Bcl-2 down-regulation and tubulin subtype composition are involved in resistance of ovarian cancer cells to vinflunine

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Abstract
Vinflunine, a new microtubule-targeting drug, has a marked antitumor activity in vitro and in vivo. Here, we studied the mechanisms mediating resistance to vinflunine. We investigated the response to vinflunine of ovarian cancer cells initially selected as paclitaxel-resistant cells (A2780-TC1 cells). By comparison with A2780-wild-type (wt) cells, we showed that A2780-TC1 cells were highly resistant to vinflunine, with resistance factors reaching 800 and 1,830 for IC50 and IC70, respectively.

Highly resistant to vinflunine, with resistance factors type (wt) cells, we showed that A2780-TC1 cells were cancer cells initially selected as paclitaxel-resistant cells. We investigated the response to vinflunine of ovarian cancer initially selected as paclitaxel-resistant cells (A2780-TC1 cells). By comparison with A2780-wild-type (wt) cells, we showed that A2780-TC1 cells were highly resistant to vinflunine, with resistance factors reaching 800 and 1,830 for IC50 and IC70, respectively. We showed that P-glycoprotein minimally participated in this cell resistance. The examination of tubulin composition revealed increased levels of acetylated α-tubulin, βIII-tubulin, and βIII-tubulin in A2780-TC1 cells before vinflunine treatment. As a consequence, vinflunine unequally affected microtubule network organization and function in A2780-wt and A2780-TC1 cells. Whereas the drug depolymerized microtubules and induced a mitotic block in A2780-wt cells, it did not depolymerize microtubules and induced a G2 block in A2780-TC1 cells. Elsewhere, the mitochondrial protein Bcl-2 was downregulated in A2780-TC1 cells. This down-regulation was related to resistance, as A2780-TC1 cells stably transfected with a Bcl-2 construct recovered a partial sensitivity to vinflunine. Lastly, we confirmed the role played by Bcl-2 by showing that the mitochondrial membrane potential was only disrupted by vinflunine in cells expressing Bcl-2. Altogether, our results indicate that modifications acquired during treatment (i.e., paclitaxel) have significant consequences on cell response to the following drug (i.e., vinflunine). Especially, this study shows that a specific pool of tubulin subtypes and a down-regulation of Bcl-2 are associated with resistance of ovarian cancer cells to vinflunine. [Mol Cancer Ther 2006;5(11):2824–33]

Introduction
Vinflunine (JAVLOR), a new anticancer agent derived from Vinca alkaloids, has a marked antitumor activity in a panel of cancer cell lines (1), murine tumors, and human tumor xenografts (2, 3). Interesting antivascular and antiangiogenic properties have also been highlighted for vinflunine (4, 5). This drug is currently in phase III clinical trials in lung and bladder cancers, as well as in phase II clinical trials in breast and ovarian cancers.

The binding site of Vinca alkaloids is at the interface between tubulin heterodimers, towards the microtubule inner lumen (6). High concentrations of vinflunine cause the dissolution of the microtubule network and formation of paracrystals by self-association of tubulin dimers (7). Lower concentrations induce microtubule depolymerization and the lowest effective concentrations suppress microtubule dynamics in cancer cells. Alterations of mitotic spindle functions by vinflunine can lead to mitotic block (8), maybe by suppressing the kinetochore-microtubule dynamics (9), but a postmitotic G1 arrest can also occur (10).

Vinflunine-induced cell death has been characterized as apoptosis but the signaling pathways are poorly understood. Vinflunine has been shown to cause c-jun NH2-terminal kinase-1 activation and caspase-3/7 cleavage (11). Recently, we have shown that mitochondria centralized the apoptotic signals triggered by vinflunine (10). These organelles play a key role in the cytotoxicity of various microtubule-targeting drugs (MTD) by releasing apoptotic factors, which lead to apoptosome formation followed by caspase activation (10, 12, 13). This process is tightly regulated by the relative levels of proapoptotic and antiapoptotic members of the Bcl-2 family that control the mitochondrial membrane permeability, thus determining the cell susceptibility to apoptosis (14).

Despite their shown effectiveness, the clinical success of the MTDs has been severely hindered by the emergence of resistant tumor cells. Interestingly, vinflunine has diminished drug resistance–inducing properties as compared with vinorelbine (15).

One common cause of cancer cell resistance to MTDs is the enhanced expression of the P-glycoprotein (P-gp).
This transmembrane drug efflux pump rapidly extrudes a variety of hydrophobic drugs, including vinflunine (16), from the targeted cells. Although the presence of P-gp has been correlated with a poor drug response, P-gp-mediated resistance is difficult to circumvent in the clinics (17). Then, it is important to understand and control the other mechanisms involved in anticancer drug resistance.

Another common mechanism of anticancer drug resistance is mediated by the modification of their cellular target. Resistance to MTDs has been closely associated with tubulin mutations and/or changes in microtubule composition (i.e., tubulin isotypes and posttranslational modifications; refs. 18–21). For example, high levels of acetylated and detyrosinated α-tubulin, markers of microtubule stability (22), have been observed in cells resistant to MTDs (20, 21, 23, 24). Increase in expression of βIII-tubulin, the main studied tubulin isotype, has been related to paclitaxel resistance, whereas its decrease has been observed during resistance acquisition to Vinca alkaloids, including vinflunine (15, 20, 25–29). With regard to the other tubulin isotypes, there is less evidence for their involvement in resistance to MTDs (20, 25, 30), especially in resistance to microtubule-depolymerizing agents.

Lastly, resistance to anticancer drugs can be mediated by alterations in apoptotic signaling pathways. In this sense, overexpression of the proapoptotic Bcl-2 family proteins, such as Bax or Bad, sensitizes cancer cells to MTDs. On the other hand, up-regulation of the prosurvival Bcl-2 family proteins, including Bcl-2 itself, has largely been described as a mechanism by which tumor cells resist to MTDs (14). However, decreased Bcl-2 has recently been associated with resistance of human ovarian cancer cells to paclitaxel (31). Thus, the role of Bcl-2 in cancer cell resistance to MTDs remains unclear and not as simple as initially thought.

In this work, we studied the mechanisms mediating resistance of human ovarian cancer cells to vinflunine. First, we showed that overexpression of the efflux pump P-gp was only responsible for a small part of the resistance to vinflunine. Second, a specific tubulin subtype composition was observed in resistant cells. Third, we also found that sensitive and resistant cells differ greatly in vinflunine-induced modifications of the microtubule network structure and function. Lastly, we showed that Bcl-2 down-regulation played a role in the resistance to vinflunine. Our results strongly suggest that both microtubules and mitochondria have key roles in human cancer cell sensitivity to vinflunine.

Materials and Methods

Drugs
Stock solutions of vinflunine (Pierre Fabre Oncologie, Toulouse, France), vinblastine (Lilly, Saint Cloud, France), doxorubicin (Dakota, Créteil, France), and verapamil (Sigma, St. Louis, MO) were prepared in distilled water. Stock solutions of paclitaxel (Sigma) and cyclosporin A (Novartis, Basel, Switzerland) were prepared in DMSO (Sigma).

Cell Culture
Culture of human ovarian cancer A2780-wild-type (wt) cells and generation of the A2780 subclone resistant to paclitaxel (A2780-TC1) were achieved as described (31). Briefly, A2780-wt cells were exposed to stepwise increasing concentrations of cyclosporin A (up to 3 μmol/L) to limit P-gp overexpression and of paclitaxel (up to 100 nmol/L). Doubling times, determined by the trypan blue exclusion method (32), were 24 and 33 hours for A2780-wt and A2780-TC1 cells, respectively.

Human full-length Bcl-2 was obtained by reverse transcription-PCR using the primers 5-TTAAGCTTAT-GGCCACACCTGGGAGACAGGT-3 (forward) and 5-CCTCTAGATTCACTTGTGGCCCAGATAGGCACC-3 (reverse) and cloned into pUSE (Upstate Biotechnology, Lake Placid, NY). A construct (pUSE-Bcl2Δ) was made in which 49 amino acids (32–80) in the loop domain were deleted by inverse PCR and replaced with a linker of four alanines. A2780-TC1 subclone was then stably transfected with pUSE-empty vector (A2780-TC1-pUSE cells), pUSE-Bcl2 vector (A2780-TC1-Bcl2 cells), or pUSE-Bcl2Δ vector (A2780-TC1-Bcl2Δ cells) by electroporation as described (31).

Exponentially growing cells (3 × 10^4/cm²) were seeded 24 hours before drug treatment. For experiments on A2780-TC1 cells, cyclosporin A was maintained in the medium at 3 μmol/L.

Growth Experiments
Cells were seeded in 96-well plates and incubated with the drugs during 72 hours. Number of viable cells was estimated with the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay or the ATPlite kit (Perkin-Elmer, Boston, MA) according to our previous works (10, 31). Absorbance was measured at 550 nm with a MR 7-000 plate reader (Dyntech, Denkendorf, Germany) for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and with a Topcount automated luminometer (Perkin-Elmer) for ATPlite experiments.

P-gp Expression
Cells were washed in PBS-1% bovine serum albumin before incubation with the monoclonal phycoerythrin-conjugated anti-P-gp-antibody (clone UIC2, Coulter Immunotech, Hamburg, Germany) for 30 minutes in the dark. Negative control was done with a phycoerythrin-conjugated secondary antibody (Jackson Immunoresearch, Baltimore, MD). Cells were then washed twice in PBS-1% bovine serum albumin before analysis by flow cytometry (FACScan, BD Biosciences, San Jose, CA).

Rhodamine 123 Uptake
Cells were incubated for 10 minutes at 37°C with various concentrations of verapamil or cyclosporin A before incubation with 0.1 μmol/L rhodamine 123 in PBS-0.2% bovine serum albumin for 1 hour at 37°C. Cells were then transferred onto ice, washed twice at 4°C, and fluorescence of accumulated rhodamine 123 was measured by flow cytometry (FACScan, BD Biosciences).

Western Blotting
Cell lysis, protein separation, and visualization were done as described (13). The primary antibodies were directed against Bcl-2 (clone 124, DakoCytomation, Glostrup, Denmark), anti-cytokeratin 1A4, Sigma), α-tubulin
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Results

A2780-TC1 Cells Are Highly Resistant to Vinflunine

In A2780-TC1 cells, the IC₅₀ for paclitaxel was 2,000-fold higher than in wt cells (5 and 10,000 nmol/L for A2780-wt and A2780-TC1 cells, respectively), as previously determined (31). For vinflunine, IC₂₀, IC₅₀, and IC₇₀ values reached 500, 20,000, and 55,000 nmol/L, respectively, in A2780-TC1 cells, whereas they were 20, 25, and 30 nmol/L in A2780-wt cells. These results clearly indicated a lower sensitivity of A2780-TC1 cells to vinflunine, illustrated by high resistance factors of 800 and 1,830 for IC₅₀ and IC₇₀, respectively (Table 1). To assess whether this resistance was specific to MTDs, we tested doxorubicin, a DNA-damaging agent, and vinblastine. A2780-TC1 cells were characterized by resistance factors to vinblastine even higher than those measured with vinflunine (Table 1). They also showed a decrease in sensitivity to doxorubicin, but much less than the one observed with MTDs (Table 1).

P-gp Overexpression Is Only Minimally Involved in Vinflunine Resistance

In our experimental conditions, A2780-TC1 cells were grown in the presence of 3 μmol/L cyclosporin A, a broad-spectrum multidrug resistance inhibitor (36). Nevertheless, their resistance to doxorubicin suggested that a multidrug resistance mechanism may be involved. We therefore assessed expression and activity of the P-gp pump. As expected, A2780-wt cells did not express P-gp, in contrast to A2780-TC1 cells (Fig. 1A). However, only 27% of P-gp remained functional in working growth conditions of A2780-TC1 cells (Fig. 1B).

To determine if the remaining P-gp activity in A2780-TC1 cells was responsible for the resistance, we measured the vinflunine-induced inhibition of cell proliferation with 10 μmol/L of verapamil. As well as high concentrations of cyclosporin A (10 or 30 μmol/L), this high concentration of verapamil totally inhibited P-gp, as shown by the maximal incorporation of rhodamine 123 (Fig. 1B). We observed that...
Vinflunine + verapamil

Vinflunine
1,830 to 1,670 for IC70 (Table 1), and it was not modified
(Table 1). Moreover, the resistance factor changed from
verapamil only partially restored A2780-TC1 cell sensitivity
and doxorubicin with or without 10

Table 1. A2780-TC1 cells are highly resistant to vinflunine, only partially through overexpression of P-gp

<table>
<thead>
<tr>
<th></th>
<th>A2780-wt (nmol/L)</th>
<th>A2780-TC1 (nmol/L)</th>
<th>Resistance factor</th>
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<tbody>
<tr>
<td>Vinflunine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50</td>
<td>20 ± 1.73</td>
<td>500 ± 19.1</td>
<td>25</td>
</tr>
<tr>
<td>IC30</td>
<td>25 ± 2.18</td>
<td>20,000 ± 2,135</td>
<td>800</td>
</tr>
<tr>
<td>IC50</td>
<td>30 ± 1.73</td>
<td>55,000 ± 3,940</td>
<td>1,830</td>
</tr>
<tr>
<td>Vinflunine + verapamil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50</td>
<td>20 ± 1.26</td>
<td>40 ± 8.11</td>
<td>2</td>
</tr>
<tr>
<td>IC30</td>
<td>25 ± 0.70</td>
<td>2,000 ± 879</td>
<td>80</td>
</tr>
<tr>
<td>IC50</td>
<td>30 ± 2.48</td>
<td>50,000 ± 4,200</td>
<td>1,670</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.03 ± 0.004</td>
<td>150 ± 31.7</td>
<td>5,000</td>
</tr>
<tr>
<td>IC30</td>
<td>0.3 ± 0.029</td>
<td>2,500 ± 750</td>
<td>8,330</td>
</tr>
<tr>
<td>IC70</td>
<td>0.7 ± 0.19</td>
<td>55,000 ± 11,500</td>
<td>78,600</td>
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<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC50</td>
<td>6 ± 1.60</td>
<td>100 ± 3.5</td>
<td>17</td>
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<tr>
<td>IC30</td>
<td>25 ± 2.08</td>
<td>400 ± 37.9</td>
<td>16</td>
</tr>
<tr>
<td>IC70</td>
<td>60 ± 13.2</td>
<td>3,000 ± 115</td>
<td>50</td>
</tr>
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NOTE: Exponentially growing cells were treated with vinflunine, vinblas-
tine, and doxorubicin with or without 10 µmol/L verapamil for 72 hours. IC50, IC30, and IC50 values (±SE) were graphically determined from at least three independent experiments for each drug and each cell line. Resistance factors were determined as ratio of IC values in A2780-TC1 cells / IC values in A2780-wt cells.

*P < 0.01; **P < 0.001.

Verapamil only partially restored A2780-TC1 cell sensitivity (Table 1). Moreover, the resistance factor changed from 1,830 to 1,670 for IC70 (Table 1), and it was not modified when higher concentrations were tested (data not shown). Thus, P-gp overexpression plays a role only for the lowest vinflunine concentrations, with a maximal 12-fold decrease in A2780-TC1 sensitivity. Even if the efflux pump participates in cancer cell resistance to vinflunine, it clearly appears that it was not the main cause. In short, other mechanisms are likely to be involved in drug resistance of A2780-TC1 cells.

Tubulin Subtype Distribution Pattern Is Modified in A2780-TC1 Cells

Modifications of the drug target are an important source of drug resistance. In A2780-TC1 cells, the cross-resistance between taxanes and Vinca alkaloids suggested that tubulin can be altered. Therefore, we investigated tubulin post-translational modifications and isotype expression levels. We first noticed that total α-tubulin and β-tubulin levels were identical in A2780-wt and A2780-TC1 cells (Fig. 2A). Similarly, there was no significant difference in β and βIV isotypes (Fig. 2C), as well as in deetyrosinated and tyrosinated α-tubulin (Fig. 2B). In sharp contrast, the acetylated α-tubulin, βII-tubulin, and βIII-tubulin expression levels were 2.24 ± 0.69-fold, 2.47 ± 0.54-fold, and 1.69 ± 0.29-fold increased in A2780-TC1 cells, respectively (Fig. 2B and C). These differences between A2780-wt and A2780-TC1 cells were statistically significant (P < 0.05). Reverse transcription-PCR experiments showed that this increase in βII-tubulin and βIII-tubulin isotypes in resistant cells was associated to an increase at the transcriptional level (factors of 2.5 and 2.1, respectively; P < 0.05; Fig. 2D). Thus, the distribution pattern of tubulin subtypes is specific for A2780-TC1 cells, suggesting that modifications in MTD target could be involved in vinflunine resistance.

Vinflunine Unequally Affects Microtubule Network Organization and Function in A2780-TC1 and A2780-wt Cells

Our observation that the cellular tubulin subset was modified in resistant cells raised the possibility that the microtubule network was also altered in response to vinflunine treatment. We observed that the microtubule network morphology did not initially differ between the two cell lines (Fig. 3). After 6 hours of treatment with a high concentration of vinflunine (20,000 nmol/L), tubulin aggregation in paracrystals appeared in both sensitive and resistant cells (Fig. 3). In agreement with P-gp experiments, it suggested that intracellular accumulation of vinflunine was similar in the two cell lines when high extracellular concentrations were used. Interestingly, 500 nmol/L vinflunine induced microtubule depolymerization in A2780-wt cells (Fig. 3A), whereas it did not modify the microtubule network in A2780-TC1 cells (Fig. 3B). Thus, differences in microtubule sensitivity to vinflunine appeared as early as 6 hours of treatment.

Microtubule network modifications by MTDs generally lead to disturbances in cell cycle progression. The distribution of cells among the different phases of the cell cycle was not significantly modified in A2780-wt cells after a 24-hour treatment with IC50 and IC70 of vinflunine. At
500 nmol/L, vinflunine caused a massive G2-M block (59%), corresponding to a mitotic block illustrated by the chromosome alignment at the metaphasic plate (Fig. 4A, DAPI). In contrast, in A2780-TC1 cells, 500 nmol/L vinflunine did not induce any modification in the cell cycle. At higher concentrations (IC50 and IC70), a G2-M block was observed in A2780-TC1 cells (Fig. 4B). Surprisingly, this blockage did not correspond to a mitotic block but to a G2 arrest, as cells contained intact nuclei with a noncondensed chromatin (Fig. 4B, DAPI). It could be attributed to tubulin paracrystal formation, induced at 6 hours of treatment (Fig. 3B) and persisting after 33 hours (data not shown), which probably hindered mitotic spindle formation. For longer time of treatment (72 hours), most of the A2780-TC1 cells was still blocked in G2 phase, whereas 20% of cells were in a polyploid state (Fig. 4C) and multinucleated (data not shown). Then, in the resistant cells, the antiproliferative effects of vinflunine are different from those observed in the sensitive cells.

Lastly, we examined whether P-gp activity in resistant cells was implicated in these differences. Similar experiments were done after complete inhibition of P-gp (10 μmol/L verapamil) in A2780-TC1 cells treated with 500 nmol/L vinflunine (IC20). In these conditions, cells accumulated in G2-M phase (Fig. 4D), indicating that P-gp inhibition was sufficient to increase vinflunine effect on cell cycle progression. Nevertheless, nuclear visualization revealed that this G2-M block still corresponded to a G2 arrest (Fig. 4D, DAPI).

Altogether, the differences observed between the two cell lines are the expression of a smaller effect of vinflunine on microtubule organization and function in resistant cells. Even if P-gp is involved in the mechanism of resistance to low concentrations of vinflunine, the microtubule network function is differentially modified by the drug, leading to G2 arrest and mitotic arrest in A2780-TC1 and A2780-wt cells, respectively.

**Bcl-2 Down-Regulation Is Associated with Vinflunine Resistance in A2780-TC1 Cells**

The apoptosis regulator Bcl-2 is consistently down-regulated in A2780-TC1 cells with respect to A2780-wt cells (Fig. 5A). Interestingly, this phenomenon has been related to paclitaxel resistance of A2780-TC1 cells (31). In this study, we hypothesized that Bcl-2 down-regulation could participate in the strong resistance of A2780-TC1 cells to vinflunine. Therefore, A2780-TC1 cells were stably transfected with a pUSE-Bcl2 construct (A2780-TC1-Bcl2 cells), restoring the Bcl-2 expression level. It led to a significant increase in A2780-TC1 cell sensitivity to vinflunine (Fig. 5B) because the IC50 value decreased by 80% as compared with the pUSE-empty vector transformed cells. Interestingly, expression of the loop-deleted Bcl-2 in A2780-TC1 cells was not as effective as expression of the entire Bcl-2 in restoring vinflunine sensitivity (Fig. 5B).

Preferentially bound to mitochondria, Bcl-2 is absent from mitochondria isolated from A2780-TC1 cells but strongly bound to mitochondria isolated from A2780-wt cells (Fig. 5C). As Bcl-2 regulates the mitochondrial ΔΨm, we evaluated the eventual effect of such variations on

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**Figure 2.** A2780-TC1 cells exhibit a specific distribution pattern of tubulin subtypes. Western blot analysis of tubulin subtypes was done on whole-cell lysates. Antibodies were directed against total α-tubulin and β-tubulin (A), α-tubulin posttranslational modifications (B), and β-tubulin isotypes (C). Expression of actin was used as a loading control. Representative of three independent experiments. D, αI-tubulin and αII-tubulin isotypes were analyzed by reverse transcription-PCR. β-Tubulin isotypes (mRNA) were normalized to β2-microglobulin transcript. Columns, mean of four independent experiments; bars, SE. *, *P < 0.05.

**Figure 3.** Vinflunine induces microtubule depolymerization only in A2780-wt cells, but formation of paracrystals in both subclones. A2780-wt cells (A) and A2780-TC1 cells (B) were treated for 6 h with vinflunine. Microtubules were visualized by fluorescent microscopy (bar, 10 μm). Higher magnifications (×4) have been added for each condition. Representative of three independent experiments.
Figure 4. Vinflunine induces mitotic block in A2780-wt cells and G2 block following by polyploidy in A2780-TC1 cells. A, B, and D, exponentially growing cells were incubated with vinflunine alone (A and B) or with 10 μmol/L verapamil (D) for 24 and 33 h for A2780-wt cells (A) and A2780-TC1 cells (B and D), respectively. Top, DNA content measured by flow cytometry. Percentages of G2-M arrested cells (G2-M) and polyploid (Poly) cells are indicated. Bottom, DAPI staining visualized by fluorescence microscopy (bar, 10 μm). Percentage of cells in mitosis (M) is indicated. C, exponentially growing A2780-TC1 cells were incubated with vinflunine for 72 h. DNA content was measured by flow cytometry. Percentage of polyploid (Poly) cells is indicated. Representative of three independent experiments.

After a 24-hour treatment with vinflunine, we detected the hyperpolarization of mitochondrial membranes in A2780-wt cells (data not shown). Because the ΔΨm increase is transient and is generally followed by a loss in ΔΨm (37), we investigated whether it could be disrupted later. After 48 hours of treatment, hyperpolarization was emphasized in A2780-wt cells and, in parallel, depolarization increased in a dose-dependent manner, as shown by the massive decrease in 3,3'-dihexyloxacarbocyanine iodide uptake (Fig. 6A). In A2780-TC1 cells, we noticed a weak hyperpolarization after 24 hours of vinflunine treatment, only for highest concentrations (data not shown). Moreover, this hyperpolarization of mitochondrial membranes was maintained at 48 hours and no depolarization occurred (Fig. 6B). Cyclosporin A did not perturb the mitochondrial ΔΨm as we obtained similar results on A2780-wt cells in presence of the P-gp inhibitor (data not shown), ruling out the possibility that cyclosporin A could be responsible for the differences observed between the two cell lines.

To further confirm the role of Bcl-2 in the effectiveness of vinflunine, we did 3,3'-dihexyloxacarbocyanine iodide measurements after a 48-hour treatment in A2780-TC1-Bcl2 cells. We showed that restoration of Bcl-2 expression levels allowed vinflunine to partially disturb mitochondrial membranes, as indicated by their massive hyperpolarization for all the concentrations and their depolarization for the highest (Fig. 6C).
From all these data, we highlight the role of Bcl-2 down-regulation in ovarian cancer cell resistance to vinflunine. Integrity of mitochondria was affected by the drug only in cells expressing Bcl-2, and down-regulation of Bcl-2 inhibited vinflunine-induced permeabilization of mitochondria.

Discussion
In the current study, we studied the response to vinflunine of ovarian cancer cells already resistant to paclitaxel (31). MTD effectiveness is generally correlated with a combination of effects on microtubule network functions and cell signaling. We show that variations in tubulin subtype levels and Bcl-2 down-regulation were both involved in the acquired resistance of A2780-TC1 cells to paclitaxel. Interestingly, these modifications had important consequences on cell sensitivity to the following vinflunine treatment.

Whereas vinflunine inhibited A2780-wt cell growth in a range of nanomolar concentrations, micromolar concentrations were required for induction of similar effects in A2780-TC1 cells. However, ovarian cancer cells were far less resistant to vinflunine than to vinblastine. These results support previous data on vinorelbine (15), indicating that vinflunine is a less potent inducer of resistance than are other Vinca alkaloids.

Vinflunine belongs to the P-gp-associated multidrug resistance group of antitumor agents (16). In our experimental conditions (3 µmol/L cyclosporin A), P-gp activity was considerably decreased (by 70%) and P-gp involvement was limited to low concentrations of vinflunine, allowing the study of the other mechanisms of resistance generally hidden behind P-gp overexpression.

In contrast with previous studies describing modifications of tubulin induced during MTD treatment, our work associates vinflunine resistance to preexisting modifications in tubulin composition. Especially, we showed that acquisition of the resistance to paclitaxel (in A2780-TC1 cells) has been accompanied by an increase in the expression level of βIII-tubulin isotype. This observation is in agreement with various works that have correlated βIII isotype overexpression and decreased sensitivity to paclitaxel (20, 25, 27–29, 38), even in the clinics (39). Surprisingly, although high βIII expression has been related to microtubule destabilization in tumor cells (20, 28, 40), it has also been described as predictive of resistance to vinorelbine in patients (41). Similarly, the increase in βIII-tubulin may contribute to the resistance of A2780-TC1 cells to vinflunine. This increase is associated to an increase at the transcriptional level, in the same range as that described in the literature (20, 27, 30, 33). A2780-TC1 cells also contained more acetylated α-tubulin. As tubulin acetylation is associated with microtubule stability (22, 24), it could,
more likely than βII-tubulin increase, contribute to resistance to the microtubule-depolymerizing agent vinflunine. This stabilization of microtubules is only related to acetylation of α-tubulin as detyrosinated α-tubulin level is the same between sensitive and resistant cells. Lastly, the most pronounced tubulin isotype modification, induced by the paclitaxel resistance acquisition, concerns the βII isotype. Its increase in A2780-TC1 cells could participate in cell resistance to vinflunine. However, whether changes in expression levels of βII-tubulin modulate cell sensitivity to MTDs remains unclear (30, 41). Altogether, our results indicate that the distribution pattern of tubulin subtypes is specific in A2780-TC1 cells. It should also be noticed that other modifications of the tubulin/microtubule system could have occurred during the acquisition of paclitaxel resistance, and thus influenced vinflunine treatment. In particular, tubulin mutations are a common mechanism of resistance to both microtubule-stabilizing and microtubule-depolymerizing agents (18, 21, 42, 43). Such mutations in tubulin can affect microtubule functions and binding of microtubule-associated proteins, thus disturbing microtubule stability (18, 20, 21, 42, 43).

Because tubulin isotype composition may be a determinant factor of microtubule functions (15, 20, 25–30, 38), it is conceivable that the changes in expression levels of tubulin subtypes in A2780-TC1 would alter the manner in which vinflunine targets microtubules. We showed that vinflunine differently affects both microtubule network organization and function in A2780-TC1 and A2780-wt cells. A same concentration of vinflunine induced microtubule depolymerization and cell cycle arrest in A2780-wt cells while having no effect on A2780-TC1 cells. Thus, differences in whole-cell sensitivity to vinflunine seem to reflect the drug effect on the microtubule network. Interestingly, when a G2-M blockage was triggered in A2780-TC1 cells, it did not correspond to a classic mitotic arrest, as in A2780-wt cells, but rather to an accumulation of cells in G2 phase. The following cell cycle led to the formation of multinucleated cells, indicating that mitosis was not successfully completed. Thus, vinflunine exhibits antimitic properties on A2780-wt cells as well as A2780-TC1 cells, but they do not lead to the same disturbances of cell cycle progression. Because tubulin composition modulates microtubule dynamics (20, 25, 28, 40), the specific distribution pattern of tubulin subtypes in A2780-TC1 cells may be responsible for the specific effects of vinflunine on the mitotic spindle function. Thus, even if it remains difficult to define the participation of each particular subtype (44), the global change in tubulin composition likely contributes to the observed resistance profile.

Mitochondria are the crossroads for intracellular signaling pathways induced by MTDs (10, 12–14, 45, 46). Inactivation of Bcl-2, through hyperphosphorylation (47), leads to mitochondrial membrane permeability and apoptosis triggering by MTDs, including vinflunine (10, 11). A relationship has been established between Bcl-2 overexpression and clinical resistance to MTDs (48). Unexpectedly, Bcl-2 recently seemed to be required for paclitaxel-mediated cytotoxicity in various ovarian cancer cell lines (31). Similarly, paclitaxel induced apoptosis in prostate cancer cells expressing Bcl-2, whereas the cells lacking Bcl-2 were resistant to the drug (49). Low Bcl-2 expression levels in patients were also associated with resistance to paclitaxel chemotherapy (31). The role of Bcl-2 in the anticancer activity of microtubule-depolymerizing agents had never been fully evaluated. In this study, we provide evidence that vinflunine effectiveness was largely decreased in the absence of Bcl-2 whereas Bcl-xL and Bax expression were unchanged (31), and that it was in part restored by reintroducing Bcl-2. Accordingly, Bcl-2 down-regulation prevented the collapse of the mitochondrial potential induced by vinflunine. Maintained hyperpolarization of the mitochondrial membrane potential in A2780-TC1 cells could be responsible for inhibition of cell proliferation without apoptosis induction by vinflunine (data not shown), as previously proposed for paclitaxel (13). In addition, the increase in vinflunine effect on mitochondria in A2780-TC1-Bcl2 cells confirmed the key role of Bcl-2 in vinflunine mechanism of action. Thus, differences in sensitivity to vinflunine measured at the cellular level, depending on Bcl-2 expression level, reflect the differences of action of vinflunine observed at the mitochondrial level. Added to the fact that Bcl-2 expression is correlated with paclitaxel resistance, our results support the hypothesis that Bcl-2 is necessary on mitochondria for maximal effectiveness of MTDs. They could explain why the link between Bcl-2 and drug susceptibility remains controversial and strongly suggest that the role of Bcl-2 as a predictor of chemotherapy response should be reexamined.

Like other MTDs (32, 34, 50, 51), vinflunine directly affects isolated mitochondria,4 leading to the release of apoptogenic factors. Bcl-2 has been described as a mitochondrial target for paclitaxel (31, 52), and a model for the interaction of paclitaxel with the Bcl-2 loop domain has been proposed (53). Whether other MTDs, including vinflunine, can bind to Bcl-2 remains unknown. Considering the role played by Bcl-2 in cell sensitivity to vinflunine, one may think that vinflunine could affect mitochondria by binding Bcl-2. Vinflunine might interact with Bcl-2, in part, through the loop domain, as A2780-TC1-Bcl2Δ are less sensitive than A2780-TC1-Bcl2, whereas Bcl-2 association with mitochondria is the same in its entire or loop-deleted form.4 Lastly, we showed that tubulin was present on mitochondrial membranes from A2780 cells, and Bcl-2 has been shown to specifically bind both mitochondrial tubulin and voltage-dependent anion channel (14, 31, 35). Such a protein complex could regulate the direct initiation by MTDs of the mitochondrial signaling pathway.

To conclude, our results support a role for both target (microtubules) and signaling (mitochondria) in human cancer cell sensitivity to vinflunine. Because diverse factors are generally responsible for the emergence of resistant

4 Personal data.
tumor cells in patients, the A2780-TC1 subclone seems to be a valuable in vitro tool to study mechanisms of multifactorial resistance. By analyzing the vinflunine response of paclitaxel-resistant cells, this study also illustrates the complexity of overcoming resistance to anticancer drugs and shows that sequential combinations of MTDs in the clinics should be carried out with caution.

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References


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