Ecteinascidin-743, a New Marine Natural Product with Potent Antitumor Activity on Human Ovarian Carcinoma Xenografts

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ABSTRACT

The antitumor activity of ecteinascidin (ET)-743, a novel marine natural product, was evaluated against a panel of human ovarian carcinoma xenografts characterized by different malignant behaviors and drug responsiveness in nude mice. These tumor models included three xenografts transplanted s.c. (HOC18, HOC22-S, and MNB-PTX-1) into nude mice, representing different levels of sensitivity to cisplatinum (DDP), which was used as reference drug for ovarian carcinoma, and two other xenografts (HOC22 and HOC8), which are highly malignant in the peritoneal cavity of nude mice, representing the growth pattern of this neoplasm. At the maximum tolerated dose of 0.2 mg/kg using an intermittent schedule of one i.v. injection every 4 days, ET-743 was highly active against HOC22-S (sensitive to DDP), inducing long-lasting, complete regressions, and against HOC18 (marginally sensitive to DDP), inducing partial tumor regressions. Moreover, significant growth delay was observed in mice bearing late-stage HOC18 tumor (400-mg tumor weight; nonresponsive to DDP). ET-743, however, was not active against MNB-PTX-1, a tumor that is highly resistant to chemotherapy, including DDP. In the i.p. ovarian carcinoma xenograft model, ET-743 at the maximum tolerated dose induced complete tumor remissions in all mice bearing HOC22 tumor, with 25% histopathologically confirmed cures, and produced marginal tumor growth delay against HOC8.

These results indicate that ET-743 is a potent drug against ovarian carcinoma xenografts, being equally as active or more efficacious than DDP in the same tumor line. Our findings with human ovarian carcinoma xenografts justify clinical assessment of this drug with this tumor target.

INTRODUCTION

In recent years, the marine ecosystem has presented a potential new source of natural products, with an increasing number of new anticancer agents being identified and developed in preclinical studies and clinical trials (1, 2). The ETs are a class of novel chemical entities that belong to the tetrahydroisoquinoline alkaloids, extracted from the Caribbean tunicate Ecteinascidia turbinata (3). Among these, ET-743 was originally selected for further development on the basis of its cytotoxic potency and selectivity in early preclinical studies, as well as for its abundance in the tunicate (4).

Initial in vitro studies identified its activity at nanomolar concentrations in the National Cancer Institute human in vitro cell line panel (5, 6). ET-743 also demonstrated potent activity in the human tumor clonogenic assay at low concentrations against a variety of freshly explanted human primary tumors (5). Early in vivo studies showed that ET-743 is active against murine P388 leukemia, B16 melanoma, and human MX-1 mammary carcinoma xenografts (6). More recently, the antitumor activity of ET-743 has been demonstrated against a human melanoma (MEXF 989) and a human non-small cell cancer (LXFL 529; Ref. 7).

The mechanism of action of ET-743 has not yet been fully elucidated, although it has been shown that ET-743 alkylates the N2 position of guanine in G+C-rich DNA sequences (8). Cells exposed to ET-743 progress through the S phase more slowly than untreated cells and are blocked in the G2-M phase, as assessed by flow cytometry analysis (9, 10). Cell death induced by ET-743 does not appear to be dependent on p53 status (4). In addition, at high concentrations over time, ET-743 first promotes a decrease in the proportion of microtubules located close

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The abbreviations used are: ET, ecteinascidin; HOCl, human ovarian carcinoma; DDP, cisplatinum; Q4×3, every 4 days, three times; MTD, maximum tolerated dose; RTW, relative tumor weight; SGD, specific growth delay; NLCK, net log cell kill; ILS, increment of life span; T/C%, optimal growth inhibition.

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3 The abbreviations used are: ET, ecteinascidin; HOC, human ovarian carcinoma; DDP, cisplatinum; Q4×3, every 4 days, three times; MTD, maximum tolerated dose; RTW, relative tumor weight; SGD, specific growth delay; NLCK, net log cell kill; ILS, increment of life span; T/C%, optimal growth inhibition.

4 E. Erba, personal communication.
1978 ET-743 Activity on Human Ovarian Carcinoma Xenografts

to the cell membrane and then a microtubule collapse around the nuclear membrane (11).

Taken together, a novel chemical structure, a potential new mechanism of action, and potent activity against human tumor models, producing long-lasting complete remissions, are the basis for the continued preclinical development of ET-743. For this purpose, we have explored, in detail, the antitumor activity of ET-743 on a panel of HOC xenografts of different origins, malignant behaviors, and drug sensitivities.

MATERIALS AND METHODS

Animals. Female NCr-nu/nu mice were obtained from the animal production colony of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). Mice were used at 8–10 weeks of age and at a mean body weight of 23 ± 2 g. Throughout this study, nude mice were housed in filtered-air laminar flow cabinets and were manipulated following aseptic procedures.

Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., Supplement 40, February 18, 1992; Circolare No. 8, G.U., July 1994) and international laws and policies (European Economic Community Directive 86/609, OJ L 358.1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

Tumor Lines. HOC xenografts HOC22, HOC8, and HOC18 were established and maintained in nude mice as described previously (12). Briefly, HOC18 was derived from a primary ovarian tumor of a treated patient and established s.c. in nude mice; HOC8 and HOC22 were derived from an untreated pleural effusion and treated ascites, respectively, and established i.p. in nude mice. MNB-PTX-1 was recently established s.c. in nude mice from malignant ascites of a patient with serous adenocarcinoma of the ovary, who had received DDP, doxorubicin, and cyclophosphamide combined chemotherapy and additional courses of Taxol, which, however, did not prevent progressive disease.

HOC18 and MNB-PTX-1 were maintained by transplanting tumor fragments s.c. in the flanks of nude mice, where they produced progressive growing tumors. HOC22 and HOC8 were transplanted as cell suspensions in the peritoneal cavity of nude mice (i.p.), where they produced ascites and solid lesions onto abdominal organs, primarily the diaphragm, omentum, pancreas, and liver. HOC22-S, derived from HOC22 cell suspension, was transplanted s.c. in nude mice.

Drug Treatment. ET-743 (Fig. 1), provided by Pharma Mar S.A. (Tres Cantos, Madrid, Spain), was prepared as a 1 mg/ml stock solution in absolute ethanol (stored at −20°C) and further diluted with phosphate buffer pH 4.0 immediately before administration. ET-743 was given i.v. to mice using the schedule of one injection Q4X3. Appropriate vehicles were injected, using the same schedule and route of injection as the drug therapies.

Treatment Evaluation. Before distribution to the various treatments, animals were randomized on the basis of body weight for i.p. tumors and on the basis of tumor size for s.c. tumors.

Mice were weighed three times a week to evaluate drug-induced toxicity. Body weight changes were recorded. The maximum body weight loss after treatment with day of nadir was reported. Animals dying within 2 weeks after the final drug administration were considered toxic deaths and were excluded from further tumor response evaluation. Two models of tumor growth were used to evaluate drug response, as described below.

s. c. Tumor Growth. HOC22-S (as a suspension of 10 × 10⁶ cells), HOC18, and MNB-PTX-1 (as 2–3-mm tumor fragments) were implanted s.c. in the flanks of nude mice. Treatments (five to six mice per group) started when tumors reached ~150 ± 50 mg (early-stage tumors) or 400 ± 100 mg (late-stage tumors).

The diameters of the tumors were measured twice a week in two dimensions with a caliper and estimates (in g) of tumor weights were calculated as [length × (width)²]/2. The end point of the experiments occurred when tumors reached a median weight of 2 ± 0.5 g or 5 weeks after the last treatment. In case of complete tumor regressions, the mice were observed for an additional 4 weeks to monitor regrowth of the tumors. Changes in tumor weight from the start of treatment (W₀) until any given time (Wₜ) were calculated for each tumor and day of measurement and expressed as the RTW (= Wₜ/W₀). The median of these values for all evaluable tumors in the control
and the treated groups was used to calculate treatment efficacy (13). Tumor doubling time was calculated as the time to achieve median RTW of twice the size of the first day of treatment for control and treated tumors (13).

Results are expressed as median tumor weight; as optimal growth inhibition (T/C%) within 5 weeks after the last drug injection, defined as [lowest ratio of the median RTW of treated over control tumors] × 100; as SGD, defined as [(doubling time of treated tumors − doubling time of control tumors)/doubling time of control tumors] (14); and as NLCK, defined as [(T − C) − duration of treatment] × 0.301/doubling time], where T and C are median times (in days) for treated and control tumors, respectively, to reach 500 mg. With T/C% ≤ 50%, SGD ≥ 1, and NLCK ≥ 1, the treatment is considered active (13). The difference in tumor regrowth (Fig. 2) was evaluated by non-parametric Mann-Whitney test. Partial regressions correspond to tumor regressions below the limit of palpation, at the end of the experiment. Differences in response were analyzed by χ² test.

i.p. Tumor Growth. HOC22 and HOC8 ascites were injected i.p. as a cell suspension in eight nude mice (10 × 10⁶ cells/animal) for each experimental group. Treatments started 10 days after tumor inoculation, when mice presented advanced tumor with ascites and microscopic tumor deposits (liver and omentum). Mice were monitored twice a week for tumor formation (abdominal distension) in the peritoneal cavity. Animals were sacrificed when they became moribund, and the day of sacrifice was considered the survival time. At autopsy, the peritoneal cavity was macroscopically examined to ascertain the presence of the tumor. Results are expressed as ILS, which was calculated as 100 × [(median survival time of treated group − median survival time of control group)/median survival time of control group]. Differences in survival time were analyzed by the log-rank test. With an ILS ≥ 40%, the treatment is interpreted as active.

Surviving animals that did not present gross evidence of tumors in the peritoneal cavity were euthanized and autopsied no earlier than 90 days after the death of the last control animal. The absence of tumor in “cured” mice was confirmed by cytohistological examination, as described previously (15). Briefly, lavages of the peritoneal cavity were spun in a cytocentrifuge, and the cells were fixed and stained with H&E. The ovary/uterus, pancreas, omentum, liver, diaphragm, and lung were collected, fixed in 10% phosphate-buffered formalin, and processed for standard histological analysis.

RESULTS

Effect of ET-743 on Ovarian Carcinoma Xenografts

The antitumor activity of ET-743 was assayed on three different HOC xenografts: HOC22-S, HOC18, and MNB-PTX-1, each with a different sensitivity to DDP (Tables 1–3). The xenografts were transplanted s.c. in the flanks of nude mice, and treatments started when all mice had tumors with a mean weight of ∼150 ± 50 mg.

Because pilot experiments have indicated the best dose schedule to be Q4×3 at the MTD of 0.2 mg/kg per injection, this treatment was used in all of the studies below.

HOC22-S. Table 1 shows the antitumor activity of ET-743 on HOC22-S, a tumor that is highly sensitive to DDP. In this tumor model, ET-743 was highly active, inducing long-lasting complete tumor regressions at the MTD (five of six complete regressions), with one toxic death. Significant growth inhibition (T/C% = 11%; one of six complete regressions and five of six partial regressions) was observed at 1/2 MTD, and minor responses were observed at 1/4 MTD (T/C% = 43%).Comparable activity was observed with DDP (T/C% = 5%) given at MTD but with complete tumor regressions in only one of six mice. The complete regressions were still present 50 days after treatment, when the experiment was terminated.

HOC18. Table 2 shows the antitumor activity of ET-743 on HOC18, a tumor that is marginally sensitive to DDP. At the MTD of 0.2 mg/kg, ET-743 exhibited a strong tumor growth inhibition (T/C% = 6%; SGD = 6.7; NLCK = 2.9). The intermediate doses showed borderline efficacy (T/C% = 49%; SGD = 0.3; NLCK = 0.5), whereas at the lowest dosages (1/4 MTD), ET-743 showed no significant activity (T/C% = 76%). Three of six partial tumor regressions were observed at the MTD of ET-743. DDP given at the MTD showed only marginal activity on HOC18 tumors (T/C% = 79%; SGD = 0.1; NLCK = −0.1) with no regression.

MNB-PTX-1. Table 3 shows the antitumor activity of ET-743 on MNB-PTX-1, a tumor that is highly resistant to DDP. On the basis of the results of HOC22-S and HOC18 xenografts, the schedule Q4×3 was chosen for all of the studies below.

Fig. 2 Antitumor effect of ET-743 on HOC18 s.c. xenograft. Nude mice were transplanted s.c. with HOC18 fragments, and treatments started 28 days (early-stage, mean tumor weight of 150 ± 50 mg) and 42 days (late-stage, mean tumor weight of 400 ± 100 mg) after tumor transplantation (n = 6). ET-743 was given i.v. Q4×3 at 0.2 mg/kg (MTD).[ ] vehicle (pool of early- and late-stage vehicles, n = 12); ▲, early treatment; △, late treatment; arrows, beginnings of treatments.
1980 ET-743 Activity on Human Ovarian Carcinoma Xenografts

Table 1  Activity of ET-743 on HOC22-S s.c. xenografts

<table>
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<tr>
<th>Compound</th>
<th>Injected dose (mg/kg, i.v.)</th>
<th>Schedule days</th>
<th>% mean body weight loss (day of nadir)</th>
<th>No. of toxic deaths/total no. of mice (day)</th>
<th>T/C% (day)</th>
<th>Complete tumor regressions</th>
<th>Partial tumor regressions</th>
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<tr>
<td>ET-743</td>
<td>0.2</td>
<td>20, 24, 28</td>
<td>13 (38)</td>
<td>1/6 (45)</td>
<td>3 (61)</td>
<td>5/6</td>
<td>1/6</td>
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<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>5 (38)</td>
<td></td>
<td>11 (61)</td>
<td>1/6</td>
<td>5/6</td>
</tr>
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<td></td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td>43 (61)</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>DDP</td>
<td>4</td>
<td>20, 24, 28</td>
<td>6 (35)</td>
<td></td>
<td>5 (70)</td>
<td>1/6</td>
<td>4/6</td>
</tr>
</tbody>
</table>

*HOC22-S was derived from a HOC22 cell suspension (10 × 10⁶) transplanted s.c. in the flanks of nude mice.

Table 2  Activity of ET-743 on HOC18 s.c. xenografts

<table>
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<tr>
<th>Compound</th>
<th>Injected dose (mg/kg, i.v.)</th>
<th>Schedule days</th>
<th>% mean body weight loss (day of nadir)</th>
<th>T/C% (day)</th>
<th>SGD</th>
<th>NLCK</th>
<th>Partial tumor regressions</th>
</tr>
</thead>
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<td>28, 32, 36</td>
<td>5 (33)</td>
<td>6 (57)</td>
<td>6.7</td>
<td>2.9</td>
<td>3/6</td>
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<td></td>
<td>0.1</td>
<td></td>
<td>5 (33)</td>
<td>49 (48)</td>
<td>0.3</td>
<td>0.5</td>
<td>0/6</td>
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<tr>
<td></td>
<td>0.05</td>
<td></td>
<td>1 (33)</td>
<td>76 (37)</td>
<td>0</td>
<td>-0.4</td>
<td>0/6</td>
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<tr>
<td>DDP</td>
<td>4</td>
<td>28, 32, 36</td>
<td>5 (36)</td>
<td>79 (43)</td>
<td>0.1</td>
<td>-0.1</td>
<td>0/6</td>
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</table>

*HOC18 was transplanted as tumor fragments s.c. in the flanks of nude mice.

Table 3  Activity of ET-743 on MNB-PTX-1 xenografts

<table>
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<tr>
<th>Experiment no.</th>
<th>Compound</th>
<th>Injected dose (mg/kg, i.v.)</th>
<th>Schedule days</th>
<th>% mean body weight loss (day of nadir)</th>
<th>T/C% (day)</th>
<th>SGD</th>
<th>NLCK</th>
<th>Partial tumor regressions</th>
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<td>72 (67)</td>
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<td>DDP</td>
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<td>35, 39, 43</td>
<td>20 (46)</td>
<td>75 (81)</td>
<td>0.2</td>
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<tr>
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<td>25, 29, 33</td>
<td>26 (40)</td>
<td>76 (40)</td>
<td>0</td>
<td>-0.4</td>
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<tr>
<td></td>
<td>ET-743</td>
<td>0.06</td>
<td>25, 26, 27, 28, 29</td>
<td>5 (36)</td>
<td>64 (54)</td>
<td>0.1</td>
<td>0</td>
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</tbody>
</table>

*MNB-PTX-1 was transplanted as tumor fragments s.c. in the flanks of nude mice. Results are from two independent experiments.

Following a second schedule of treatment, ET-743 was administered daily for 5 consecutive days (Table 3, experiment 2). ET-743 given at 0.06 mg/kg per injection (total dose = 0.3 mg/kg) was not significantly active (T/C% = 64%; SGD = 0.1; NLCK = 0) against MNB-PTX-1 xenografts. No advantage of the daily treatment over the intermediate schedule was observed, although a lower total dose was administered. The dose of 0.12 mg/kg per injection (total of 0.6 mg/kg) was toxic in all of the mice (data not shown). It is worth noting that an unexpectedly high drug toxicity was always observed in this tumor model, due perhaps to the combination of treatment and tumor-induced toxicity (Table 3).

Effect of ET-743 on Late-Stage Tumors

On the basis of the encouraging results obtained with ET-743 in early-stage xenografts (Tables 1 and 2), we investigated whether ET-743 was able to inhibit tumor growth even in mice bearing heavy tumor burdens. Fig. 2 shows a comparison of ET-743 efficacy with treatments starting on HOC18 xenografts at different tumor burdens.

Treatments were started on day 28 (180 ± 23 mg tumors, early stage) and on day 42 (484 ± 86 mg tumors, late stage) in the two groups. ET-743 was given Q4X3 at the MTD of 0.2 mg/kg per injection. As shown before, early-stage treatment was highly active (T/C% = 6%; SGD > 6; NLCK = 2.9), and a similar antitumor effect (T/C% = 9%; SGD > 4; NLCK = 2.6) was observed when treatment with ET-743 started on day 42, the time at which mice presented heavier tumor burden. Both treatments were able to induce partial regressions in 50% of the tumors (on days 57 and 64 for early and late-stage treatment, respectively); however, early-stage treatment delayed tumor regrowth for a longer period (77 days from first day of regression) compared to late-stage treatment (21 days; P < 0.05; Fig. 2).

Effect of ET-743 on Ovarian Carcinoma Xenografts Growing in the Peritoneal Cavity

In patients, ovarian carcinomas disseminate in the peritoneal cavity, where they produce ascites and diffuse carcinoma-tosis. We, therefore, studied the effect of ET-743 in models of HOC xenografts that grow in the peritoneal cavity of nude mice, forming ascites and disseminating tumors and, thus, resembling....
Fig. 3 Antitumor effect of ET-743 on HOC22 i.p. xenograft. Nude mice were inoculated i.p. (day 0) with 10 × 10⁶ cells, and treatments were started (arrows) at day 10 after tumor transplantation (n = 8). Drugs were given i.v. Q4×3 at the following doses: ET-743, 0.2, 0.1, and 0.05 mg/kg; DDP, 4 mg/kg.

Fig. 4 Antitumor effect of ET-743 on HOC8 i.p. xenograft. Nude mice were inoculated i.p. (day 0) with 10 × 10⁶ cells, and treatments were started (arrows) at day 10 after tumor transplantation (n = 8). Drugs were given i.v. Q4×3 at the following doses: ET-743, 0.2, 0.1, and 0.05 mg/kg; DDP, 4 mg/kg.

**DISCUSSION**

ET-743 is a natural product of marine origin that has shown potent *in vitro* antitumor activity in a variety of human tumor types (5–7). Antitumor activity has also been measured against *in vivo* murine and human tumor models (6, 7), but no extensive studies on the potential of this compound as an antitumor agent have been reported. For this reason, this study was undertaken, and it shows the remarkable activity of ET-743 against HOC xenografts that are progressively growing in nude mice.

The i.v. administration of ET-743 was significantly effective against HOC22-S and HOC18 HOC xenografts transplanted s.c. in nude mice. Both xenografts were highly responsive to ET-743, with remarkable long-lasting objective responses (partial and complete regressions). Significant growth delay with partial responses was achieved at 1/2 MTD and 1/4 MTD, thus clearly showing a dose-response relationship and providing evidence of a good therapeutic index.

DDP-based combinations are the most efficient therapies for ovarian cancers, but resistance and toxicity often limit the continuation of treatments with this agent. We, therefore, used DDP as a reference drug throughout our study. We found that ET-743 is active not only on DDP sensitive tumors (HOC22-S) but also on a tumor (HOC18), which is only marginally sensitive to DDP. When compared at equitoxic doses, ET-743 was at least as active as DDP on HOC22-S xenograft, although more complete regressions were observed (P < 0.05), and it was more active than DDP on HOC18 xenograft. In contrast, ET-743 was not active against MNB-PTX-1, a tumor that is highly resistant to chemotherapy, both in patients and in nude mice. It is worth considering that the three xenografts HOC22-S, HOC18, and MNB-PTX-1 were obtained from patients previously treated with a combination of drugs, including DDP, and who were undergoing tumor progression. ET-743 was active on two of these patients’ tumor-derived xenografts. Although a wider spectrum of tumors with different sensitivities to chemotherapy is necessary to make stronger conclusions, our data do support an interesting use of ET-743 for platinum-pretreated patients. This data are consistent with the finding that ET-743 acts by a different mechanism of action from that of DDP. Recent studies...
have indicated that ET-743 forms adducts at the position of guanine N2, in the minor groove of DNA. Therefore, although the mechanism of action of ET-743 involves DNA damage, it appears that the DNA lesions caused by this drug are different from those produced by DDP or by conventional alkylators, which form mainly in the major groove of DNA. (8, 16, 17). These mechanistic differences provide a rationale for combining DDP and ET-743 in future preclinical studies to verify the efficacy and toxicity of this drug combination.

Routine analysis of the majority of the preclinical studies based on xenograft response, ET-743 treatment is started in mice bearing ~150 mg of tumor, a size representative of early-stage tumor or a residual tumor after surgery, but it does not reflect a common condition of cancer patients who often receive chemotherapy when the tumor burden is larger. ET-743 given at the optimal dose produced a significant tumor growth delay and partial responses in mice bearing late-stage HOC18 tumor (~400 mg), suggesting that this compound may still be potentially active when the tumor burden is large. With this tumor burden, treatment with DDP was completely inactive (T/C% > 50%; data not shown). Once again, these data support the potent antitumor effect of ET-743 and indicate its use in DDP refractory tumors.

Because the natural anatomical localization of the ovarian carcinoma is the peritoneal cavity (18), ET-743 was also tested on an i.p. tumor model that mimics the growth pattern of the human disease. The treatment was started on late-stage tumor, 10 days after tumor transplant (15). In fact, at this time, all mice developed visible ascites and involvement of the organs of the peritoneal cavity, a common condition in refractory ovarian cancer patients (12, 19–21). ET-743 was highly active on HOC22 xenograft growing in the peritoneal cavity, producing an increment of survival time that was dose dependent. At the highest dose of ET-743, all mice showed complete response and 25% were tumor-free (cured) when examined histologically. At all of the doses (MTD, 1/2 MTD, and 1/4 MTD), ET-743 was more active than DDP tested at its MTD. HOC22 (i.p. variant) and HOC22-S (s.c. variant) are derived from the same patient's tumor. Although both models are responsive to ET-743 treatment, some discrepancy is observed because the HOC22 growing i.p. is significantly more sensitive to ET-743 than to DDP. Whether these findings are due to the selection of the xenograft, the site of growth, or the tumor burden needs further clarification. Nevertheless, from this preclinical study, ET-743 seems to be a very promising treatment for ovarian cancer confined to the peritoneal cavity and suggests its use as a consolidation therapy after DDP-based therapy. The same treatment on another tumor (HOC8) transplanted i.p. in nude mice was effective but to a lesser extent, with no complete responses. On HOC8, ET-743 was less active than DDP. Interestingly, HOC8 was derived from a patient who went into partial remission after DDP-based treatment (22), and this might explain the sensitivity of this tumor model to DDP. Given the mechanistic difference discussed above, it is not surprising that the spectrum of activity of ET-743 does not overlap with that of DDP.

On the basis of its potential new mechanism of action and its activity against in vivo human tumor models, ET-743 is currently undergoing Phase I clinical investigation in Europe and in the United States. By showing that ET-743 is highly active against HOC xenografts, we indicate ovarian carcinoma patients as candidates for Phase II trials. The HOC xenografts described here with their pattern of sensitivity to ET-743 can be a useful model to study the mechanism of action of this compound and to optimize combination therapies of ET-743 with other chemotherapeutic agents.

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