Apoptosis as a Determinant of Tumor Sensitivity to Topotecan in Human Ovarian Tumors: Preclinical in Vitro/in Vivo Studies

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ABSTRACT
Preclinical and clinical studies have documented the pharmacological interest in camptothecin derivatives in the treatment of resistant tumors. In particular, topotecan, a water-soluble derivative, exhibited promising activity in pretreated ovarian carcinoma. The present study investigated the pattern of tumor response in two human ovarian carcinoma xenografts and in their cisplatin-resistant sublines characterized by different mechanisms of drug resistance. In IGROV-1/Ptl cells, cisplatin resistance has been ascribed to a reduced susceptibility to apoptosis as a consequence of p53 mutation and inactivation of its function. In the A2780 cisplatin-resistant subline, which retained the wild-type p53 gene status, the development of resistance has been possibly related to increased cell ability to repair drug-induced DNA damage. The in vivo results of the present study showed that topotecan could overcome the resistance in A2780/CP but not in IGROV-1/Ptl tumor xenografts. The pattern of tumor response following in vivo topotecan treatment correlated with drug ability to induce apoptosis but not with its in vitro antiproliferative activity. The antitumor efficacy of topotecan in the four tumors reflected a different cell response to drug-induced DNA damage, as suggested by different perturbations of cell cycle progression. Indeed, only in the subline refractory to topotecan in vivo, IGROV-1/Ptl, did we observe a persistent arrest of the cells in the S-phase, resulting in a cytostatic and not a cytotoxic effect, since a low level of apoptosis was induced by the drug. In conclusion, the current results support that determination of drug-induced apoptosis is a useful predictor of tumor response to topotecan in ovarian carcinomas and suggest that p53 gene status may be a critical determinant of cell response to topoisomerase inhibitors.

INTRODUCTION
Camptothecins are cytotoxic agents known to be DNA topo I inhibitors (1). The antitumor activity found in preclinical models (2, 3) was confirmed in clinical studies (4). Topotecan (10-hydroxy-9-dimethylaminomethyl-(S)-camptothecin) is a water-soluble camptothecin analogue currently under clinical investigation. It has been recently approved for ovarian carcinoma treatment based on the results of a Phase III study in cisplatin-pretreated patients (5). A lack of cross-resistance between topotecan and cisplatin in ovarian tumors had already been documented in Phase II clinical studies (6). We previously showed that topotecan is extremely active against i.p. growing human ovarian tumor xenografts, including cisplatin-resistant tumors (7).

The cellular basis of sensitivity to topo I inhibitors remains unclear. A better understanding of the critical events involved in drug-induced cytotoxicity may have clinical implications for optimization of therapy with topo I inhibitors and rational development of effective drug combinations. Although multiple factors may contribute to the development of drug resistance of ovarian carcinoma cells, resistance to apoptosis induction has been proposed as a critical mechanism of drug resistance. Indeed, apoptosis is a major mode of cell death in response to drug treatment (8, 9), and a correlation between apoptotic death and chemosensitivity has been documented in preclinical studies with ovarian carcinomas (10). In this regard, the status of p53, a gene involved in cell cycle control and regulation of apoptosis (11, 12), has been implicated as a critical determinant of the response of ovarian cancer to cisplatin (13, 14).

The aim of the present investigation was to evaluate the cytotoxic and antitumor activity of topotecan against four human ovarian tumor cell lines and their corresponding tumor xenografts that exhibit differential response levels and different mechanisms of resistance to cisplatin. In IGROV-1/Ptl, the development of drug resistance has been ascribed to p53 mutation, resulting in a reduced cell ability to activate the apoptotic response (13). In contrast, A2780/CP retained a wild-type p53 gene status. The cellular basis of reduced sensitivity of the latter line to cisplatin is likely related to increased cell ability to repair drug-induced DNA damage, since high levels of topo I expression were found (7).

Our results in cell cultures showed a marginal cross-resistance to topotecan in the A2780 cisplatin-resistant cell line and not in the IGROV-1/Ptl cells. However, the pattern of in vitro
Topotecan and Apoptosis Induction

cell response did not reflect the in vivo tumor response, since only the A2780 tumor xenograft selected for resistance to cisplatin exhibited lack of cross-resistance to topotecan. In contrast, in the IGROV-1/Pt1 tumor, complete cross-resistance to topotecan was observed in vivo. These cells, carrying a mutant p53 gene, were not susceptible to topotecan-induced apoptosis, suggesting a major role for the p53-dependent pathway of apoptosis in topotecan efficacy in ovarian tumors.

MATERIALS AND METHODS

Drugs. Topotecan (Smith-Kline Beecham Pharmaceutical, Reigate, Surrey, United Kingdom) was dissolved in water, and cisplatin (Bristol-Meyers Squibb, Wallingford, CT) was dissolved in saline immediately before use.

Human Tumor Cell Lines and Cytotoxicity Studies. The IGROV-1 and the A2780 cell lines originated from ovarian carcinomas of two untreated patients (15, 16). The resistant cell lines, IGROV-1/Pt1 and A2780/CP, were selected in our laboratory in the presence of increasing cisplatin concentrations (13). All of the cell lines were cultured in RPMI 1640 (Biowhittaker, Verviers, Belgium) containing 10% FCS (Life Technologies, Inc., Gaithersburg, MD). The resistant sublines exhibited karyotypic features similar to those of the parental cell lines, but a somewhat reduced proliferation rate (doubling times 28 ± 2 versus 33 ± 1 h in the IGROV-1 cell systems and 20 ± 1 versus 22 ± 1 in the A2780 cell systems). This change was also reflected in in vivo growth (see below).

For cytotoxicity studies, cells (5 × 10⁴/ml) were seeded in 6-well plates (Costar Corporation, Cambridge, MA) and after 24 h were treated for 1 h with the drugs. Cell growth was evaluated at 72 h after treatment by cell counting (Coulter Electronics Ltd., Luton, United Kingdom). The results are expressed as IC₅₀ values (drug concentration inhibiting 50% of cell growth) and are the means of at least three independent experiments.

In Vivo Studies. Six- to 10-week-old female athymic Swiss nude mice were used in the study. Animals, obtained from Charles River Italy (Calcio, Italy), were maintained in laminar air-flow rooms. Sterilized cages, bedding, and acidified water were used for mouse care. The air was kept at a temperature of 24–26°C with 50% humidity. The experiments were approved by the Institutional Committee for Animal Experimentation. Because the IGROV-1/Pt1 tumor did not grow in the ascitic form (7), s.c. growing tumor models were used to allow a direct comparison of response to in vivo treatment among the four tumor systems. Previous studies indicated a parallelism of tumor responsiveness between s.c. and i.p. growing ovarian carcinoma models (7).

All tumor lines originated from s.c. injection of 5–10 × 10⁶ cells/flank of mice. For line maintenance and experimental purposes, tumor fragments were grafted s.c. into both flanks of athymic mice by a 13-gauge trocar. Growth of s.c. tumors was followed by biweekly caliper measurement of tumor length and width. Tumor growth (TW) was calculated in milligrams using the formula: TW = width² × length/2 according to Geran et al. (17). For chemotherapy studies, each experimental group consisted of at least eight assessable tumors. Drugs were delivered i.v. in a volume of 10 ml/kg of body weight. Two different treatment schedules were used, i.e., every 7 days for 3 times (q7d×3) or every 4 days for 3 times (q4d×3), which in our experience represent the optimal schedules for cisplatin and topotecan, respectively. Treatments started with just measurable tumors (50–70 mg). The effects of drug treatments were expressed at a specified day (see Table 1) as percentage of TW in treated versus control mice, calculated as: 100 – mean TW in treated/mean TW in control. Moreover, the LCK produced by the treatment was calculated from the days the tumors took to reach a mean specified weight (see Table 1) in treated and control mice, according to the formula: days in treated – days in control/3.32 × tumor doubling time (calculated by linear regression analysis of control tumor growth).

No control mouse died because of tumor burden in the experimental frame. Death of treated mice was ascribed to lethal toxicity and was recorded throughout all of the experimental frame.

Statistical comparison between topotecan-treated versus cisplatin-treated mice was evaluated by Student’s t test (two tailed).

Biochemical Studies. The GSH level and GST activity were assessed by processing fragments from the four tumor lines as described previously (18). The GSH level was analyzed according to Tietze (19), and results were expressed as nmol/mg protein. GST activity was assayed according to Habig et al. (20) and was expressed as nmol/min/mg protein.

Analysis of p53 Gene. Single-strand conformation polymorphism analysis was performed as described previously (21), for exons 5–9, because they are localized in the region of the p53 gene most often mutated. PCR-amplified exons were also subjected to direct DNA sequencing with an Amplicycle Sequencing kit (Perkin-Elmer Corp., Branchburg, NJ). Each sequencing reaction was performed at least twice, analyzing separate amplifications. The results for IGROV-1 and IGROV-1/Pt1 cells have been already reported (13). As shown in Fig. 1, no mobility shift, for all of the exons tested, was observed for A2780 and A2780/CP cells in comparison to the human cervical carcinoma cell line (SiHa) carrying a wild-type p53 gene (22). To confirm the wild-type status of exons 5–9, all of them were subjected to direct DNA sequencing. No mutations were revealed.

Cellular Studies. Apoptosis was assessed by fluorescence microscopy as already described (13). Briefly, ethanol-fixed cells were stained with propidium iodide solution (50
Table I: Efficacy of systemic topotecan on s.c. growing human ovarian tumor xenografts.

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Treatment schedule (1st day)</th>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>% TWI (day)</th>
<th>Toxic/total</th>
<th>LCK</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>q7d × 3 (3)</td>
<td>Topotecan</td>
<td>7.5</td>
<td>79 (24)</td>
<td>0/4</td>
<td>1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(1.9 ± 0.5)</td>
<td>Topotecan</td>
<td>10</td>
<td>89 (24)</td>
<td>0/5</td>
<td>2.1</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>6</td>
<td>99 (24)</td>
<td>0/5</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Topotecan</td>
<td>10</td>
<td>85 (24)</td>
<td>0/5</td>
<td>1.5</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>A2780/CP</td>
<td>(3.1 ± 0.9)</td>
<td>Topotecan</td>
<td>7.5</td>
<td>55 (21)</td>
<td>0/5</td>
<td>1</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Topotecan</td>
<td>10</td>
<td>75 (21)</td>
<td>0/5</td>
<td>1.55</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>6</td>
<td>61 (24)</td>
<td>1/5</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGROV-1</td>
<td>q4d × 3 (6)</td>
<td>Topotecan</td>
<td>7.5</td>
<td>39 (34)</td>
<td>1/4</td>
<td>0.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>(3.5 ± 0.9)</td>
<td>Topotecan</td>
<td>10</td>
<td>85 (34)</td>
<td>1/4</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>5</td>
<td>23 (34)</td>
<td>1/4</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGROV-1/PtI</td>
<td>q4d × 3 (15)</td>
<td>Topotecan</td>
<td>7.5</td>
<td>66 (21)</td>
<td>1/5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(5.1 ± 1.5)</td>
<td>Cisplatin</td>
<td>5</td>
<td>23 (34)</td>
<td>1/4</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Doubling time of control tumor, calculated by linear regression analysis of the growth curve.

Table 2: Sensitivity of ovarian tumors to cisplatin and topotecan: comparison of in vitro and in vivo results.

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Optimal TWI (%) induced by Cisplatin</th>
<th>Topotecan</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>99</td>
<td>89</td>
<td>1.4</td>
</tr>
<tr>
<td>A2780/CP</td>
<td>61</td>
<td>85</td>
<td>4.3</td>
</tr>
<tr>
<td>IGROV-1</td>
<td>66</td>
<td>75</td>
<td>26.0</td>
</tr>
<tr>
<td>IGROV-1/PtI</td>
<td>23</td>
<td>39</td>
<td>226</td>
</tr>
</tbody>
</table>

* Data from Table 1.

Table 3: Biochemical and molecular characterization of human ovarian tumor lines.

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>topo I expression*</th>
<th>GSH content b (nmol/mg protein)</th>
<th>GST activity b (nmol/min/mg protein)</th>
<th>p53 status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>0.083</td>
<td>7.2 ± 3.3</td>
<td>188 ± 71</td>
<td>Wild-type</td>
</tr>
<tr>
<td>A2780/CP</td>
<td>0.150</td>
<td>38 ± 0.02</td>
<td>120</td>
<td>Wild-type</td>
</tr>
<tr>
<td>IGROV-1</td>
<td>0.027</td>
<td>3.9 ± 2</td>
<td>295 ± 59</td>
<td>Wild-type</td>
</tr>
<tr>
<td>IGROV-1/PtI</td>
<td>0.033</td>
<td>17.9 ± 0.8</td>
<td>278</td>
<td>Mutant</td>
</tr>
</tbody>
</table>

* By Northern blot analysis. Values were normalized to g-actin (from Ref. 7).

RESULTS

The effects of topotecan and cisplatin on the tumor growth of two human ovarian carcinoma xenografts, IGROV-1 and A2780, and their cisplatin-resistant variants are reported in Table 1. Cisplatin was more toxic when given according to an “every 4 days” compared to an “every 7 days” schedule (5- and 6-mg/kg/injections were the respective maximal tolerated doses). Conversely, no differences in the maximal tolerated doses were observed for topotecan according to the two treatment schedules investigated. Cisplatin, given according to a weekly schedule, was very effective against the A2780 tumor, almost completely inhibiting tumor growth (99% TWI), and much less effective against the A2780/CP variant (61% TWI and LCK <1). The two doses (including the maximum tolerated dose) of topotecan investigated in the study were active on both tumor xenografts, causing comparable LCK and tumor growth inhibition. Against the parent IGROV-1 tumor, which was moderately responsive to cisplatin (66% TWI and LCK of 1.3), topotecan was somewhat more effective than cisplatin (75% TWI and LCK of 1.6), although the difference was not significant. In
contrast, both drugs were inactive against the resistant IGROV-1/Pt1 tumor, as documented by marginal effects on tumor growth and LCK. Therefore, from these in vivo results, topotecan showed cross-resistance to cisplatin in the IGROV-1/Pt1 but not in the A2780/CP resistant tumor line.

These findings did not reflect the pattern of antiproliferative activity of the two drugs in these cell lines in vitro, where the cytotoxic effect of topotecan was similar in IGROV-1 and IGROV-1/Pt1 cells (Table 2). Conversely, in the A2780/CP cell system an appreciable resistance to topotecan was observed (resistance index, 5).

Different mechanisms of resistance are likely to be involved in the cisplatin-resistant phenotype of these cells. Our results showed that the cellular response to topotecan was not
related to p53 status. In addition, the pattern of cell response to the drug was not explained by topo I expression, nor by GSH content or GST activity, since no correlation was found between topotecan IC_{50} and these parameters (Table 3).

Since p53 status may influence the apoptotic response (12), we investigated the induction of apoptosis after topotecan treatment in the four cell lines (Fig. 2). The level of basal apoptosis in control cells never exceeded 10% in all of the cell lines. At 24, 48, and 72 h after treatment, equitoxic concentrations of topotecan (IC_{50} and IC_{80}) induced a similar increase in apoptosis in the A2780 and A2780/CP cells. In contrast, topotecan was a potent inducer of apoptosis only in IGROV-1 but not in IGROV-1/Pt1 cells (85% versus 20% cells in apoptosis at the respective IC_{50}). Twenty-four and 48 h after topotecan treatment, lower levels of apoptosis induction were observed in all of the cell lines compared to the levels observed after 72 h. Apoptosis induction was dose and time dependent in the four ovarian carcinoma cell lines.

In an attempt to find a cellular basis for differential induction of apoptosis in the examined cell systems, perturbations of cell cycle progression were analyzed. The effects of equitoxic concentrations (IC_{80}) of topotecan on cell cycle distribution are presented in Fig. 3. The flow cytometric profiles in IGROV-1 and IGROV-1/Pt1 cells were quite different at all of the examined time points (from 24 to 72 h after treatment): in the parental cells, a minimal modification of the distribution along the cell cycle was observed, with a reduction of 30–40% of cells in G_{1} and an increase of cells in the S-phase and G_{2}. Conversely, in the resistant cells, G_{1} disappeared almost completely with an increase in the S-phase and, to a lesser extent, in G_{2}. A differential cell cycle perturbation after topotecan treatment (IC_{80}) was observed also in the two A2780 cell systems. No modifications were observed in the A2780 cell cycle, whereas in A2780/CP a decrease in G_{1} and an increase in G_{2} were observed up to 48 h. At 72 h, cell cycle distribution was similar to that of untreated cells.

**DISCUSSION**

Preclinical and clinical evidence supports the pharmacological interest of topo I inhibitors in the treatment of cisplatin-resistant tumors. A relevant mechanism of cisplatin resistance has been ascribed to increased DNA repair. Indirect evidence suggests a contribution of topo I in the repair mechanism of DNA-damaging agents, including cisplatin and ionizing radiations (23, 24). Indeed, overexpression of the DNA topo I gene has been reported in cisplatin-resistant cells (7, 25). In addition, potentiation of cisplatin cytotoxicity by 9-aminocamptothecin was found to be associated with persistence of specific cisplatin-induced DNA lesions (26). Comparing topotecan antitumor activity in cisplatin-sensitive and -resistant human ovarian tumor xenografts, the present study showed lack of cross-resistance in one of the two cisplatin-resistant tumors, A2780/CP. High responsiveness of this tumor to topotecan treatment, including cured mice, has already been reported in a study dealing with local-regional treatment of the i.p. growing xenograft (7).
The lower antitumor efficacy achieved by i.v. topotecan in the present study (no cured mice were observed) indicated a more favorable pharmacological profile for local-regional versus systemic treatment with the drug. Such an observation may be explained by a short half-life of the lactone form of the molecule, which is known to be the active form (27). A clinical trial of i.p. topotecan in ovarian carcinoma patients is suggested by these results.

In an attempt to establish a relationship between the efficacy of topo I inhibitors in cisplatin-resistant tumors and cellular and molecular bases, we have chosen two tumor cell systems characterized by different mechanisms of drug resistance. In both systems, resistance to cisplatin was associated with resistance to apoptosis induction. In IGROV-1/Ptl cells, a markedly reduced ability to activate apoptosis (evident at equitoxic drug levels) has been related to inactivation of p53 function following gene mutation in both alleles (13). In contrast, a reduction of apoptotic response in A2780/CP cells following exposure to cisplatin apparently did not reflect an inactivation of p53 function due to mutation of the hot spot region. In these cell lines growing in vitro, a complete lack of cross-resistance has been documented only in IGROV-1/Pt1 cells using an antiproliferative test to evaluate the pattern of cell response after a short-term exposure to topotecan (1 h). However, the pattern of tumor response after in vivo treatment of the same tumor lines xenografted into nude mice did not reflect the relative sensitivity observed in vitro. Indeed, IGROV-1/Pt1 tumor carrying mutant p53 was refractory in vivo to topotecan, whereas A2780/CP tumor exhibited responsiveness comparable to that of the sensitive tumor. Thus, in these models, the relative therapeutic efficacy of topotecan reflects the differential drug ability to activate apoptosis between resistant cells and parental cells (Fig. 2) rather than drug antiproliferative activity. The loss of wild-type p53 function has been correlated with acquired resistance to cisplatin (13). Based on the present findings, the presence of the wild-type p53 gene seems to be required also for topotecan-induced apoptosis and tumor response.

A different cell cycle control following drug exposure in the four cell lines is possibly responsible for the lack of a correlation between the antiproliferative activity and antitumor efficacy of topotecan. Indeed, only minimal and transient perturbations of the cell cycle were observed in the treated cells from responsive tumors, whereas a persistent accumulation in the S-phase was detected in the resistant IGROV-1/Pt1 cells up to 72 h. A markedly different perturbation of the cell cycle following exposure to cisplatin in resistant IGROV-1 cells (with loss of G1 delay) has been ascribed to the lack of WAF-1 expression (13). Therefore, in this cell system the ability to regulate cell cycle progression could result in cytostasis, possibly providing the cell with time required for DNA repair, because a very low level of apoptosis was induced by the drug over 72 h. This observation may have relevant implications, since apoptosis induction by topo I inhibitors could be a more useful predictor of tumor responsiveness than a simple drug-induced reduction of proliferative activity. The role of cell cycle control and apoptosis activation as determinants of sensitivity to topo I inhibitors has been reported for other tumor cells (28).

The current study showed that topotecan may be effective in the treatment of cisplatin-resistant tumors. However, a lack of cross-resistance between cisplatin and topotecan could not be regarded as a general phenomenon, since p53 status may influence the relative sensitivity to both agents, as observed in the IGROV-1-resistant tumor, suggesting that p53 status may be a critical determinant of the sensitivity of ovarian carcinoma to DNA-damaging agents. In addition, this is the first study supporting that level of apoptosis induction may be a useful predictor of tumor responsiveness in vivo. Thus, these results may have implications for the clinical use of topo I inhibitors and for rational development of combinations including these agents.

REFERENCES

Apoptosis as a determinant of tumor sensitivity to topotecan in human ovarian tumors: preclinical in vitro/in vivo studies.

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