these

compounds exhibit in vitro an antitumor effect similar to that of vinblastine.

In the case of resistant cells, the effect of 17-desacetylvinblastine is dramatically decreased, and the activity of both the Trp derivative and its octaarginine conjugate is similar in the case of HL-60/MRP1. The HL-60/MDR1 cells pump out the Trp derivative so effectively that its activity decreases to one tenth, while the octaargininеTrp conjugates show comparable effect with 17-desacetylvinblastine. Interestingly, the effect of vinblastine sulfate is reduced only by MDR1 proteins and only to a little extent. The biggest effect is measured in the presence of the MDR1 protein when the cells are treated by vinblastine derivatives. In the case of conjugates, the MRP1 proteins result in a bigger reduction in the activity. These results show that 17-desacetylvinblastine is a very good substrate of the transporter proteins. Considering the decreased effect of the conjugates, it is attractive to speculate that the transport proteins can also pump out the conjugated 17-desacetylvinblastine from the cells. The extent of efflux increased in the case of conjugates with a shorter oligoarginine, which could suggest that the character of conjugates became similar to that of the drug. On the other hand, the efficacy of the MRP1 protein to 17-desacetylvinblastineTrp is increased by the conjugation. In the resistant cells, the proteins pump out the compounds and the resulting concentration reduction inhibits the effect of the drugs. Because the cytotoxicity of 17-desacetylvinblastine and the octaargininеTrp conjugate are almost similar on resistant cells, it can be supposed that the reason for the significant difference in the case of the sensitive cells is the difference between the concentrations of active 17-desacetylvinblastine in the cells. In the case of 17-desacetylvinblastine, the achieved effective intracellular concentration is determined only by the diffusion rate. However, with the conjugates at least two different processes are involved, namely, the internalization of the conjugates and the interaction of the released (free) or conjugated drug with the microtubular system at the site of action. In order to analyze the influence of the presence of oligoarginine, the effect of the conjugates was studied on tubulin polymerization in vitro and in vivo.

In Vitro and in Vivo Effect of Vinblastine Derivatives on Tubulin Polymerization. In order to characterize the effect of vinblastine and its conjugate derivatives on a microtubule assembly, the polymerization of tubulin heterodimers was investigated in vitro at different drug concentrations.

Figure 4 shows that vinblastine and its derivatives inhibit paclitaxel-induced tubulin polymerization; however, this effect was dependent upon the nature of the derivatives. The dose−response curves obtained by plotting the polymerization rate as a function of drug concentrations reveal that the inhibitory effects of 17-desacetylvinblastineTrp and the 17-desacetylvinblastineTrp-Arg_8-2 conjugates are comparable to those of vinblastine in the polymerization assay. However, the inhibitory potency of the 17-desacetylvinblastineTrp-Arg_8-1 conjugate on the microtubule assembly was less pronounced. It should be noted that with the mixture of the two Trp-Arg_8-containing isomers (before their HPLC separation) we obtained a lower inhibitory effect than with 17-desacetylvinblastineTrp-Arg_8-2, but a higher effect than with 17-desacetylvinblastineTrp-Arg_8-1. This result suggests that the 17-desacetylvinblastineTrp-Arg_8-1 conjugate is not a potential antimitotic agent. However, the effect observed in an in vitro system with purified tubulin cannot provide information on the specificity of the drugs. Specificity, i.e., the antimicrotubular activity of the drug, was studied at a cellular level using HeLa cells by investigating both the interphase and spindle microtubules in parallel.

The majority of the eukaryote cells are at interphase, and only the minority are in mitosis—at a certain point in time—even

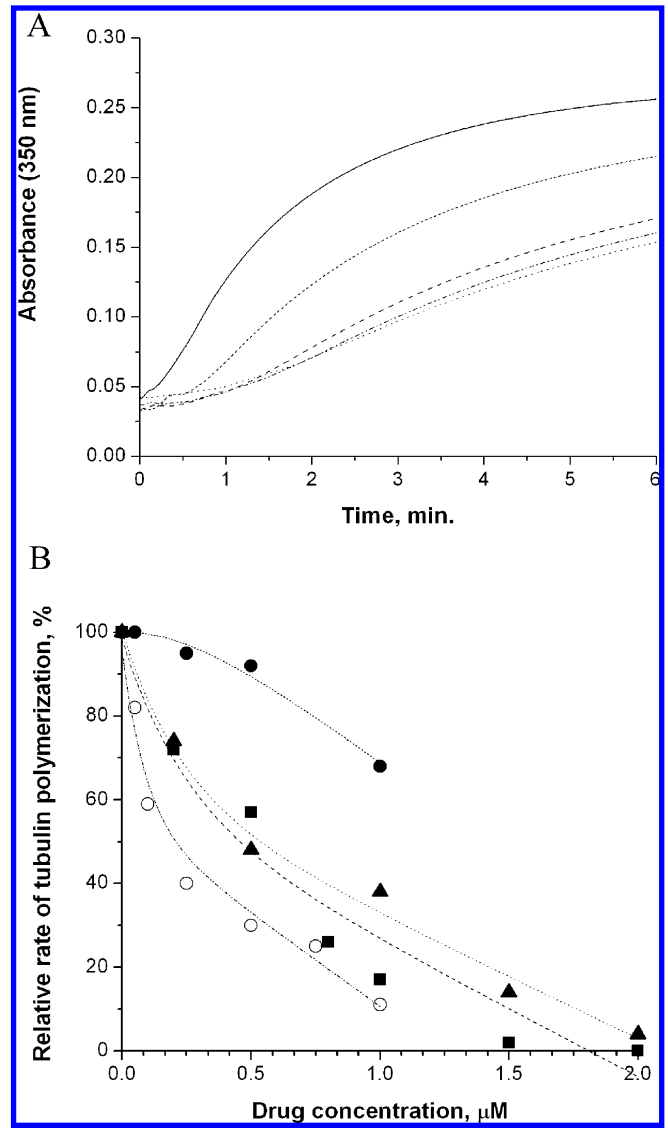

Figure 4. Effect of vinblastine and its conjugate derivatives on tubulin polymerization. (A) Turbidimetric assay. The paclitaxel-induced tubulin polymerization was followed at $\lambda = 350$ nm. The concentration of vinblastine and its derivatives was 1 μM. Tubulin control (solid line), vinblastine (dashed line), 17-desacetylvinblastineTrp (dotted line), 17-desacetylvinblastineTrp-Arg_8-1 (short dashed line), 17-desacetylvinblastineTrp-Arg_8-2 (dash-dot-dotted line). (B) The relative effect of vinblastine and its derivatives on tubulin polymerization as a function of drug concentration. (■, dashed line) vinblastine, (▲, dotted line) 17-desacetylvinblastineTrp, (●, short dashed line) 17-desacetylvinblastineTrp-Arg_8-1, (○, dash-dot-dotted line) 17-desacetylvinblastineTrp-Arg_8-2.

in cancer cells, which are characterized by rapid proliferation. Therefore, if a drug specifically targets the mitotic microtubule spindle, this effect is not necessarily reflected at a cellular level by the cytotoxicity index.

In order to characterize the antimicrotubule activities of vinblastine and its conjugate derivatives at a cellular level, we performed comparative studies in human cervical carcinoma (HeLa) cells. The drugs were added to the medium of the growing cells at different concentrations. After 24 h, the microtubular network of the cells was immunostained with antitubulin antibody and visualized by immunofluorescence microscopy.

Figure 5 shows that the interphase microtubular network can be characterized by three well-defined stages: (i) Normal stage (Figure 5a): the microtubular network is assembled into bundled fibers of highly ordered fashion. The fibers originate from the

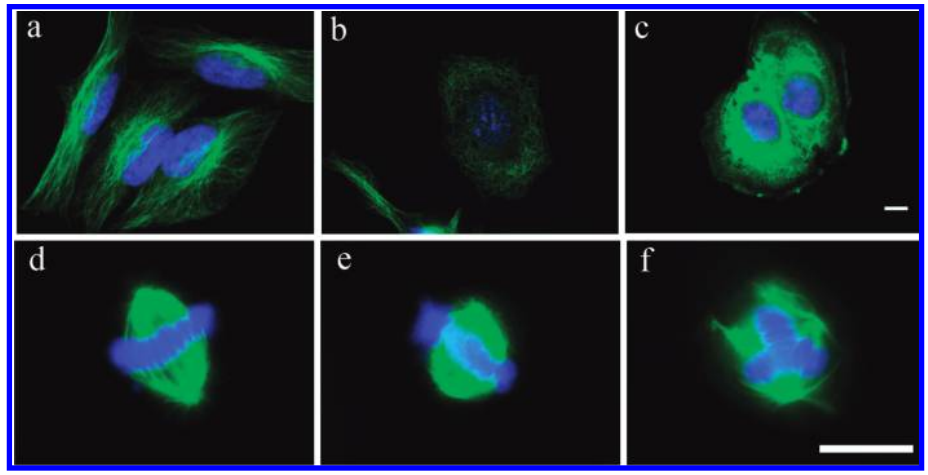

Figure 5. Characteristic pictures for interphase (a−c) and mitotic (d−f) microtubule network formations in HeLa cells immunostained for α-tubulin (green); normal, control (a and d), fragmentated (b) induced by 2.5 μM 17-desacetylvinblastineTrp, depolymerized (c) induced by 2.5 μM vinblastine, asymmetric (e) induced by 0.25 μM, and multipolar (f) induced by 2.5 μM 17-desacetylvinblastineTrp treatment. Blue: DAPI. Bar: 5 μm.

Table 2. Effect of Vinblastine and 17-Desacetylvinblastine Derivatives on the Spindle and Interphase Microtubule Ultrastructures[a]

	vinblastine		17-desacetylvinblTrp		17-desacetylvinblTrpArg$_8$-**2**		17-desacetylvinblTrpArg$_8$-**1**	
	aberrant mitosis (%)	interphase microtubules	aberrant mitosis (%)	interphase microtubules	aberrant mitosis (%)	interphase microtubules	aberrant mitosis (%)	interphase microtubules
control	2	normal	2	normal	2	normal	2	normal
0.25 μM	100	depolymerized	47	normal	22	normal	n. d.[b]	n. d.
1 μM	100	depolymerized	100	fragmented	75	normal	45	normal
2.5 μM	100	depolymerized	100	fragmented	98	fragmented	75	normal
5 μM	100	depolymerized	100	depolymerized	100	fragmented	100	normal

[a] HeLa cells were treated with the drugs at different concentrations for 24 h. The percentage distribution of the aberrant mitotic spindle among all cells in the phase of mitosis was quantified by investigating random microscopic fields. The structure of the interphase and mitotic microtubules was visualized by immunofluorescence microscopy using monoclonal antitubulin antibody. [b] n.d: not defined.

microtubule organizing center and are concentrated around the nucleus. Their free ends radiate outward toward the cell membrane, forming a network. In drug-treated cells, microtubules undergo significant reorganizations. (ii) Fragmented stage (Figure 5b): long bundles of microtubules are fragmented into short, disorganized fibers which radiate to the cell periphery in a criss−cross fashion. In the vicinity of nuclei, the microtubules acquire an amorphous appearance. The microtubule organizing centers are no longer apparent. (iii) Depolymerized stage (Figure 5c): all microtubules are disassembled; the depolymerized microtubules show homogeneous cytosolic distribution.

We also studied the changes of the spindle structures in parallel with those of the interphase at various drug concentrations. Treatment of the cells with vinblastine or its derivatives caused significant alterations in the spindle structures. Typical structures visualized in the drug-treated samples are shown in Figure 5: normal, symmetric (Figure 5d), asymmetric (Figure 5e), and multipolar (Figure 5f).

For evaluation of the specificity of the vinblastine derivatives, we quantified the relative amount (%) of the aberrant mitotic spindles as compared to all mitotic cells. In addition, the ultrastructures of the interphase microtubules were visualized and ranked according to the categories presented in Figure 5.

According to the data summarized in Table 2, vinblastine, even at the lowest concentration used in this study, depolymerized the microtubular network completely; the tubulin homogeneously distributed within the cytosol corresponding to Figure 5c. 17-DesacetylvinblastineTrp causes much less damage to the microtubular network than vinblastine, and 47% of the mitotic cells are aberrant at 0.25 μM. The two Trp-Arg$_8$ conjugates displayed distinct effects, while the 17-desacetylvinblastineTrp-Arg$_8$-2 isomer conjugate caused significant fragmentation of the interphase microtubule at 2.5 μM, the 17-desacetylvinblastineTrp-Arg$_8$-1 isomer conjugate did not affect them at all even at a 5 μM concentration. These results clearly show that the 17-desacetylvinblastineTrp-Arg$_8$-1 isomer conjugate is highly selective against the mitotic spindles and destroys only these structures in the mitotic cells. As among tumor cells there are more cells in mitosis at a certain time point than among normal cells, these data suggest that the 17-desacetylvinblastineTrp-Arg$_8$-1 isomer conjugate may have a selective cytotoxic effect against tumor cells and may thus have lower side effects. As the isomers are different in the configuration of Trp, further studies are needed to rationalize this selectivity.

CONCLUSIONS

Vinblastine is an efficient antitumor agent, but its side effects and resistance can restrict its use. A promising approach to avoid the harmful effects is its conjugation with an appropriate partner compound. The coupling of 17-desacetylvinblastineTrp with cell-penetrating oligoarginines results in conjugates whose biological effectiveness correlates with the chain length of peptides. The octa- and hexaarginine conjugates preserve the cytotoxicity of the original drug on both sensitive and resistant HL-60 cells. Moreover, the 17-desacetylvinblastineTrp-Arg$_8$-1 isomer conjugate preferentially destroys the mitotic spindle, at least in the case of cancerous HeLa cells, resulting in cell cycle inhibition. Further studies should be performed to demonstrate that this conjugate can exclusively target the tumor cells and is less toxic to normal cells in many other cases.

ACKNOWLEDGMENT

These studies were supported by grants from the Hungarian Research Fund (OTKA, no. K68285 and K68734), Hungarian Ministry of Education (NKFP Medichem 2), and from the Hungarian Ministry of Health (ETT 209/2009). The authors are grateful to Dr. Szilvia Bősze for the helpful discussion and to

Gedeon Richter Plc. (Budapest, Hungary) for sponsorship. Moreover, one of the authors (Á. G.-K.) is indebted to the József Varga Foundation for financial support.

Supporting Information Available: NMR data and spectra, analytical HPLC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Owellen, R. J., Jr., Owens, A. H., and Donigian, D. W. (1972) The binding of vincristine, vinblastine and colchicine to tubulin. *Biochem. Biophys. Res. Commun. 47*, 685–691.

(2) Owellen, R. J., Hartke, C. A., Dickerson, R. M., and Hains, F. O. (1976) Inhibition of tubulin-microtubule polymerization by drugs of the vinca alkaloid class. *Cancer Res. 36*, 1499–1502.

(3) Toso, R. J., Jordan, M. A., Farrell, K. W., Matsumoto, B., and Wilson, L. (1993) Kinetic stabilization of microtubule dynamic instability in vitro by vinblastine. *Biochemistry 32*, 1285–1293.

(4) Prakash, V., and Timasheff, N. S. (1991) Mechanism of interaction of vinca alkaloids with tubulin: catharanthine and vindoline. *Biochemistry 30*, 873–880.

(5) Barnett, J. C., Cullinan, J. G., Gerzon, K., Hoying, R. C., Jones, W. E., Newlon, W. M., Poore, G. A., Robison, R. L., Sweeney, M. J., Todd, G. C., Dyke, R. W., and Nelson, R. L. (1978) Structure-activity relationships of dimeric Catharanthus alkaloids. 1. Deacetylvinblastine amide (vindesine) sulfate. *J. Med. Chem. 21*, 88–96.

(6) Conrad, A. R., Cullinan, J. G., Gerzon, K., and Poore, A. G. (1979) Structure-activity relationships of dimeric catharanthus alkaloids. 2. Experimental antitumor activities of N-substituted deacetylvinblastine amide (Vindesine) su1fates. *J. Med. Chem. 22*, 391–400.

(7) Bhushana Rao, K. S. P., Collard, M-P. M., Dejonghe, J. P. C., Atassi, G., Hannart, J. A., and Trouet, A. (1985) Vinblastin-23-oyl amino acid derivatives: chemistry, physicochemical data, toxicity, and antitumor activities against P388 and L1210 leukemias. *J. Med. Chem. 28*, 1079–1088.

(8) Laguzza, C. B., Nichols, L. C., Briggs, L. S., Cullinan, J. G., Johnson, A. D., Starling, J. J., Baker, L. A., Bumol, F. T., and Corvalant, F. R. J. (1989) New antitumor monoclonal antibody-vinca conjugates LY203725 and related compounds: design, preparation, and representative in vivo activity. *J. Med. Chem. 32*, 548–555.

(9) Apelgren, D. L., Zimmerman, L. D., Briggs, L. S., and Bumol, F. T. (1990) Antitumor activity of the monoclonal antibody-vinca alkaloid immunoconjugate LY203725 (KSl/4−4-Desacetylvinblastine-3-carboxhydrazide) in a Nude Mouse Model of human ovarian cancer. *Canser Res. 50*, 3540–3544.

(10) Starling, J. J., Maciak, S. M., Hinson, A. N., Nichols, L. C., Briggs, L. S., Laguzza, C. B., Smith, W., and Corvalan, F. R. J. (1992) In vivo antitumor activity of a panel of four monoclonal antibody-vinca alkaloid immunoconjugates which bind to three distinct epitopes of carcinoembryonic antigen. *Bioconjugate Chem. 3*, 315–322.

(11) Gutowski, C. M., Briggs, L. S., and Johnson, A. D. (1991) Epidermal growth factor receptor-reactive monoclonal antibodies: xenograft antitumor activity alone and as drug immunoconjugates. *Cancer Res. 51*, 5471–5475.

(12) Johnson, A. D., and Laguzza, C. B. (1987) Antitumor xenograft activity with a conjugate of a vinca derivative and the squamous carcinoma-reactive monoclonal antibody PF1/D. *Cancer Res. 47*, 3118–3122.

(13) Vlahov, R. I., Santhapuram, R. K. H., Kleindl, J. P., Howard, J. S., Stanford, M. K., and Leamon, P. C. (2006) Design and regioselective synthesis of a new generation of targeted chemotherapeutics. Part 1: EC145, a folic acid conjugate of desacetylvinblastine monohydrazide. *Bioorg. Med. Chem. Lett. 16*, 5093–5096.

(14) Leamon, P. C., Reddy, A. J., Vlahov, R. I., Kleindl, J. P., Vetzel, M., and Westrick, E. (2006) Synthesis and biological evaluation of EC140: a novel folate-targeted vinca alkaloid conjugate. *Bioconjugate Chem. 17*, 1226–1232.

(15) Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G., and Rothbard, J. B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res. 56*, 318–325.

(16) Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem. 276*, 5836–5840.

(17) Hudecz, F., Bánóczi, Z., and Csík, G. (2005) Medium-sized peptides as built in carriers for biologically active compounds. *Med. Res. Rev. 25*, 679–736.

(18) Rothbard, J. B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P. L., Wender, P. A., and Khavari, P. A. (2000) Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat. Med. 6*, 1253–1257.

(19) Miklán, Zs., Szabó, R., Zsoldos-Mády, V., Reményi, J., Bánóczi, Z., and Hudecz, F. (2007) New ferrocene containing peptide conjugates: Synthesis and effect on human leukemia (HL-60) cells. *Biopolymers. 88*, 108–114.

(20) Bánóczi, Z., Peregi, B., Orbán, E., Szabó, R., and Hudecz, F. (2008) Synthesis of Daunomycin containing oligoarginine conjugates and their effect on Human leukemia cells (HL-60). *Arkivoc iii*, 140–153.

(21) Enkvist, E., Lavogina, D., Raidaru, G., Vaasa, A., Viil, I., Lust, M., Viht, K., and Uri, A. (2006) Conjugation of adenosine and hexa-(D-arginine) leads to a nanomolar bisubstrate-analog inhibitor of basophilic protein kinases. *J. Med. Chem. 49*, 7150–7159.

(22) Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970) Color test of detection of free amino groups in the solid phase synthesis of peptides. *Anal. Biochem. 34*, 595–598.

(23) The ethyl ester derivative of 3 is known as an antitumor agent vintriptol investigated in phase I and II clinical studies, see also Oosterkamp, H. M., Vlasveld, L. Th., Wanders, J., Beijnen, J. H., Van Tellingen, Dubbelman, O., A. C., Simonetti, G. P. C., Franklin, H. R., Vermorken, J. B., and Ten, W. W. (1991) Phase I study of vintriptol, a tryptophan ester of vinblastin. *Eur. J. Cancer Clin. Oncol. 27*, 1222–1226.

(24) Na, C. N., and Timasheff, S. N. (1986) Interaction of vinblastine with calf brain tubulin: multiple equilibria. *Biochemistry 25*, 6214–6222.

(25) Lehotzky, A., Tirián, L., Tökési, N., Lénárt, P., Szabó, B., Kovács, J., and Ovádi, J. (2004) Dynamic targeting of microtubules by TPPP/p25 affects cell survival. *J. Cell Sci. 117(Pt 25)*, 6249–6259.

(26) Hannart, J. A., Bhushana Rao, K. S. P., and Trouet, A. (1981) *N*-(Vinblastinoyl-23) derivatives of amino acids and peptides and their preparation, EP0041935.

(27) Brady, S. F., Pawluczyk, J. M., Lumma, P. K., Feng, D.-M., Wai, J. M., Jones, R., DeFeo-Jones, D., Wong, B. K., Miller-Stein, C., Lin, J. H., Oliff, A., Freidinger, R. M., and Garsky, V. M. (2002) Design and synthesis of a pro-drug of vinblastine targeted at treatment of prostate cancer with enhanced efficacy and reduced systemic toxicity. *J. Med. Chem. 45*, 4706–4715.

BC100028Z