Voacamine Modulates the Sensitivity to Doxorubicin of Resistant Osteosarcoma and Melanoma Cells and Does Not Induce Toxicity in Normal Fibroblasts

Maria Condello,†‡ Dario Cosentino,† Silvia Corinti,§ Gabriella Di Felice,§ Giuseppina Multari,⊥ Francesca Romana Gallo,⊥ Giuseppe Arancia,† and Stefania Meschini*†

†Department of Technology and Health, Italian National Institute of Health, Rome, Italy
‡Institute of Chemical Methodologies, National Research Council (CNR), Rome, Italy
§Department of Infectious, Parasitic and Immune-Mediated Diseases, Italian National Institute of Health, Rome, Italy
⊥Department of Therapeutic Research and Medicines Evaluation, Italian National Institute of Health, Rome, Italy

Supporting Information

ABSTRACT: In previous studies it has been demonstrated that the plant alkaloid voacamine (1), used at noncytotoxic concentrations, enhanced the cytotoxicity of doxorubicin and exerted a chemosensitizing effect on cultured multidrug-resistant (MDR) U-2 OS-DX osteosarcoma cells. The in vitro investigations reported herein gave the following results: (i) the chemosensitizing effect of 1, in terms of drug accumulation and cell survival, was confirmed using SAOS-2-DX cells, another MDR osteosarcoma cell line; (ii) compound 1 enhanced the cytotoxic effect of doxorubicin also on the melanoma cell line Me30966, intrinsically drug resistant and P-glycoprotein-negative; (iii) at the concentrations used to sensitize tumor cells, 1 was not cytotoxic to normal cells (human fibroblasts). These findings suggest possible applications of voacamine (1) in integrative oncologic therapies against resistant tumors.

Since multidrug resistance (MDR) adversely affects the efficacy of many cancer chemotherapeutic agents, several studies have been performed to reverse the resistant phenotype and develop effective curative strategies. In most of these attempts, P-glycoprotein (P-gp) inhibitors are used, as the overexpression of this molecular pump is often related to the occurrence of MDR. Such agents used to obtain data do not appear very promising in clinical practice as a result of their severe side effects. Therefore, a priority in anticancer pharmacology is a search for additional chemosensitizing substances, effective against resistant tumors and with a low degree of systemic toxicity.

In 1994 You and colleagues reported found that three indole alkaloids, including voacamine (1), obtained from Peschiera laeta, were able to enhance the cytotoxic effect of vinblastine on drug-resistant human oral epidermal carcinoma cells.1 Moreover, in other studies,2,3 the bisindole alkaloid voacamine (1), isolated from the plant Peschiera fuchsiaefolia Miers (Apocynaceae),4 exerted a chemosensitizing effect on cultured MDR osteosarcoma cells (U-2 OS/DX) exposed to doxorubicin. Pretreatment with 1, at noncytotoxic concentrations, inhibited P-gp action in a competitive way, accounting for the enhancement of intracellular content and cytotoxic effect of doxorubicin induced on MDR cells. Voacamine (1) is also used with chloroquine and artemisinin for malaria treatment in vivo.5

The aims of this study were to verify (i) whether the chemosensitizing effect of voacamine (1) is specific to the U-2 OS/DX osteosarcoma cell line or is also exerted on other human osteosarcoma cells of different origin; (ii) that voacamine enhances the cytotoxic effect of doxorubicin also on melanoma cells, intrinsically drug resistant and surface P-gp-negative; and (iii) that voacamine chemosensitizing concentrations are not cytotoxic for normal cells (human fibroblasts), in view of its possible clinical application.

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RESULTS AND DISCUSSION

Characterization of the Tumor Cell Lines Used. Cell lines (SAOS-2-WT, SAOS-2-DX, and Me30966) were characterized for their morphology, P-gp expression, doxorubicin uptake and efflux and susceptibility to doxorubicin treatment. Under the scanning electron microscope, SAOS-2-WT and SAOS-2-DX cells (Figure 1A and B, respectively) showed a similar morphology characterized by a flat aspect and a smooth surface with scarce microvilli and numerous long and thin protrusions. Me30966 melanoma cells were shown to be polygonal in shape, with short microvilli randomly distributed on the surface (Figure 1C).

The P-gp cytofluorimetric intensity was much higher in SAOS-2-DX cells than in SAOS-2-WT cells (Figure 2A), accounting for the resistant phenotype of the former. The fluorescence profile of control cells coincided with that of SAOS-2-DX cells than in their sensitive counterparts as well as in control cells. (B) Me30966 melanoma cells were completely negative for P-gp labeling.

The localization of surface P-gp by laser scanning confocal microscopy confirmed the flow cytometric data. Numerous fluorescent spots were visible on the surface of SAOS-2-DX cells (Figure 3A), whereas SAOS-2-WT cells (Figure 3B) appeared negative.

The intracellular accumulation of doxorubicin was evaluated by flow cytometry after treatment for 1 h with 1.0 μg/mL of this compound and its efflux 2 h after the end of the treatment. Figure 4A shows that the intracellular content of doxorubicin was lower in resistant cells (about 40%) than in their parental counterparts. Melanoma cells, which do not express surface P-gp, accumulated a slightly greater amount of doxorubicin than SAOS-2-DX cells, but a lower amount than SAOS-2-WT cells. Concerning the capability of extruding doxorubicin molecules, flow cytometric determination revealed a noticeable drug efflux in SAOS-2-DX cells, a very low efflux in their sensitive counterparts, and an intermediate value in Me30966 cells (Figure 4B). These observations account for the MDR phenotype of SAOS-2-DX cells and seem to suggest that the intrinsic resistance of Me30966 melanoma cells could be due to mechanisms alternative to the expression of P-gp.

The cytotoxicity induced on the cell lines by 1.0 μg/mL doxorubicin for 24 h was evaluated using the MTT assay. As shown in Figure 5, the percent survival of SAOS-2-DX cells was much higher (about 80%) than that of SAOS-2-WT cells (about 20%), while about 40% of Me30966 cells maintained their viability.

In a previous study the enhancement of the cytotoxic effect of doxorubicin exerted by 1 on U-2 OS/DX cells was shown.
The use of a resistant cell line, SAOS-2-DX, derived from the same tumor but from different patients, is a strategy to obtain a better understanding of osteosarcoma biology and to identify novel targets for specific therapies. Also examined were Me30966 cells, which are intrinsically doxorubicin resistant and not P-gp mediated. Doxorubicin accumulation, efflux analysis, and the MTT test revealed alternative MDR mechanisms in melanoma cells. Me30966 cells showed intermediate values of doxorubicin uptake, doxorubicin efflux, and cell survival when compared to SAOS-2-WT and SAOS-2-DX. Previous studies carried out on stabilized melanoma cell lines have suggested a functional role of intracytoplasmic P-gp in the transport and sequestration of drugs. The presence of P-gp in the membrane of intracytoplasmic vesicles may reduce the doxorubicin content inside the nucleus, as a result of the trapping of drug molecules into acidic compartments.

**Figure 3.** Immunofluorescence detection of the surface P-gp obtained by laser scanning confocal microscopy. Numerous fluorescent spots were visible on the surface of resistant SAOS-2-DX cells (A), whereas sensitive SAOS-2-WT cells (B) were negative for the P-gp labeling.

**Figure 4.** Doxorubicin (DOX) accumulation and efflux evaluated by flow cytometry in three cell types. (A) The intracellular content of the fluorescent compound, after treatment for 1 h at 1.0 μg/mL concentration, was much lower in resistant SAOS-2-DX cells than in the sensitive SAOS-2-WT cells. Me30966 melanoma cells, even though they do not express P-gp, accumulated a similar amount of doxorubicin as MDR osteosarcoma cells. (B) Quantification of doxorubicin, determined 2 h after the end of treatment, revealed a high drug efflux in resistant SAOS-2-DX cells, a low efflux in their wild-type counterparts, and an intermediate value in Me30966 melanoma cells.

**Figure 5.** Cytotoxic effect of doxorubicin (DOX) treatment on osteosarcoma and melanoma cells. After treatment with doxorubicin (1.0 μg/mL for 1 h), about 80% of MDR SAOS-2-DX cells maintained their viability, whereas the percent survivals of intrinsically resistant Me30966 melanoma cells and sensitive SAOS-2-WT cells were about 40% and 20%, respectively.

**Cytotoxic Effect of Treatment with Voacamine (1) on Tumor and Normal Cell Lines.** The possible cytotoxicity induced by 1 was evaluated by flow cytometry after staining with trypan blue. Figure 6 shows the percentages of dead cells after treatment with concentrations of 1 ranging from 0.1 to 5.0 μg/mL, for 4 h (Figure 6A) and 24 h (Figure 6B).

No cell line was affected by a 4 h voacamine treatment. Only after 24 h treatment with the highest concentration (5.0 μg/mL) was a significant cytotoxic effect observed. The cytotoxic effect of voacamine (1) was also evaluated on nontumor AG1522 cells, a human fibroblast cell line. Figure 6C shows that normal cells responded to treatment with 1 in a similar manner to tumor cells; thus only after exposure to 5.0 μg/mL of this compound for 24 h was there a significant increase in cell death. The lack of cytotoxicity by treatment with voacamine given alone on osteosarcoma, melanoma, and normal human fibroblasts was demonstrated. These results pointed to a right strategy in the use of this alkaloid as chemosensitizing agent against doxorubicin.
Effects of Voacamine (1) on Intracellular Concentration Levels and Distribution of Doxorubicin. The intracellular concentrations of doxorubicin, in both the uptake and efflux phases, were determined using flow cytometry, and the intracellular distribution was analyzed using a laser scanning confocal microscope.

The uptake and efflux phases parameters were evaluated on cells treated with doxorubicin alone or with the combinations voacamine (1) + doxorubicin and cyclosporin A + doxorubicin (as control). Compound 1 was administered at a concentration of 1.0 μg/mL. Neither 1 nor cyclosporin A had any significant effect on doxorubicin accumulation in sensitive SAOS-2-WT
cells or P-gp-negative melanoma cells (Figure 7A), whereas in resistant SAOS-2-DX cells, the presence of voacamine induced an increase in drug accumulation, similar to that induced by cyclosporin A. The role of 1 in regulating doxorubicin transport was investigated further by determining the percentage of efflux (Figure 7B). The drug efflux rates in SAOS-2-WT and melanoma cells were not affected by the presence of voacamine or cyclosporin A: in resistant cells a noticeable increase in drug retention was observed in the presence of both MDR-modulating agents.

The observations carried out by laser scanning confocal microscopy on living SAOS-2-WT and SAOS-2-DX cells indicated a different effect of voacamine (1) on the intracellular distribution of doxorubicin (Figure 8).

In fact, in sensitive cells treated with doxorubicin alone, the fluorescent test compound molecules were located mainly inside the nuclei, which appeared to be strongly positive (Figure 8A). In resistant cells, the intensity of the fluorescent signal was much lower, confirming the flow cytometric results (Figure 4A) and, above all, showing a very different distribution (Figure 8B), since the nuclei were negative and a diffuse cytoplasmic fluorescence could be detected. The antitumor activity of anthracyclines is mainly ascribed to their intercalation between the base pairs of the nuclear DNA molecule, which can alter the conformation of the nucleic acid and cause DNA fragmentation and inhibition of RNA and DNA synthesis. Multidrug-resistant tumor cells are generally able to strongly reduce the intranuclear concentration of the cytotoxic agents, thus becoming unresponsive to many antineoplastic drugs. This property is often associated with increased efflux of the cytotoxic compounds, due to the activation of mechanisms of intracellular transport and to the overexpression of proteins, such as P-gp, which act as ATP-dependent molecular pumps. During the efflux phase, drug molecules are transported from the nucleus to the cell periphery utilizing the cytoplasmic vesicular apparatus. These findings explain the different intracellular doxorubicin distribution observed by laser scanning confocal microscopy among sensitive and resistant cells (Figure 8A and B, respectively). When treatment with doxorubicin of SAOS-2-DX cells was carried out in the presence of voacamine (1) (Figure 8C) or the P-gp inhibitor cyclosporin A (Figure 8D), the intracellular content and distribution of the fluorescent drug were similar to those observed in SAOS-2-WT cells. These observations are in agreement with our previous results, suggesting that 1 reacts with P-gp producing conformational changes with consequent epitope modulation. Since 1 is a substrate for the transport protein, it interferes with the P-gp-mediated drug export, acting as a competitive antagonist of doxorubicin. For this reason, neither voacamine (1) nor cyclosporin A had any effect on drug uptake and efflux in either SAOS-2-WT or Me30966 cells (data not shown) because both of them do not express surface P-gp.

**Voacamine (1)-Induced Enhancement of the Cytotoxic Effect of Doxorubicin on MDR Cells.** SAOS-2-DX cells were treated for 72 h with doxorubicin concentrations of...
In resistant osteosarcoma cells (Figure 9A), the MTT test demonstrated clearly the chemosensitizing action of the pretreatment with voacamine (1), besides confirming that up to a 1.0 μg/mL concentration of this compound does not affect cell survival even after a long period of exposure and that doxorubicin administration (0.5 or 1.0 μg/mL) was barely cytotoxic for this cell type. After treatment with four voacamine and doxorubicin combinations, SAOS-2-DX cells exhibited a significant reduction of survival as compared with treatment with doxorubicin alone. Pretreatment with 1.0 μg/mL 1 induced about 50% cell survival also when followed by a low doxorubicin concentration (0.5 μg/mL).

The MTT test on melanoma cells gave the following results (Figure 9B): (i) voacamine (1) at both concentrations did not affect cell survival; (ii) 0.1 μg/mL doxorubicin alone was not cytotoxic, whereas at a concentration of 0.5 μg/mL the cell survival was reduced to about 58%; (iii) the combined voacamine and doxorubicin treatments induced a significant reduction in cell survival when doxorubicin was used at the noncytotoxic concentration of 0.1 μg/mL.

Cell survival analysis on SAOS-2-DX and Me30966 cells revealed that the combined treatment produced a significant cytotoxic effect, even when doxorubicin was used at the noncytotoxic concentrations of 0.5 μg/mL for osteosarcoma-resistant cells and 0.1 μg/mL for intrinsically resistant melanoma cells. Considering the serious side effects (hepatotoxicity and cardiotoxicity) induced in vivo by doxorubicin treatment, the possibility of obtaining the same cytotoxic effect against tumor cells by using lower concentrations seems to be very promising. In fact, under the experimental conditions used, the drastic reduction of doxorubicin can be obtained by the apparently harmless pretreatment with the alkaloid 1.

**Morphological Changes Evaluated by Phase Contrast Microscopy.** Figure 10 shows the monolayer of control SAOS-2-DX cells (Figure 10A) and Me30966 cells (Figure 10C) as compared with those observed after combined voacamine (1) and doxorubicin treatment (Figure 10B and D, respectively).

Since after exposure to either voacamine (1) or doxorubicin alone the morphology of both cell types appeared to be unaltered (not shown), the synergistic cytotoxic effect of the two compounds was supported by these observations.

In conclusion, the observations herein reported showed that the plant alkaloid voacamine (1), used at noncytotoxic concentrations, is capable of overcoming the multidrug-resistant phenotype of cultured tumor cells by acting as a competitive antagonist against the P-gp-mediated mechanism of extrusion of selected cytotoxic drugs. Also, voacamine (1) administered at higher concentrations (>3.0 μg/mL) has been demonstrated to be an autophagy inducer able to exert apoptosis-independent cytotoxic effects on both wild-type and MDR tumor cells.15

**EXPERIMENTAL SECTION**

**Isolation of Voacamine (1).** The root bark of *Peschiera fuchsiaefolia* was provided by Mr. S. Rossi from CIBECOL Ltda. (Porto Alegre, Brazil). The dried plant material (root bark) was ground to a powder. A reference sample (Product No. SNV-100) of the powered plant material has been deposited in the laboratory of Natural Substances and Traditional Medicine in the Italian National Institute of Health, Rome, Italy. The powder (630 g) was percolated three times with 2% aqueous acetic acid (AcOH), overnight. The resulting combined acidic extracts were made alkaline to pH 9 by Na₂CO₃ and extracted three times with CH₂Cl₂. The combined

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organic layers were evaporated under vacuum to dryness to afford the crude total tertiary alkaloids as free bases. The dried organic extract of the tertiary alkaloids (about 15 g) was separated by countercurrent distribution (CCD) in a Craig–Post apparatus between CH2Cl2 (stationary lower phase) and phosphate/citric acid buffer solutions (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0).20 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).4 The separation by CCD was monitored by TLC plates (silica gel 60 F254; elution with the upper phase of the system solvent (mobile upper phase) at discontinuously decreasing pH (from 6.0 to 2.0)).

**Cell Cultures.** The established human osteosarcoma cell line (SAOS-2-WT) and its derived MDR variant (SAOS-2-DX) were kindly provided by Dr. K. Scotlandi, Rizzoli Orthopedic Institute, Bologna, Italy. The SAOS-2-DX cell line was obtained through exposure of the parental sensitive line to sublethal concentrations of doxorubicin increasing up to 580 ng/mL.22 Both cell lines were grown as a monolayer in IMDM medium (Gibco Life Technologies, Paisley, U.K.).

The human metastatic melanoma line (Me30966) and the human skin fibroblasts (AG1522) were provided by Dr. F. Lozupone and Dr. A. Tabocchini, respectively, National Institute of Health, Rome, Italy. They were grown as monolayers in RPMI medium (Gibco Life Technologies, Paisley, U.K.).

All media used for cell culturing were supplemented with 10% fetal bovine serum (FBS) (Euroclone), 1% penicillin (50 U/mL)–streptomycin (50 μg/mL) (Euroclone), and 1% nonessential amino acids (Euroclone) in a humidified atmosphere of 5% CO2 in a water-jacketed incubator at 37 °C.

**Scanning Electron Microscopy.** For scanning electron microscopy, cells were labeled with IgG2a (Sigma Chemical Co.) and processed as described in the flow cytometry description. The confocal observations were carried out using a Leica TCS SP2 spectral confocal microscope (Leica Microsystems, Wetzlar, Germany).3

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay.** The effect of doxorubicin, and combined treatment (1 plus doxorubicin) on the viability of SAOS-2-DX and
Me30966 cells was evaluated by the MTT assay. After 24 h of seeding cells in a 96-well plate (1 × 10^4 SAOS-2-DX cells or 5 × 10^3 Me30966 cells), treatment with voacamine (1) (0.5 or 1.0 μg/mL, doxorubicin (0.1, 0.5, or 1.0 μg/mL), or 1 (administered 30 min before) plus doxorubicin was carried out. The effect of treatment at 24 h for Me30966 cells or 72 h for SAOS-2-DX cells was evaluated using MTT salt. 25

Phase Contrast Microscopy. SAOS-2-DX cells, untreated and treated with 1.0 μg/mL voacamine (1) plus 1.0 μg/mL doxorubicin, and Me30966 cells, untreated and treated with 1.0 μg/mL 1 plus 0.5 μg/mL doxorubicin, were observed by phase contrast microscopy (Zeiss, Axiovert200, Göttingen, Germany).

Statistical Analysis. The values shown in the figures represent the averages ± standard deviations of three independent experiments. Student’s t test was used for statistical analysis. Differences were considered significant at p values of ≤0.05.

ASSOCIATED CONTENT

Supporting Information

Spectroscopic data of voacamine (1). The information is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +39 06 4990 2783. Fax: +39 06 4990 3563. E-mail: stefania.meschini@iss.it.

Notes

The authors declare no competing financial interest.

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