

Therapeutic Targeting of the Endothelin A Receptor in Human Ovarian Carcinoma¹

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ABSTRACT

Fn1-2 The endothelin A receptor (ET_AR) autocrine pathway is overexpressed in many malignancies, including ovarian carcinoma. In this tumor, engagement of ET_AR triggers tumor growth, survival, neoangiogenesis, and invasion. To evaluate whether ET_AR represents a new target in cancer treatment, we examine *in vitro* and *in vivo* the effect of the selective ET_AR antagonist ABT-627 (atrasentan), a small p.o. bioavailable molecule, in mono- and combination therapy with taxane. ABT-627 effectively inhibits cell proliferation, vascular endothelial growth factor (VEGF) secretion of ovarian carcinoma cell lines, and primary cultures. ET_AR blockade also results in the sensitization to paclitaxel-induced apoptosis. In ovarian carcinoma xenografts, in which the ET-1/ET_AR autocrine pathway is overexpressed, tumor growth was significantly inhibited in ABT-627-treated mice compared with control. The therapeutic efficacy of ABT-627 was associated with a significant reduction in microvessel density, expression of VEGF, and matrix metalloproteinase-2, and increased the percentage of apoptotic tumor cells. Combined treatment of ABT-627 with paclitaxel produced additive antitumor, apoptotic, and antiangiogenic effects. These findings demonstrate that the small molecule ABT-627 is a candidate for clinical testing as an antitumor agent in ovarian cancer patients, especially in combination with taxane therapy. Interruption of ET_AR signaling therefore, represents, a promising therapeutic strategy in ovarian carcinoma.

INTRODUCTION

Ovarian cancer is the leading cause of gynecologic cancer-related deaths. About 26,500 women are diagnosed yearly with an overall 5-year survival rate of only 47%. Despite recent advances in cytoreductive surgery and combination chemotherapy, improvement in long-term survival of these patients has been slight (1, 2).

Fn3 The ET³ family is composed of three isopeptides, ET-1, -2, -3, which are potent mitogens for several human tumors including carcinoma of the prostate (3, 4), ovary (5), colon (6), cervix (7), breast (8), endometrium (9), as well as melanoma (10) and Kaposi's sarcoma (11). ETs and their receptors have been implicated in cancer progression through autocrine and paracrine pathways.

ET-1, which is the most common circulating form of ETs, is produced by many epithelial tumors. The peptide signals through two G protein-coupled receptors, ET_A and ET_B, that have different affinities for ETs (12). The ET_B receptor (ET_BR) binds the three peptide isoforms with equal affinity. In contrast, ET_AR binds ET-1 with higher affinity than the other isoforms (13). The ET-1/ET_AR autocrine path-

way has a key role in the development and the progression of prostatic, ovarian, and cervical cancers (14).

We have previously demonstrated that ET-1 and the ET_AR are overexpressed in primary and metastatic ovarian carcinomas, as compared with normal ovaries (5). In ovarian tumor cells, ET-1 acts as an autocrine growth factor selectively through the ET_AR (15). Ligand binding to the receptor results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca²⁺ levels, activation of protein kinase C, MAPK and p125 focal adhesion kinase phosphorylation (13). Among downstream events after ET_AR activation in ovarian carcinoma, ET-1 causes epidermal growth factor receptor transactivation, which is partly responsible for MAPK activation, suggesting that the coexistence of ET-1 and epithelial growth factor autocrine circuits in these tumor cells could enhance their growth potential (16).

Neovascularization is an early and critical event in ovarian cancer progression (17, 18). In this regard, we previously demonstrated that elevated expression of ET-1 and its cognate receptor was significantly associated with MVD and VEGF expression (19). ET-1 modulates various stages of neovascularization, including endothelial cell proliferation, migration, invasion, protease production, and tube formation, and stimulates neovascularization *in vivo* (20, 21). Furthermore, activation of ET_AR by ET-1 stimulates VEGF production by increasing levels of the transcription factor HIF-1 α , a critical regulator of tumor growth and angiogenesis (22).

High levels of ET-1 were detected in the majority of ascitic fluids of ovarian cancer patients and were significantly correlated with VEGF ascitic concentrations, suggesting that ET-1 enhances the secretion of extracellular matrix-degrading proteinases. Thus, ET-1 acting through the ET_AR consistently induces the activity of two families of metastasis-related proteinases, MMPs and the urokinase type plasminogen activator system. Interestingly, we found that the addition of a specific ET_AR antagonist blocked ET-1-induced migration and invasion of ovarian carcinoma cells (23).

ET-1 acts as an antiapoptotic factor, suggesting that the peptide may also modulate cell survival pathways (24, 25). This is further supported by the demonstration that ET-1 is effective in inhibiting PAC-induced apoptosis, and that an ET_AR antagonist completely blocks the ET-1-induced survival effect. Engagement of the ET_AR by ET-1 triggers activation of antiapoptotic signaling through Bcl-2-dependent and phosphatidylinositol 3-kinase-mediated Akt pathway (26).

In view of the above findings, the ET_AR has been proposed as a potential target for anticancer therapy (27). The recent identification of low-molecular-weight compounds that inhibit ligand-induced activation of the ET_AR now offers the possibility of testing this therapeutic approach in a clinical setting. Among various ET_AR antagonists, ABT-627 (atrasentan) is a p.o. bioavailable ET antagonist that potently ($K_i = 34$ pM) and selectively binds to the ET_AR, blocking signal transduction pathways implicated in cancer cell proliferation and in other host-dependent processes that promote cancer growth (28).

In the present study, we tested *in vitro* and *in vivo* the antiprolif-

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³ Abbreviations used are: ET, endothelin; ET_AR, ET receptor A; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; HIF-1, hypoxic inducible factor-1; MMP, matrix metalloproteinase; MVD, microvessel density; PAC, paclitaxel; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling; Ab, antibody.

erative, antiangiogenic, and proapoptotic activities of ABT-627 on human ovarian carcinoma, in which the ET_AR is frequently overexpressed and the ET-1/ET_AR autocrine pathway is biologically active. A large body of experimental and clinical evidence has shown that the cytotoxic activity of certain compounds, such as taxanes, can be enhanced by combination with agents that block growth factor receptors involved in antiapoptotic signaling pathways (29, 30). We, therefore, determined whether ABT-627 has a cooperative effect with PAC, which is currently used in the treatment of human ovarian carcinoma.

MATERIALS AND METHODS

Materials. Clinical grade ABT-627 (atrasentan) was provided by Abbott Laboratories (Abbott Park, IL), and PAC was provided by Bristol Myers Italy.

Primary cell cultures and cell lines. Two primary ovarian carcinoma cells (PMOV1 and PMOV2) were derived from ascitic fluids that were freshly obtained after informed consent from two untreated patients bearing serous ovarian carcinoma (stage III) at the Regina Elena Cancer Institute.

Briefly, cells were harvested by centrifugation at 200 g for 5 min at room temperature, resuspended in Dulbecco's PBS, and then centrifuged through F₁ cell-Histopaque 1077 (Sigma, St. Louis, MO).

Interface cells were washed in culture medium, and 5×10^6 viable cells were seeded in 75 cm² culture flasks. All of the experiments herein reported were conducted between the first and second *in vitro* passage. The purity of primary cultures was assessed by immunophenotyping with a panel of monoclonal Abs recognizing ovarian tumor-associated antigens, such as MOV 18, keratin 7, and CA 125 (kindly provided by Professor M. I. Colnaghi). The human ovarian carcinoma cell line OVCA 433 and HEY cell lines were generous gifts from Prof. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). OVCA 433 was established from ascites obtained from patient with advanced serous ovarian adenocarcinoma (31). The HEY cell line was derived from a xenograft of a peritoneal deposit of a cystoadenocarcinoma of the ovary (32). Cervical carcinoma-derived cell line C33A, purchased from American Type Culture Collection, is human papillomavirus negative, does not produce ET-1, and expresses only mRNA for ET_BR (7). All of the cell lines were cultured in DMEM/10% FCS at 37°C in 5% CO₂/95% air.

Cell Proliferation Assay. Cell lines or primary cultures cultured in DMEM were harvested at 80% confluence and plated at 5×10^4 cells/well in 12-well plates, were cultured for 24 h, and then were incubated in serum-free medium (HEY and OVCA 433) or in low-serum (0.5%) condition (PMOV1, PMOV2, and C33A) for 72 h with different concentrations of the selective non-peptide ET_AR antagonist, ABT-627. For time-dependent studies, ovarian cancer cells were incubated as long as 5 days in the absence or in the presence of ET_BR antagonist BQ 788 (1 μM; Peninsula Laboratories, Belmont, CA) or of ABT-627 (1 μM). At the indicated times, cells were harvested by trypsinization and counted in a hemocytometer. The experiments were performed in triplicate and repeated twice.

Apoptosis Assay. The induction of programmed cell death was determined as described previously (24) by the cell death detection ELISA Plus kit (Boehringer Mannheim, Indianapolis, IN). Briefly, 5×10^4 cell/well were seeded into 12-well plates. After a 48-h treatment, cells were washed once with PBS, and 0.5 ml of lysis buffer was added. After a 30-min incubation, the supernatant was recovered and assayed for histone-associated DNA fragments, as recommended by the manufacturer, at 405 nm by the use of a microplate reader. The experiments were performed in quadruplicate and repeated twice.

Evaluation of VEGF Secretion. HEY cells were plated at 1×10^6 cells/dish and treated for 24 h with 1 μM ABT or 60 nM PAC, alone and in combination in serum-free medium. The concentration of VEGF in the conditioned media obtained from HEY cells was determined three times in duplicate by an ELISA kit using the reagents and protocol supplied with the quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, MN). The intraassay variations were 6.5%.

HEY Xenografts in Nude Mice. Female athymic (nu⁺/nu⁺) mice, 4–6 week of age, were purchased from Charles River Laboratories (Milan, Italy). The treatment protocol followed the guidelines of animal experimentation adopted by the Regina Elena Cancer Institute under the control of the Ministry of Public Health. Mice were given injections s.c. into one flank with 1.5×10^6

viable HEY cells, as determined by trypan blue staining, resuspended in 200 μl of PBS. After 7 days, when established tumors of ~0.2–0.3 cm in diameter were detectable, mice were randomized in groups ($n = 10$) to receive different treatments. One group was treated i.p. for 21 days with ABT-627 at the indicated daily doses (2 mg/kg/day or 10 mg/kg/day). PAC was used as reference compound (20 mg/kg/dose given i.v. every 4 days for three doses). To determine the effects of combined treatment, three different experiments with a total of 40 mice for each experiment were performed. In each experiment, each group consisted of 10 mice. One group was treated i.p. for 21 days with ABT-627 (2 mg/kg/day) dissolved in NaHCO₃ 0.25 N; one group was treated i.v. with PAC (20 mg/kg/dose, given three times, once a day on days 1, 5, and 9) and one group was treated i.v. with PAC in combination with ABT-627 (given i.p. on days 1–21). Control mice were given injections in the same way with 200 μl of drug vehicle. One experiment was terminated after 40 days to allow the harvesting of tumor xenografts for immunohistochemical and Western blot analysis. Tumor size was measured with calipers and was calculated using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$.

Immunohistochemical Analysis. Indirect immunoperoxidase staining was carried out on acetone-fixed 4-μm frozen tissue sections stored at –20°C until use with no loss of activity. The avidin biotin assays were performed using the Vectastain Elite kit (for nonmurine primary Abs) and the MOM kit (for murine primary Abs) obtained from Vector Laboratories. Mayer's hematoxylin was used as nuclear counterstain. Negative control stain was represented by sections in which the incubation with the primary Ab, polyclonal or monoclonal, was either omitted or substituted by isotype-matched immunoglobulins, respectively. The primary Abs used were as follows: anti-ET-1 murine monoclonal Ab (clone TR.ET.48.5; Affinity Bioreagents, Golden, CO) 1:200; rabbit anti-ET_AR and anti-ET_BR (Abbott Laboratories, IL) 1:20; rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) 1:200; monoclonal rat antimouse CD31 (PECAM-1; generously donated by Dr. A. Mantovani, Mario Negri Institute, Milan, Italy); and a monoclonal anti-MMP-2 (Oncogene Research Products, Cambridge, MA) 1:20. The VEGF (147) Ab is a rabbit polyclonal IgG raised against a NH₂-terminal epitope (1–140) common to all splice variants of VEGF. For ET_AR, an Ab was raised against a decapeptide (DNPERYSTNL) of its extracellular NH₂-terminal domain, and for ET_BR an Ab was raised against a peptide (CGLSRIWGEERGFPDRTP) of its NH₂-terminal domain. Vessel count was performed by two independent observers on a $\times 200$ field, according to the criteria of Weidner *et al.* (33). To quantify apoptosis, TUNEL assay was performed using a commercially available *in situ* apoptosis detection kit (Boehringer Mannheim). For the quantification of total TUNEL expression, the number of apoptotic cells was counted in 10 randomly selected fields ($\times 200$) as a percentage of total cells using an immunofluorescence microscopy.

Western Blot Analysis. Total cell lysates were obtained from homogenized HEY tumor specimens. The protein extracts were resolved by 7.5% SDS-PAGE and probed with an anti-VEGF polyclonal Ab (Santa Cruz Biotechnology). Immunoreactive proteins were visualized by enhanced chemoluminescence (Amersham International) according to the manufacturer's instructions.

Statistical Analysis. Statistical evaluations of data were made by the two-sided Student's test with Bonferroni corrections. Time course of tumor growth was compared across the treatment groups with the use of two-way ANOVA, with group and time as the variables.

RESULTS

Inhibition of Ovarian Carcinoma Cell Proliferation and Potentiation of PAC-induced Apoptosis by ET_AR Blockade. To evaluate the effect of ABT-627 on the proliferation of various ovarian carcinoma cells, we used two primary cultures (PMOV1 and PMOV2) and two established cell lines (HEY and OVCA 433). All of these cells express functional ET_AR, ranging from ~20,000 (PMOV1 and PMOV2) to 35,600 (HEY) and 43,600 (OVCA 433) ET-1 binding sites/cell, and secrete high levels of ET-1 (15, 34). We treated these ovarian cancer cells with different concentrations of ABT-627 for 72 h and measured the effect of ABT-627 on cell viability. Treatment with ABT-627 at doses ranging between 0.01 and 2 μM determined a dose-dependent inhibition of spontaneous growth rate with a compa-

able IC_{50} of $\sim 1 \mu\text{M}$ in all of the tested cell lines. The inhibitory effect of the ABT-627 was absent in C33A cells, a cervical cell line not expressing ET_AR (7, 35), indicating that only ET_AR-expressing cells respond to growth inhibition by ABT-627. To determine whether there was a time-dependent effect of the ABT-627 treatment on the growth inhibition, primary cell cultures and cell lines were incubated for up to 5 days in the absence or presence of ET_AR (ABT-627, $1 \mu\text{M}$) and ET_BR (BQ-788, $1 \mu\text{M}$) antagonists. In all of the ovarian carcinoma cells, spontaneous growth was significantly inhibited in the presence of ABT-627. The addition of the ET_BR antagonist did not affect the basal growth rate of the cells, even in PMOV1 cells, which coexpressed mRNA for ET_A and ET_B receptors (34). These studies demonstrate that endogenous ET-1 acts as an autocrine modulator of ovarian carcinoma cell proliferation only through ET_AR, which was selectively inhibited by ABT-627 (Fig. 1).

To determine whether the antiproliferative effect of ABT-627 resulted in the induction of programmed cell death, we evaluated the percentage of dying cells in ABT-627-treated and control cultures. As shown in Fig. 2, ABT-627 ($1 \mu\text{M}$) treatment increased the percentage of apoptotic HEY and OVCA 433 ovarian cancer cells after 48 h of treatment ($P \leq 0.05$). In these cells, activation of ET_AR by ET-1 prevents PAC-induced apoptosis (26). We, therefore, evaluated the potential combined proapoptotic effect of treatment with ET_AR antagonist and PAC. HEY and OVCA 433 cells were incubated with 60

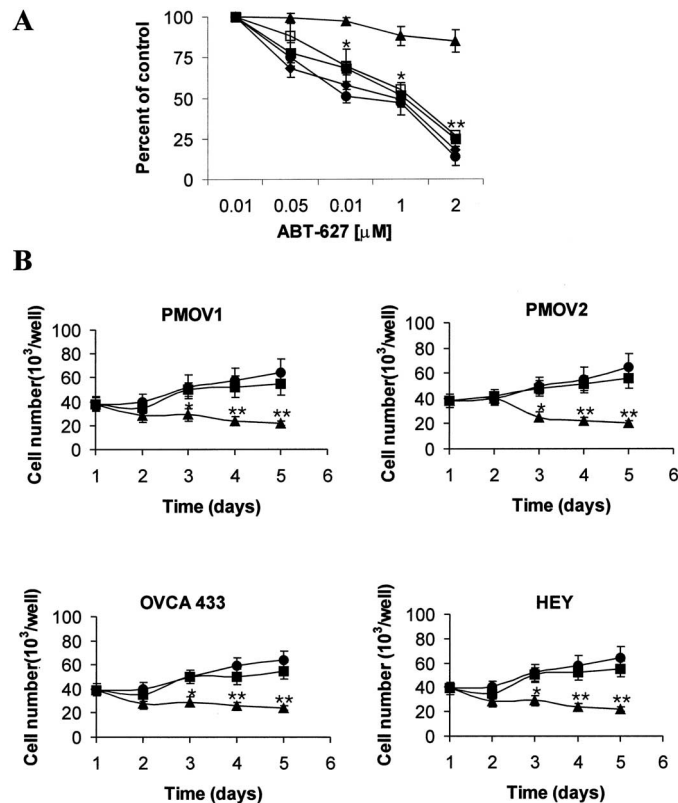


Fig. 1. Effect of ABT-627 on the spontaneous growth rate of primary cultures and cell lines of ovarian carcinoma. A, dose-dependent effect of ABT-627. PMOV1 (○), PMOV2 (□), HEY (■) and OVCA 433 (●) ovarian cancer cells and C33A (▲) cervical cancer cells were seeded at 5×10^4 cells/well in 12-well plates and were incubated for 72 h in the presence of different concentrations of ABT-627. Data are the average of two different experiments, each performed in triplicate. Error bars, mean \pm SD. *, $P < 0.01$; **, $P < 0.001$ B, time-dependent effect of ABT-627. Ovarian carcinoma cells were seeded at 5×10^4 cells/well in 12-well plates. PMOV1 and PMOV2 cells were cultured in 0.5% FCS medium and HEY and OVCA 433 cells were cultured in serum-free medium in the absence (●) or presence of $1 \mu\text{M}$ ABT-627 (▲) or $1 \mu\text{M}$ BQ 788 (■), and cell numbers were measured at the indicated times in a hemocytometer. Error bars, mean \pm SD of data from two independent experiments, each of which was performed in triplicate. *, $P < 0.05$; **, $P < 0.001$ compared with control.

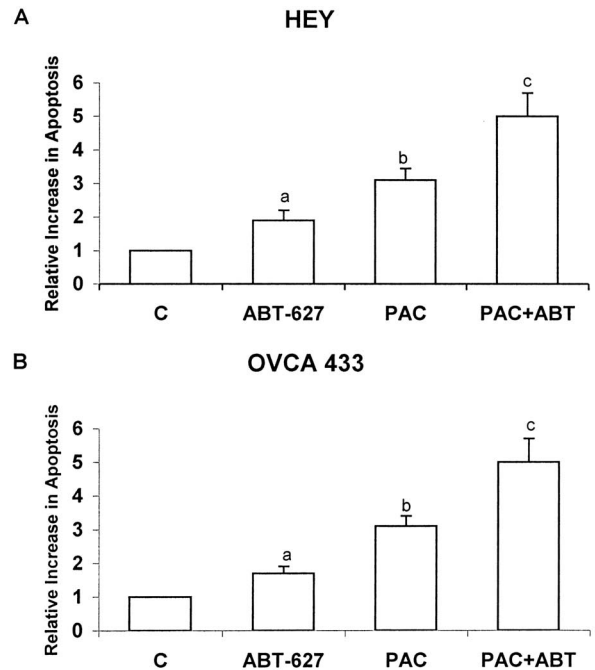


Fig. 2. Induction of apoptosis by treatment with ABT-627 alone and in combination with PAC in ovarian carcinoma cells. HEY (A) and OVCA 433 (B) cells were serum-starved for 24 h and then untreated or treated for 48 h with ABT-627 alone ($1 \mu\text{M}$) or with PAC (60 nM) alone or in combination (PAC+ABT). Data are expressed in arbitrary units as relative increase compared with untreated cells considered as 1. The absolute percentage of apoptotic cells in the control is 17% for HEY and 15% for OVCA 433. Data represent the averages of quadruplicate determinations of two separate experiments; error bars, mean \pm SD. a, $P < 0.05$; b, $P < 0.001$; c, $P < 0.0001$.

nM PAC alone or in combination with ABT-627 ($1 \mu\text{M}$). As expected, the addition of ABT-627 significantly increased PAC-induced apoptosis ($P \leq 0.0001$) in both cell lines (Fig. 2). These results established that ET_AR-activated autocrine survival pathways were affected by treatment with ABT-627.

Effect of ET_AR Antagonist on VEGF Production. ET_AR overexpression can promote tumor development through its stimulatory action on cancer cell growth. However, ET_AR may also regulate angiogenesis by promoting the tumor production of VEGF (19, 22). Endogenous levels of the angiogenic factor, VEGF, were measured by ELISA in the conditioned medium of HEY cells incubated in serum-free medium for 24 h. Untreated HEY cells secreted ~ 375 pg VEGF/ 10^6 cells/24 h. Treatment with either ABT-627 ($1 \mu\text{M}$) or PAC (60 nM) alone caused an $\sim 45\%$ inhibition of VEGF secretion ($P \leq 0.001$, compared with control). The combination of ABT-627 with PAC exerted a marked inhibitory effect, reaching almost 60% reduction of VEGF secretion to 150 pg/ 10^6 cells/24 h ($P \leq 0.001$; Fig. 3).

Inhibition of Growth of Human HEY Ovarian Carcinoma in Nude Mice. The potential antitumor effect of ABT-627 *in vivo* was assessed in murine tumor xenografts. Human ovarian carcinoma cells HEY, which overexpress ET_AR and secrete high levels of ET-1 (34), were grown as s.c. tumors in nude mice. Seven days later, when well-established HEY xenografts were palpable with a tumor size of ~ 0.25 cm³, mice were randomized into treatment and vehicle control groups of 10 animals each. The treated mice were given injections i.p. for 21 days with two different concentrations of ABT-627, 2 mg/kg/day and 10 mg/kg/day. Treatment with ABT-627 produced a 65% inhibition of HEY tumor growth on day 40 after tumor injection with either low (2 mg/kg/day) or high (10 mg/kg/day) doses ($P < 0.001$ compared with control; Fig. 4A). ABT-627 treatment was generally well tolerated with no detectable signs of acute or delayed toxicity, even at the highest ABT-627 dose (10 mg/kg/day). Tumor

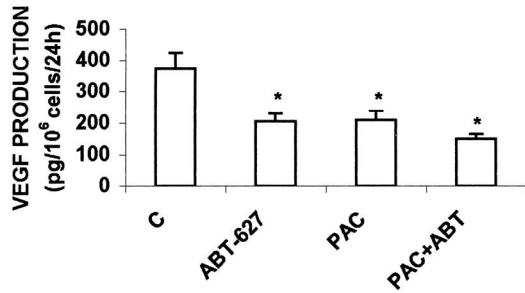


Fig. 3. Effects of ABT-627, PAC, or ABT-627 plus PAC in the conditioned media of HEY cells. HEY cells were incubated with 1 μ M ABT-627, 60 nM PAC, or in combination (PAC+ABT). VEGF production is reported as means of results from three ELISA determinations, each performed in duplicate. Error bars, \pm SD; *, $P \leq 0.001$.

growth suppression by treatment with 2 mg/kg/day ABT-627 was comparable with that achieved by treatment with PAC (20 mg/kg i.v. given three times, once a day on days 1, 5, and 9). Immunohistochemical analysis using specific anti-ET-1, anti-ET_AR Abs demonstrated that tumor xenografts expressed detectable levels of ET-1 and ET_AR (data not shown). These results are consistent with the concept that ABT-627 suppresses the growth of ET_AR-expressing tumors in nude mice. The comparison of time course of tumor-growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.0001$; Fig. 4). Furthermore, the tumor growth inhibition obtained with ABT-627 persisted for up to 4 weeks after the termination of treatment.

We next evaluated whether the cooperative proapoptotic effect of ABT-627 and PAC that was observed *in vitro* could also be obtained *in vivo*. For combined treatment, a 2-mg/kg/day dose of ABT-627 was selected because it induced a 65% inhibition of tumor growth, was well tolerated, and corresponded to that used in human clinical trials (28). This dose of ABT-627 was given i.p. for 21 days in combination with three i.v. administrations of PAC (20 mg/kg) given three times,

once a day on days 1, 5, and 9. More marked tumor growth inhibition (90% of controls) was elicited by combined treatment with ABT-627 and PAC ($P < 0.0001$; Fig. 5). HEY tumor xenografts, freshly excised on day 40 after tumor cell injection, were analyzed for tumor growth inhibition and immunohistochemically. As shown in Table 1, the combined treatment was highly effective with no histological evidence of HEY tumors in 4 of 10 mice. The dual treatment at the dose and schedule tested were well tolerated, as judged by the absence of weight loss or other signs of acute or delayed toxicity. We, therefore, by maintaining the treated animal cohort up to 66 days, determined the duration for which bioactivity persisted with combined PAC and ABT-627 treatment. As compared with control tumor xenografts, the growth delay in established tumors persisted for up to 4 weeks after the termination of treatment with ABT-627 combined with PAC (Fig. 5)

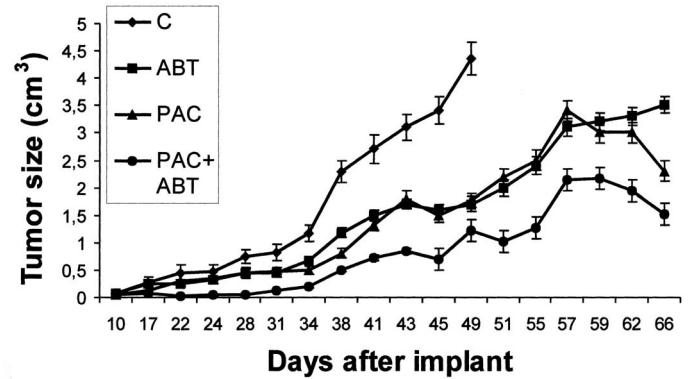


Fig. 5. Antitumor activity of ABT-627 treatment in combination with PAC on established HEY human ovarian carcinoma xenografts. Mice were given injections of 1.5×10^6 HEY cells s.c. in the dorsal flank. After 7 days, the mice were treated i.p. for 21 days with vehicle (\diamond , control) or with ABT-627 (2 mg/kg/day) alone (\blacksquare), or with PAC alone (\blacktriangle , 20 mg/kg i.v. every 4 days for three doses), or in combination with PAC (\bullet). Three different experiments with a total of 40 mice for each experiment were performed. In each experiment, each group consisted of 10 mice. Data points, averages; error bars, \pm SD.

Fig. 4. A, antitumor activity of ABT-627 treatment on established HEY human ovarian carcinoma xenografts. Mice were given injections of 1.5×10^6 HEY cells s.c. into the dorsal flank. After 7 days, mice were treated i.p. for 21 days with vehicle (control, open bars), two doses of ABT-627 (2 mg/kg/day, closed bars; or 10 mg/kg/day, hatched bars); error bars, \pm SD. B, antitumor effect of ABT-627 (2 mg/kg/day, \bullet). PAC was used as reference compound (20 mg/kg i.v. every 4 days for three doses). Control mice were treated with vehicle (\blacksquare). Each group consisted of 10 mice. Error bars, \pm SD. C, representative example of nude mice 5 weeks after tumor implant showing a vehicle-treated mouse (left) and a mouse that received 2 mg/kg/day of ABT-627 for 21 days (right).

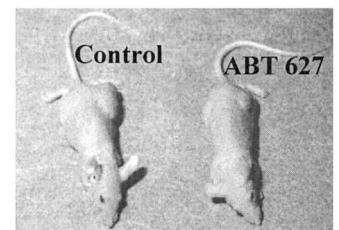
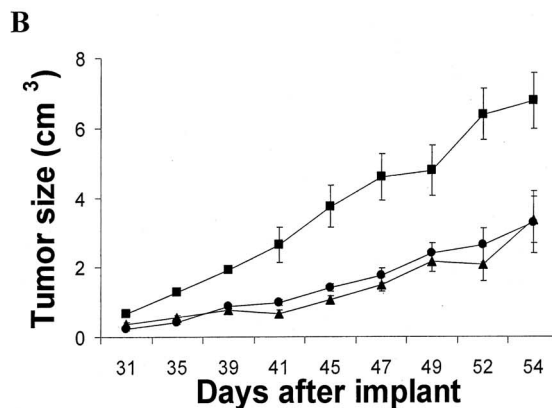
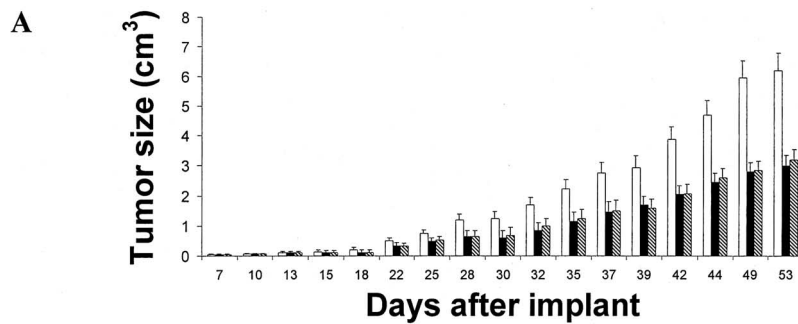


Table 1 Immunohistochemical analysis of HEY ovarian carcinoma xenograft after treatment with ABT-627 and PAC

Nude mice were given a s.c. injection of 1.5×10^6 viable HEY cells on day 0. Treatment was started on day 7 after tumor injection. Mice were treated for 21 days i.p. with ABT-627 (2 mg/kg/day) or with PAC (20 mg/kg i.v. every 4 days for 3 doses). Mice were sacrificed on day 40 after tumor injection, and immunohistochemical analysis was performed. Each group consisted of 10 mice. No histological evidence of HEY tumor was detected in 4 of 10 mice treated with the combination of PAC and ABT-627.

Treatment	Tumor volume (cm ³ ± SD)	Mice tumor-free	Median vessel density ^a	Apoptotic cells ^b	% positive cells ^c	
					VEGF	MMP-2
Control	2.6 ± 1.29	0/10	152.5 ± 10	2.4 ± 1.5	58.3 ± 5	74.1 ± 2
ABT-627, 2 mg/kg	0.87 ± 0.36 ^d	0/10	80 ± 4.9 ^e	12.9 ± 3.3 ^e	31.9 ± 2 ^e	23.9 ± 4 ^e
PAC, 20 mg/kg	0.82 ± 0.57 ^d	0/10	76 ± 9.9 ^e	13.3 ± 2.2 ^e	36 ± 3 ^e	25 ± 6 ^e
ABT-627 + PAC	0.26 ± 0.1 ^e	4/10	51.5 ± 2.1 ^e	21.1 ± 3.3 ^e	21.9 ± 4 ^e	18.8 ± 4 ^e

^a Vessel counts were assessed by light microscopy after staining for CD31. Areas containing the highest numbers of capillaries and small venules were identified by scanning at low power, and individual vessel counts were performed at $\times 200$.

^b The relative number of apoptotic cells was quantitatively assessed in 10 randomly selected fields ($\times 200$). TUNEL staining was expressed as a percentage of positive cells ± SD.

^c The percentage of positive cells was scored by the averaging of five field counts ($\times 200$).

^d $P < 0.001$.

^e $P < 0.0001$.

Effect of ABT-627 Treatment on Angiogenesis and Apoptosis *in Vivo*. Because HEY cells express ET_AR and various autocrine and paracrine angiogenesis-related factors including ET-1, VEGF, and MMP-2, we evaluated the expression of these factors *in vivo* after ABT-627 treatment at a lower dosage (2 mg/kg/day). Immunohistochemical evaluation of the expression of VEGF, performed on HEY tumors on day 40 after tumor cell injection, revealed a marked reduction (45%) in the percentage of VEGF-positive HEY cells in ABT-627-treated mice ($P \leq 0.0001$; Table 1; Fig. 6). Tumor-induced

vascularization, which was quantified as MVD using Ab against CD31, was directly proportional to the expression of VEGF. There was a parallel reduction in MVD in tumors after treatment with ABT-627 (45% inhibition compared with control tumors; $P \leq 0.0001$; Fig. 6; Table 1).

Activation of ET_AR in HEY cells leads to up-regulation of MMP secretion and activation, as well as promotion of cell invasion (23). In this context, we evaluated by immunohistochemistry whether ET_AR blockade inhibits MMP-2 expression. ABT-627 treatment signifi-

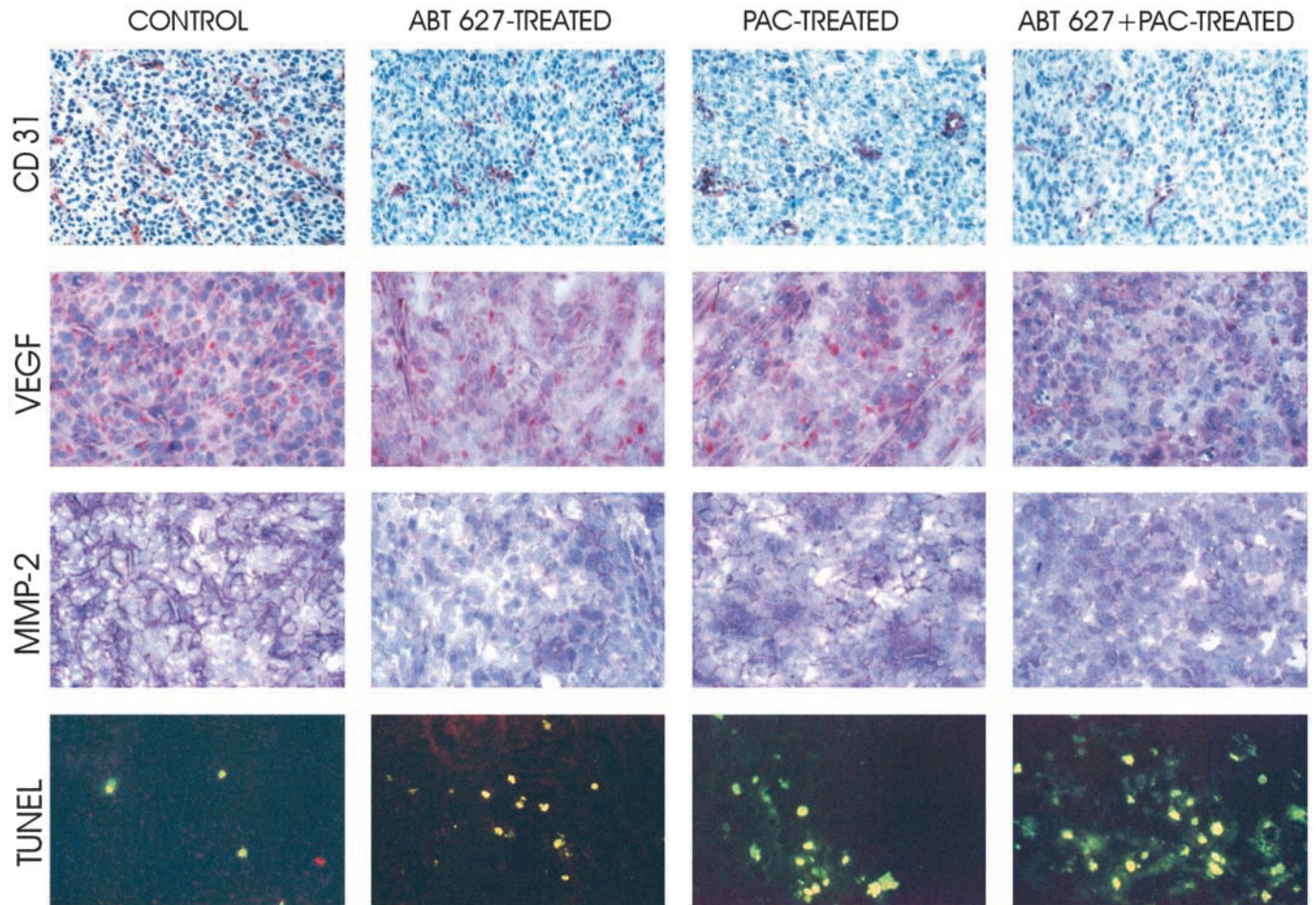


Fig. 6. Immunohistochemical analysis. Mice bearing HEY tumor xenografts were treated i.p. for 21 days with ABT-627 (2 mg/kg/day) alone, or with PAC (20 mg/kg/day i.v. every 4 days for three doses), or with ABT-627 plus PAC. Tumors were removed from controls and from treated mice and were snap-frozen in liquid nitrogen on day 40 after tumor injection. Cryostat sections were immunostained for expression of CD31, VEGF, MMP-2, and apoptotic tumor cells as described in experimental procedures. The panels demonstrate that maximal inhibition of MVD, decrease in VEGF and MMP-2 expression, and increase in apoptotic tumor cells were achieved by combined ABT-627 and PAC treatment compared with single-agent administration (CD31, VEGF, MMP-2, or TUNEL. $\times 200$).

cantly reduced the percentage of HEY cells positive for MMP-2 (67% inhibition compared with control; $P \leq 0.0001$; Fig. 6; Table 1).

On the basis of the knowledge that ABT-627 induces apoptosis by blocking the ET-1/ET_AR antiapoptotic pathway, we also evaluated the effect of such treatment on the induction of apoptosis in HEY xenografts, by using the *in situ* TUNEL method. A significant increase in the percentage of TUNEL-positive cells was found in HEY tumors treated with ABT-627 ($P \leq 0.001$; Fig. 6; Table 1).

Finally, because ABT-627 treatment significantly enhances the antitumor activity of the cytotoxic drug PAC, which has been shown to affect tumor neovascularization *in vivo* (36), we tested whether its therapeutic efficacy was directly correlated with a decrease in proangiogenic molecules (VEGF, MMP-2), in MVD, and with an increase in apoptosis of tumor cells. Under our experimental conditions, PAC treatment caused a reduction in MVD, VEGF, and MMP-2 expression, and an increase in the tumor apoptotic index similar to that induced by ABT-627 treatment. Almost complete inhibition of VEGF, MMP-2 expression, and tumor neovascularization, and an increase in apoptosis, were observed after combined treatment of ABT-627 (2 mg/kg/day) with PAC (20 mg/kg/day; $P \leq 0.0001$; Fig. 6; Table 1). HEY tumor xenografts, freshly excised on day 40 after tumor cell injection, were also analyzed for VEGF expression by Western blots (Fig. 7). We observed a marked reduction of VEGF expression in animals treated with ABT-627 or PAC. Approximately 70% inhibition was obtained when ABT-627 was combined with PAC.

DISCUSSION

The ET_AR autocrine pathway contributes to ovarian cancer progression by inducing cell proliferation, survival, angiogenesis, and metastatic spread (5, 26, 19, 23). These results suggest that the pharmacological inhibition of the ET_AR signaling pathway may improve cancer treatment (37). A promising approach in this context has been the development of small molecules capable of inhibiting the binding of the endogenous ligand to the ET_AR. Among these novel compounds, ABT-627 is a potent and p.o. bioavailable antagonist that reverses or blocks the ET-1-mediated effects *in vitro* and *in vivo* (27).

In view of this, the therapeutic potential of ABT-627 was evaluated by analyzing its activity on cell proliferation, neovascularization, induction of apoptosis, and tumor growth inhibition in ovarian carcinoma cells with a functional ET_AR-driven autocrine pathway. ABT-627 treatment inhibited cell proliferation and increased programmed cell death in primary cultures and ovarian carcinoma cell lines. Furthermore, ABT-627 treatment with no associated toxicity displayed antitumor activity *in vivo* against established HEY cancer xenografts in nude mice. The extent of tumor inhibition was similar to that obtained using the cytotoxic drug PAC. Of major interest, enhancement of this antitumor activity was observed when mice were treated with ABT-627 in combination with PAC. The combination treatment produced complete clinical and cytological tumor regression in 4 of 10 mice on day 40 after tumor injection. Furthermore, in the remaining animals, this schedule caused almost complete suppression of tumor growth.

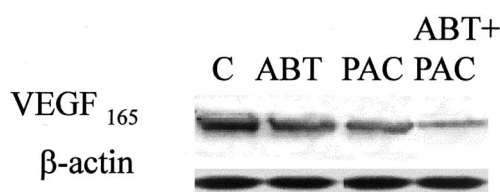


Fig. 7. Effects of the different treatments on VEGF expression in tissue specimens. VEGF protein levels were evaluated in tissue lysates by Western blotting.

Elevated expression of ET-1 and its receptors have been shown to be significantly associated with increased vascularization, as assessed by MVD and VEGF production (19). These effects are attributable to the ability of ET-1, acting through the ET_AR, to stimulate VEGF production by increasing HIF-1 α to an extent comparable with that induced by hypoxia (22). Because increased HIF-1 activity may also influence tumor progression independent of its regulation of VEGF expression (38), the inhibition of HIF-1 α could have therapeutic activity especially in those malignancies, such as ovarian tumors, in which HIF-1 overexpression correlates with mortality (39). Furthermore, ET_AR activation by ET-1 promotes ovarian carcinoma cell migration and invasion by up-regulating the secretion and activation of multiple tumor proteinases (23). Because the above pleiotropic activities of ET-1 are inhibited *in vitro* by ET_AR antagonists, we determined whether treatment with ABT-627 that resulted in tumor growth inhibition was associated *in vivo* with the inhibition of the expression of factors that are relevant for tumor progression, including invasion, angiogenesis, and metastasis. Immunohistochemical analysis of the tumor xenografts, indeed, demonstrated that ABT-627 treatment *per se* is capable of producing a significant decrease in VEGF expression and MVD. The selective ET_AR antagonist, therefore, appears to exert an *in vivo* antitumor effect that may be attributable to direct blockade of the ET_AR-dependent mitogenic pathway and, at least in part, to the inhibition of VEGF secretion that sustains the proliferation of tumor blood vessels.

Tumors of ABT-627-treated animals exhibited also a significant decrease in MMP-2 expression, which is a critical mediator of invasiveness in ovarian carcinoma (40). Although other proteases are likely to be involved, the inhibition of MMP-2 activity is likely to represent a relevant mechanism by which ABT-627 could inhibit tumor invasiveness.

Recent findings have shown that in tumor vascularization, “vasculogenic mimicry” (*i.e.*, the presence of vascular channels, cord, and sinuses that are formed by tumor cells and that lack endothelial cell lining, and mosaic vessels that are lined by endothelial and tumor cells) may occur (41). This is also the case for ovarian tumors, because invasive ovarian cancer cells have been shown to form microvascular channels (42). In high-stage, high-grade ovarian cancers, 7–10% of channels containing RBCs were lined by tumor cells. Interestingly, the invasive ovarian cancer cells that are capable of generating tubular networks *in vitro* expressed both MMPs and ET_AR.⁴ Tumor cells displaying a molecular vasculogenic phenotype may serve as an important indicator of tumor plasticity in a growing tumor mass, requiring an aggressive treatment regimen. Therefore, the antiangiogenic effect of ABT-627 treatment in our model might also be explained by its interference with the formation of microvascular channels lined by tumor cells overexpressing ET_AR and MMP-2, in the absence of endothelial cells expressing ET_BR (43–45).

The enhancement of antitumor activity was also accompanied by a significant decrease in the expression of VEGF and MMP-2 and, hence, a decrease in mean MVD and an increase in apoptotic tumor cells (TUNEL +). ET-1 acts through ET_AR as a survival factor by protecting HEY and OVCA 433 cells against PAC-induced apoptosis via activation of antiapoptotic signaling pathways (26). Because impairment of apoptotic pathways is a major molecular mechanism leading to chemoresistance (46), ET_AR blockade by ABT-627 leading to the sensitization of tumor cells to PAC-induced apoptosis could produce an additive therapeutic effect. This, indeed, seems to occur *in vivo* after combined treatment with ABT-627 and PAC, because the inhibition of tumor growth achieved with this protocol correlates with the highest tumor apoptotic index.

Preliminary results of two independent Phase II studies performed in

⁴ Unpublished observations.

prostate cancer-bearing patients have shown the feasibility of oral administration of ABT-627 for prolonged periods with no major toxic effects at doses that provided steady-state plasma level within the biologically effective concentrations demonstrated in the present study (28, 47–49).

In conclusion, these findings demonstrate the antitumor activity of ABT-627 and provide a rationale for the clinical evaluation of this molecule, alone and in combination with cytotoxic drugs such as taxanes in patients with ovarian tumors and, potentially, in other epithelial tumors that overexpress functional ET_AR (50).

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