Anti-Cancer Drugs 2000, **11,** pp. 471–478

Report

Ex vivo activity of XR5000 against solid tumors

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Topoisomerases I and II unravel DNA during transcription, DNA replication and DNA repair. Inhibitors of both enzymes are important anticancer drugs, but only now are combined inhibitors becoming available for clinical use. In this study we have used an ATP-based chemosensitivity assay to determine the activity of XR5000 and possible combinations against ovarian cancer, a tumor sensitive to current topoisomerase inhibitors, and melanoma, an insensitive tumor. A further six tumors of other types were also tested. The results from 20 ovarian cancer and 18 melanoma biopsies show remarkably little difference between the tumor types in terms of IC_{50}, IC_{90} or two summary indices of chemosensitivity based on all of the concentrations tested. XR5000 on its own shows a steep concentration–response curve in most tumors, only achieving high reduction (above 95%) of ATP levels at 2440 ng/ml (6 μM). The results were often similar to the combination of etoposide and topotecan, particularly at the higher concentrations tested. The combinations with greatest activity in ovarian cancer were with paclitaxel or cisplatin, while melanoma showed greatest improvement with paclitaxel or treosulfan. The results are encouraging for the clinical introduction of this agent, and suggest that it will be effective in combination with currently available drugs for both ovarian cancer and melanoma. [© 2000 Lippincott Williams & Wilkins.]

Key words: ATP, chemosensitivity, chemotherapy, luminescence, topoisomerase, XR5000.

Introduction

Topoisomerase inhibitors are an important class of anticancer agent with the potential for treating a variety of tumors. Topoisomerases are nuclear enzymes required for the maintenance of DNA structure, and for the relief of the torsional stress which occurs in double-standard DNA during both transcription and replication. Two major human topoisomerases have been identified. Type I topoisomerase transiently breaks one strand of the duplex DNA, whereas type II topoisomerase (α and β isoforms) transiently breaks both strands of DNA. These enzymes are now recognized as important cellular targets for a number of successful chemotherapeutic agents.[1] Drugs that target topoisomerase II (e.g. doxorubicin and etoposide) have been widely used for many years,[2] whereas those that specifically target topoisomerase I (e.g. topotecan and CPT-II) have made an important impact more recently.[3] Many of the currently identified topoisomerase inhibitors do not act as direct enzyme inhibitors but act through stabilization of the transient topoisomerase–DNA cleavable complex resulting in DNA strand breakage and cell death.

Several groups have suggested that the combination of both a topoisomerase I and a topoisomerase II inhibitor might be beneficial in cancer therapy. As topoisomerase I and topoisomerase II bind to different sites on DNA and act at different parts of the cell cycle,[4] joint inhibition of both enzymes should hit a larger population of cells in any asynchronous population.[5] Furthermore, tumors treated with topoisomerase active agents can develop resistance due to either alteration in levels of expression and catalytic activity of topoisomerase I and/or II.[1] Thus by simultaneously inhibiting both topoisomerase I and II it may be possible to circumvent this acquired resistance due to alteration of topoisomerase I or II. Both *in vitro* and *in vivo* data have been reported which support a synergistic effect of sequential exposure to a topoisomerase I and a topoisomerase II inhibitor.[6,7] However, the clinical utility of this sequential administration of topoisomerase I and II inhibitors has been complicated by severe toxicity.[8,9] Thus, at present, the benefit of combination over single-agent administration has not been proven. A second approach has been to combine topoisomerase I and topoisomerase II inhibition in a single molecule. Recently described joint inhibitors of topoisomerase I

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and II include intoplicine,[5] F11782,[10] TAS-103[11] and XR5000, previously known as DACA.[12–14] XR5000 is an acridine derivative which is currently in clinical evaluation. Phase I studies where XR5000 was given as a 120 h i.v. infusion[14] have recently been completed and will be reported elsewhere. XR5000 has the advantage of avoiding topoisomerase-dependent multidrug resistance as well as circumventing multidrug resistance associated with the overexpression of the transmembrane transporters P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP).

The introduction of new agents to clinical practice is a difficult and expensive process. We have recently begun to use a new approach[15] based on chemosensitivity testing of tumor-derived cells at an early stage of the clinical development of new agents to speed up this process and direct clinical trials towards tumors for which the agents show *ex vivo* activity. The ATP-based tumor chemosensitivity assay (ATP-TCA), a new-generation chemosensitivity assay, was developed from previous ATP cytotoxicity assays[16–18] by Drs Andreotti and Cree, and published in 1995.[19] Subsequent development studies showed first that the results matched clinical outcome with 75–80% accuracy, comparable to microbial antibiotic or estrogen receptor testing.[15,19,20] On the basis of these results, Dr Kurbacher and colleagues conducted a phase II study of ATP-TCA directed therapy in recurrent ovarian cancer.[21] A non-randomized control group showed a 37% overall response rate (OR), with a median progression-free survival (PFS) of 20 weeks. The assay-directed group had a 64% OR with a median PFS of 50 weeks. A phase III study based on these encouraging results is now in progress.[22] In addition to its potential for individualization of chemotherapy, the ATP-TCA has already contributed to the assessment of new agents,[23] to the design of new regimens[20,24,25] and to investigation of the effects of oncogene expression on chemosensitivity.[26]

In this study we report the *ex vivo* activity of XR5000 against ovarian cancer, a tumor which often shows anthracycline sensitivity both *ex vivo* and in clinical practice, and against melanoma, a solid tumor which does not benefit from anthracycline therapy.

Methods

A test drug concentration (TDC) was established for XR5000 based on the plasma concentrations attained clinically and the degree of protein binding. This approach has been used successfully in predicting clinical outcome in the ATP-TCA with other drugs.[19] Following initial studies showing efficacy of XR5000 alone in melanoma and ovarian tumors, we went on to test the drug in combination with other agents with which it might be usefully combined, using direct addition of drugs together at standard TDCs.

Tumor tissue

Tissue from solid tumors surplus to diagnostic requirements or clinical trials of the ATP-TCA was used for testing with XR5000. Local ethics committee approval for the use of this tissue was obtained. The study included 18 untreated metastatic cutaneous melanomas, 20 recurrent ovarian carcinomas and six solid tumors of other types. All of the ovarian carcinomas had previously been treated with platinum-containing regimens and five had also received anthracyclines. Full details of the tumors tested are given in Table 1.

XR5000 and other drugs

Aliquots of XR5000 were made up according to the manufacturer's instructions and frozen at $-20^{\circ}C$.[27] No loss of activity was noted in aliquots stored for 7.5 months compared with fresh drug (n=3). Aliquots of other drugs were stored as previously published[27] or recommended by the manufacturer. The TDCs used for each agent are shown in Table 2.

Table 1. Tumors tested with XR5000, showing the average age (range), sex ratio and previous treatment

Tumor type	Number	Age	Sex (M:F)	Previous treatment
Ovarian carcinoma	20	59 (43–74)	0:20	all previous platinum, anthracycline in three cases
Melanoma	18	51 (32–69)	10:8	none
Unknown primary	2	52 (43–60)	1:1	none
Fallopian carcinoma	1	64	0:1	previous platinum
Renal carcinoma	1	82	0:1	none
Medullary carcinoma (thyroid)	1	52	1:0	none
Leiomyosarcoma	1	33	0:1	previous platinum

Table 2. TDCs used

Drug	TDC (μg/ml)	Storage (mg/ml)
XR5000	1.2	25 (−20°C)
Cisplatin	3.0	1 (−20°C)
Etoposide	16.0	20 (−20°C)
Gemcitabine	12.0	40 (−20°C)
Paclitaxel	13.6	6.0 (RT[a])
Topotecan	16.0	1 (−20°C)
Treosulfan	20.0	50 (−20°C)
Vinblastine	0.5	1 (−20°C)

[a]Room temperature.

ATP-TCA

The ATP-TCA was performed as published.[19] Briefly, tumor tissue was minced, dissociated overnight and the cells resuspended (after washing) to 400 000 cells/ml. Alternatively, ascites were centrifuged and the cells resuspended after washing to 200 000 cells/ml. If there was significant erythrocyte contamination or debris in the sample, the cell suspension was purified by density centrifugation over Histopaque-1077 (Sigma, Poole, UK), washed 3 times and resuspended to 400 000 cells/ml (solid tumor) or 200 000 cells/ml (ascites). Drugs were prepared as 8 × 100% TDC in a proprietary serum-free medium (CAM; DCS Innovative Diagnostik Systeme, Hamburg, Germany). The TDC for each drug was determined by pharmacokinetic data, taking into account the peak plasma concentration, the degree of protein binding and the toxicity of the agent. Where clinical response data to the single agent is available, this is regarded as the 'gold standard' for TDC determination. Doubling dilutions of the drugs (100 μl/well) were made in triplicate wells in six rows of a 96 U-well polypropylene plate (Corning-Costar, High Wycombe, UK). The remaining two rows were reserved for a no drug control (MO, medium alone) and a maximum inhibitor (MI). Finally, 100 μl of tumor cells was added to each well across the plate. The final drug concentration within the plate is 200–6.25% TDC. Following incubation of the plate at 37°C in 5% CO_2 and 99% humidity for 6–7 days, ATP was extracted from the cells by addition of 50 μl of a somatic cell extractant (DCS). Aliquots of 50 μl were transferred to a white 96-well polystyrene plate (Dynex Labsystems, Ashford, UK) and 50 μl luciferin–luciferase reagent added. The light output (luminescence) from each well was read in a 96-well microplate luminometer (MPL1; Berthold Diagnostic Systems, Pforzheim, Germany). The results were expressed as:

$$\% \text{ Inhibition} = 1 - \frac{(\text{test} - \text{MI})}{(\text{MO} - \text{MI})} \times 100$$

Data analysis

The raw luminescence counts from the ATP-TCA were entered into a spreadsheet (Excel 97) for analysis. The spreadsheet automatically provides graphs of the percent inhibition at each concentration against % TDC and calculates several summary parameters, including IC_{90} and IC_{50}. However, these parameters ignore much of the information present and comparison between drugs is usually accomplished by the use of the area under the inhibition–TDC curve ($Index_{AUC}$) or by a natural log Index which skews the assessment towards lower concentrations ($Index_{SUM}$). Combination effects are examined in the spreadsheet by the method of Poch *et al.*[28]

Results

The concentration–inhibition curves for a series of ovarian carcinomas, melanomas and other tumors are shown in Figure 1. There is a clear dose–response effect, with higher concentrations of XR5000 producing inhibition around the 90–100% mark in most tumors. The concentrations required to produce 50% and 90% inhibition (IC_{50} and IC_{90}) are shown with the two summary indices in Table 3(a). An $Index_{SUM} < 300$ indicates strong activity against the tumor and $Index_{SUM} > 450$ is usually indicative of resistance. XR5000 achieved sensitivity on this basis as a single agent in three of 20 ovarian carcinomas and two of 18 melanomas, but there is considerable heterogeneity with a wide range of observed results (Table 3). There was resistance to cytotoxicity in nine of 20 ovarian tumors and seven of 18 melanomas. Of the other tumors tested, one of six showed $Index_{SUM} < 300$ with XR5000, but there was resistance ($Index_{SUM} > 450$) in three of six tumors, two unknown primaries and a medullary C-cell carcinoma of thyroid (Figure 1). However, the summary indices take no account of the shape of the concentration–response curve, which conforms to a steep sigmoid pattern in most tumors (Figure 1). It is notable that XR5000 alone was able to induce greater than 95% inhibition, usually at the 200% TDC level (2.4 μg/ml) in 11 of 20 (53%) ovarian carcinomas, 11 of 18 (61%) melanomas and five of six (83%) other tumors.

We used the combination of topotecan (a topoisomerase I inhibitor) and etoposide (a topoisomerase II inhibitor) for comparison with XR5000. XR5000 was usually better than each of the single agents and nearly equivalent to them in combination, although it tended to be less active at lower concentrations (Figure 2 and Table 3).

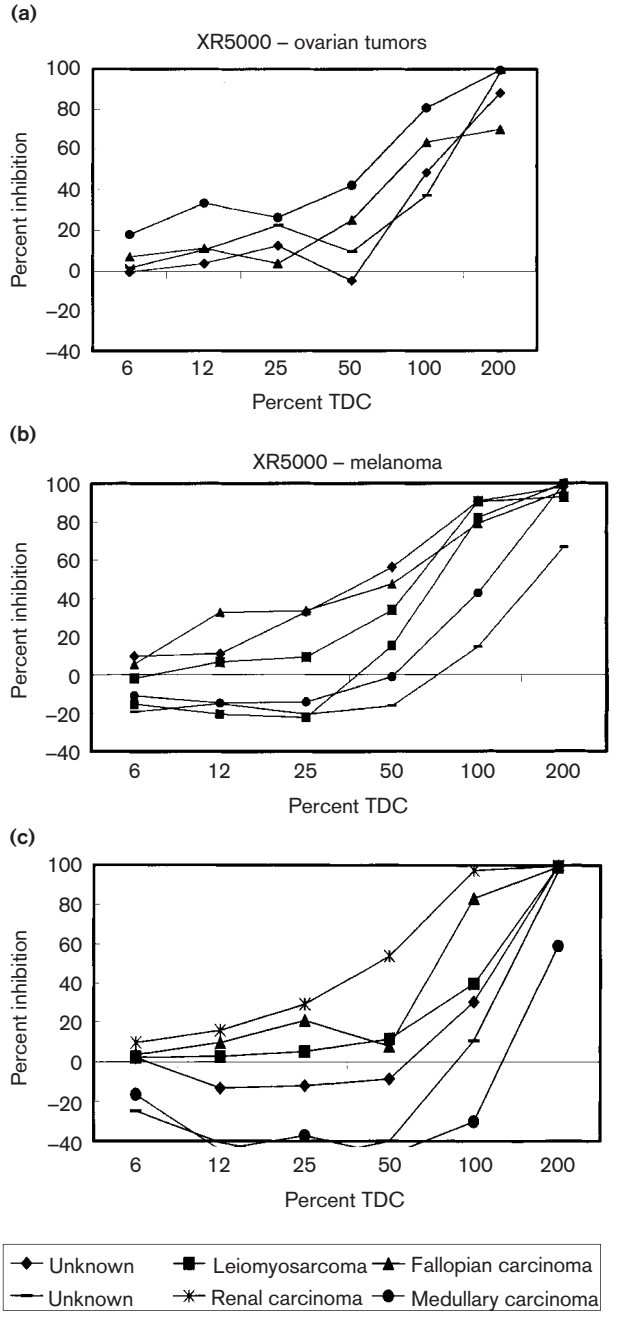

Figure 1. The activity of XR5000 against from (a) six ovarian tumors, (b) six melanomas and (c) other tumors showing examples of the concentration–response curves obtained. The 100% TDC level represents 1221 ng/ml, equivalent to the C_{max} achieved *in vivo*.

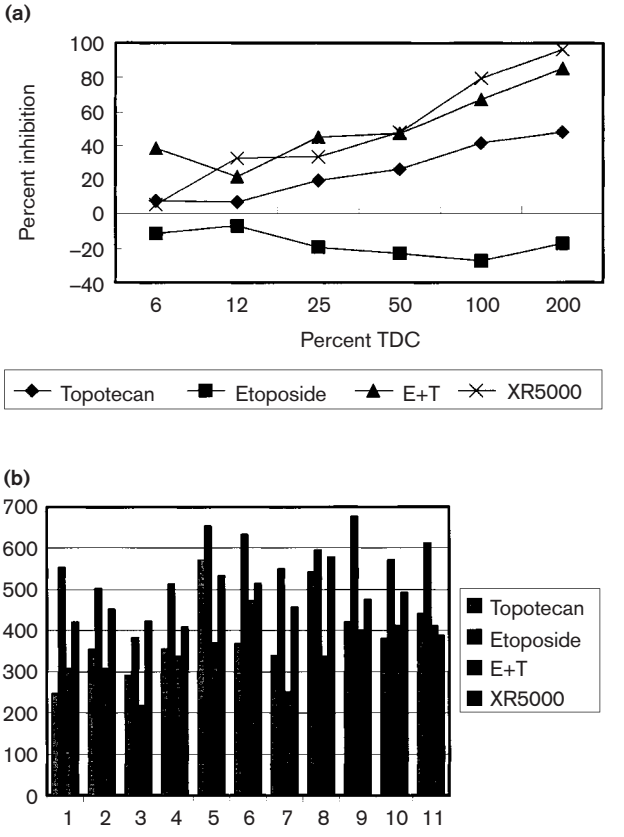

Figure 2. (a) Comparison of XR5000 with etoposide+topotecan in a melanoma showing similar effects the combination in comparison with XR5000. (b) Bar graphs of the $Index_{sum}$ index showing the effect of XR5000 in comparison with etoposide+topotecan in 11 ovarian cancer biopsies.

We went on to test a number of different agents in combination with XR5000 (Figure 3 and Table 3). The results for pretreated ovarian cancer show improved activity for the combination of XR5000 with paclitaxel (Table 3). There is also some increase with cisplatin, despite previous failure of platinum-based therapy in all of the patients. The combination of XR5000 with gemcitabine was generally disappointing in ovarian cancer with little enhancement of the IC_{90} or in the percentage of tumors reaching greater than 95% inhibition (Table 3), although there was improved activity of this combination at lower concentrations, reflected in the improved IC_{50} and summary indices (Figure 3a and Table 3).

The results for combinations of XR5000 in melanomas are similar (Figure 3 and Table 3). Again, the most promising agent in combination with XR5000 was paclitaxel (Table 3). There was also considerable enhancement of inhibition for XR5000+treosulfan. The combinations with cisplatin and gemcitabine were again disappointing, although there was an interesting increase in activity on the basis of index with vinblastine.

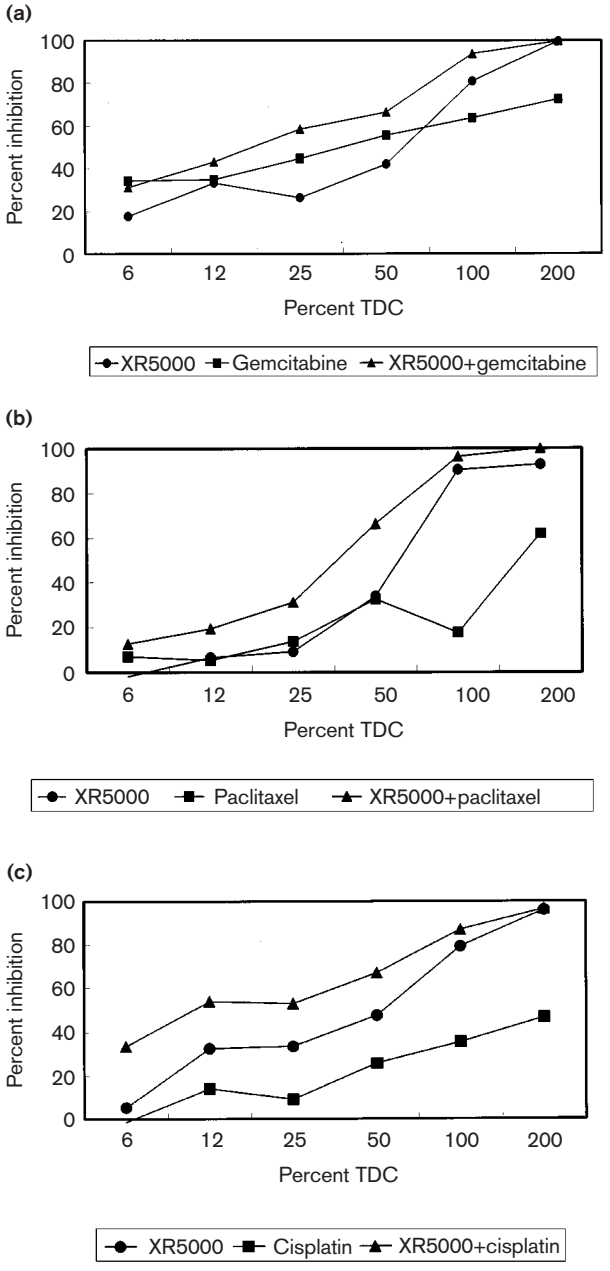

Figure 3. The effect of XR5000 can be modulated by combination with other agents. (a) The combination of XR5000 with gemcitabine in a recurrent ovarian carcinoma shows little enhancement over the response with XR5000 alone. (b) The combination of XR5000 with paclitaxel in a melanoma. (c) The combination of XR5000 with cisplatin in a melanoma.

Discussion

XR5000 shows a similar degree of *ex vivo* activity in both melanoma and ovarian cancer, despite the fact that melanoma is generally more chemoresistant.[19,29] This suggests that it is likely to find wide application against solid tumors and that it would be worth testing a larger number of tumor types. The few that we were able to test in this study gave encouraging results. XR5000 has a steep dose–response curve, which tends not to plateau until 100% tumor cell inhibition has been achieved. The concentration required to achieve 100% tumor cell inhibition is therefore often in excess of the clinically achievable peak plasma concentration using the standard dosage employed in clinical trials. However, since the drug is given over 5 days at this dose, the cumulative effect may obviate the need for an increase in the dose given. It may also be possible to achieve dose intensification by the use of modified preparations and this may enhance clinical efficacy by analogy with doxorubicin (Neale *et al.*, unpublished).

It is interesting that XR5000 is superior to either topotecan or etoposide alone in the assay, and that it often produces similar results to those seen with the combination of these two agents. There is mounting evidence that cells circumvent topoisomerase I inhibitors by down-regulating this enzyme while upregulating topoisomerase II, and vice versa both *in vitro* and *in vivo*.[5–9,30–34] Exposure of the cells *ex vivo* to the combination of topotecan+etoposide, or XR5000, avoids this resistance mechanism by inhibiting both topoisomerase I and II at the same time. However, both etoposide[2] and topotecan[35,36] are sensitive to drug efflux pump-mediated resistance and this may affect the efficacy of the combination. Nevertheless, there is clinical data showing that topotecan+etoposide can be an effective regimen in some patients with ovarian carcinoma.[37] This is of course encouraging for XR5000, which is not susceptible to drug efflux mechanisms.[38]

Once it became clear that XR5000 had activity in the ATP-TCA against both melanoma and ovarian carcinoma, we decided to test its efficacy in combination with a number of different agents. The best combinations for ovarian cancer and melanoma differed, although the XR5000+paclitaxel combination was effective in both. The degree of enhancement of XR5000 activity in combination with cisplatin in ovarian cancer is surprising as all of these were recurrent tumors following platinum-containing therapy. However, topoisomerases are involved in DNA repair,[39–41] and enhanced repair is a major mechanism for resistance to cisplatin and other DNA-damaging agents.[41,42] Furthermore, it has been noted that inhibition of DNA repair is greatest for combinations of topoisomerase I and II inhibitors *in vitro*.[41] Inhibition of such repair by combined topoisomerase inhibitors such as XR5000 may therefore be able to reverse cisplatin

Table 3. Results of XR5000, topotecan + etoposide and combinations of XR5000 chemosensitivity testing in the tumor types tested (median + range), with percentage showing greater than 95% inhibition at any concentration tested

Drug/combination	*n*	IC_{50}	IC_{90}	Index $_{AUC}$	Index $_{SUM}$	> 95% Inhibition
(a) Ovarian carcinoma						
XR5000	20	95 (26–145)	187 (130–258)	8599 (4326–15434)	441 (238–578)	11 (53%)
topotecan	15	75 (9–144)	246 (184–284)	10840 (5893–13295)	355 (248–571)	0 (0%)
etoposide	17	442 (66–3196)	796 (272–5756)	1620 (−3238–10914)	553 (322–677)	0 (0%)
topotecan + etoposide	13	68 (19–95)	160 (89–222)	12551 (9332–16060)	338 (219–472)	1 (8%)
gemcitabine	18	23 (4–256)	256 (149–461)	11679 (3687–17151)	284 (75–529)	0 (0%)
paclitaxel	18	86 (4–149)	192 (64–337)	10532 (5710–18003)	363 (95–489)	8 (44%)
cisplatin	18	102 (37–546)	254 (169–982)	8329 (637–14094)	326 (277–486)	0 (0%)
XR5000 + gemcitabine	16	56 (5–126)	167 (88–221)	13334 (7799–16642)	288 (153–469)	10 (63%)
XR5000 + paclitaxel	8	55 (22–90)	96 (81–177)	14590 (10571–16655)	286 (218–583)	8 (100%)
XR5000 + cisplatin	9	54 (35–84)	99 (91–234)	13838 (10483–15411)	317 (259–423)	7 (78%)
(b) Melanoma						
XR5000	18	91 (25–168)	182 (99–269)	9459 (1920–15406)	448 (301–590)	11 (61%)
topotecan	4	64 (−116–207)	254 (−209–373)	8505 (−11287–11780)	413 (336–828)	0 (0%)
etoposide	7	274 (−587–594)	509 (−1096–1070)	3244 (−4269–8133)	534 (388–706)	0 (0%)
topotecan + etoposide	2	39 (28–56)	88 (149–210)	14243 (12235–16252)	257 (220–295)	1 (50%)
gemcitabine	18	244 (5–526)	439 (202–946)	5236 (−5887–15881)	462 (153–714)	0 (0%)
paclitaxel	17	99 (6–263)	186 (91–473)	10413 (−1405–16263)	351 (193–683)	11 (65%)
cisplatin	18	72 (14–287)	239 (96–517)	6318 (−11773–16320)	431 (184–806)	2 (11%)
vinblastine	17	509 (55–1528)	742 (341–2752)	2781 (−17029–10107)	511 (317–1062)	0 (0%)
treosulfan	18	87 (6–1018)	205 (34–1832)	10158 (−2034–18526)	427 (87–656)	5 (28%)
XR5000 + gemcitabine	12	76 (10–158)	172 (74–199)	11055 (2704–17291)	348 (153–585)	8 (67%)
XR5000 + paclitaxel	6	41 (10–121)	91 (85–187)	15204 (9310–17080)	309 (166–500)	6 (100%)
XR5000 + cisplatin	13	67 (6–134)	133 (80–277)	12700 (5281–16641)	337 (163–551)	8 (62%)
XR5000 + vinblastine	7	74 (12–153)	168 (96–200)	11614 (4812–14950)	423 (217–570)	6 (86%)
XR5000 + treosulfan	4	58 (14–67)	94 (61–119)	14277 (13885–17501)	287 (170–339)	4 (100%)

resistance in some patients. Inhibition of DNA repair underlies the combination of cytosine analogs such as gemcitabine with DNA-damaging agents and appears to be able to reverse resistance to these agents in a large number of different tumor types.[42,43] Although one would expect XR5000 to produce significant DNA

damage, concomitant addition of XR5000 with gemcitabine *ex vivo* did not greatly enhance cell inhibition, except at lower concentrations. This is consistent with other studies and clinical experience in which the combination of topoisomerase inhibitors with gemcitabine has had at best additive effects.[44]

Platinum-containing regimens are also effective against melanoma, but in this study the combination of XR5000 with cisplatin was not as effective as its combination with treosulfan. This drug alkylates DNA at the O^7 position, and is therefore not susceptible to resistance mediated by up-regulation of O^6-methylguanylmethyltransferase (MGMT).[45] Treosulfan has recently been shown to have efficacy against melanoma both by chemosensitivity testing and in phase II clinical studies.[15,42,46,47] Chemosensitivity testing suggested that it might benefit from combination with gemcitabine[42,46] and early clinical results are encouraging (Reinhold *et al.*, unpublished). The fact that XR5000+treosulfan is effective in this study may result from similar inhibition of DNA repair, but this is speculative and will require molecular pharmacological confirmation. Once again, combination of XR5000 with paclitaxel was effective and the combination with vinblastine also showed good activity. The mechanism involved is difficult to understand, since there is no direct relationship between microtubules and topoisomerase. However, it may be that the enhancement observed is based on intracellular pharmacology, rather than any molecular mechanism.

Clearly our observations here are a first step: they require examination of the molecular pharmacology of these combinations, together with further chemosensitivity studies aimed at establishing the efficacy of XR5000 against a wider group of tumor types, and the optimal sequence for combination of existing drugs such as paclitaxel, cisplatin and treosulfan. The steep concentration–response is an issue for this drug, but the cumulative effect of a 5-day infusion or enhanced delivery technology may permit much higher concentrations of XR5000 to be reached within the tumor. XR5000 may have a role in melanoma as well as more conventional tumor targets for topoisomerase inhibitors.

Acknowledgments

We wish to thanks all of the oncologists and surgeons who submitted material for testing, particularly Drs Lamont, Hindley, Khoury and Weaver. We are grateful to members of the Pre-clinical Therapeutic Models Group of the EORTC for their advice and encouragement.

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(Received 27 April 2000; revised form accepted 11 May 2000)