Therapeutic Targeting of the Endothelin-A Receptor in Human Ovarian Carcinoma: Efficacy of Cytotoxic Agents is Markedly Enhanced by Co-administration with Atrasentan

Laura Rosanò, Francesca Spinella*, Valeriana Di Castro*, Pier Giorgio Natali†, and Anna Bagnato**

Abstract: The endothelin-1/endothelin-A receptor autocrine pathway is overexpressed in ovarian carcinoma. We explored the efficacy of atrasentan (ABT-627), a small orally active endothelin-A receptor antagonist, in monotherapy and combination therapy on HEY ovarian carcinoma xenografts. Atrasentan (2 mg/kg per 24 hours i.p. for 21 days) induced similar inhibition of tumor growth as paclitaxel (20 mg/kg i.v. three times a day every 4 days) with a reduction of 65% compared to control. The co-administration of atrasentan enhanced the efficacy of cytotoxic agents, such as taxanes or platinum compounds. Administration of atrasentan in combination with paclitaxel caused a strong antitumor effect. Remarkably, four of ten mice bearing HEY xenografts had no histological evidence of tumors. Tumor growth inhibition was accompanied by a significant decrease of molecular effectors involved in angiogenesis and invasion and by enhanced tumor cell apoptosis. Moreover, although cisplatinum as a single agent (5 mg/kg i.p. on day 1) markedly inhibited HEY tumors, atrasentan was very effective in potentiating this effect, with partial or complete tumor regression. The antitumor, anti-angiogenic, and apoptotic activities obtained with atrasentan and the enhanced efficacy of cytotoxic agents provide a rationale for its clinical evaluation in ovarian carcinoma.

Key Words: endothelin-1 (ET-1), ABT-627, paclitaxel, cisplatinum, ovarian carcinoma

(*J Cardiovasc Pharmacol*™ 2004;44(suppl 1):S132–S135)

The endothelin (ET) family is composed of three isopeptides, endothelin-1 (ET-1), the most common circulating form of ET, endothelin-2 (ET-2) and endothelin-3 (ET-3), which are potent mitogens for several human tumors.[1] The peptides signal through two G-protein-coupled receptors, endothelin-A (ET_A) and endothelin-B (ET_B), which have different affinities for ET. The ET_B receptor binds the three peptide isotypes with equal affinity. In contrast, the ET_A receptor binds ET-1 with higher affinity than the other isoforms.[2] Endothelins and their receptors have been implicated in cancer progression through autocrine and paracrine pathways.[3] In particular, the ET-1/ET_A receptor autocrine pathway has a key role in the development and the progression of prostatic, ovarian and cervical cancers.[4–7]

We have previously demonstrated that ET-1 and the ET_A receptor are overexpressed in primary and metastatic ovarian carcinomas when compared with normal ovaries.[6] In ovarian tumor cells, ET-1 acts as an autocrine growth factor selectively through the ET_A receptor.[8] Ligand binding to the receptor results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca^{2+} levels, activation of protein kinase C, mitogen-activated protein kinase and p125 focal adhesion kinase phosphorylation.[9] Among downstream events after ET_A receptor activation in ovarian carcinoma, ET-1 causes epidermal growth factor receptor transactivation, which is partly responsible for mitogen-activated protein kinase activation, suggesting that the coexistence of ET-1 and epidermal growth factor autocrine circuits in these tumor cells could enhance their growth potential.[10] Activation of ET_A receptor by ET-1 stimulates vascular endothelial growth factor (VEGF) production by increasing levels of the transcription factor hypoxia-inducible factor-1α, a critical regulator of tumor growth and angiogenesis.[11,12]

High levels of ET-1 were detected in the majority of ascitic fluids of ovarian cancer patients suggesting that ET-1 enhances the secretion of extracellular matrix-degrading proteinases.[11] Thus, ET-1 acting through the ET_A receptor consistently induces the activity of two families of metastasis-related proteinases, matrix metalloproteinases (MMP) and the urokinase-type plasminogen activator system. Interestingly, we found that addition of a specific ET_A receptor antagonist blocked ET-1-induced migration and invasion of ovarian carcinoma cells.[13] Furthermore ET-1/ET_A receptor induces loss of gap junction intercellular communication (GJIC) through a phosphorylation of the GJIC protein, connexin 43.[14]

Laboratories of *Molecular Pathology and Ultrastructure, and †Immunology, Regina Elena Cancer Institute, Rome, Italy

Address correspondence and reprint requests to Anna Bagnato, Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute, Via delle Messi D'Oro 156, 00158 Rome, Italy. E-mail bagnato@ifo.it

Endothelin-1 acts as an antiapoptotic factor, suggesting that the peptide may also modulate cell survival pathways. This is further supported by the demonstration that ET-1 is effective in inhibiting paclitaxel-induced apoptosis, and that an ET_A receptor antagonist completely blocks the ET-1-induced survival effect. Engagement of the ET_A receptor by ET-1 triggers activation of antiapoptotic signaling through Bcl-2-dependent and phosphatidylinositol 3-kinase-mediated AKT pathway.[15]

In view of the above findings, the ET_A receptor has been proposed as a potential target for anticancer therapy.[1] The recent identification of low molecular weight compounds that inhibit ligand-induced activation of the ET_A receptor now offers the possibility of testing this therapeutic approach in a clinical setting. Among various ET_A receptor antagonists, ABT-627 (atrasentan) is an orally active antagonist that potently (K_i = 34 p*M*) and selectively binds to the ET_A receptor, blocking signal transduction pathways implicated in cancer cell proliferation and other host-dependent processes promoting cancer growth.[16]

Ovarian cancer has the highest mortality rate among gynecological cancers. 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.[17,18] In the present study, we determined whether ABT-627 has a cooperative effect with paclitaxel and cisplatinum, which are currently used in the treatment of human ovarian carcinoma.

METHODS

Materials

Clinical grade ABT-627 (atrasentan) was provided by Abbott Laboratories (Abbott Park, IL, USA), and paclitaxel was provided by Bristol Myers (Sermoneta, Italy), and cisplatinum by Teva Pharma BV (Mijdrecht, Netherlands).

Cell Lines

The human ovarian carcinoma cell line HEY was a generous gift from Professor Giovanni Scambia (Catholic University School of Medicine, Rome, Italy) and was derived from a peritoneal deposit of a cystoadenocarcinoma of the ovary.[19] All cell lines were cultured in Dulbecco's modified Eagle's medium/10% fetal calf serum at 37°C in 5% CO_2/95% air.

HEY Xenografts in Nude Mice

Female athymic (nu^+/nu^+) mice, 4–6 weeks of age, were purchased from Charles River Laboratories (Milan, Italy). The treatment protocol followed the guidelines of animal experimentation of the Italian Ministry of Health.

Mice were injected subcutaneously on one flank with 1.5×10^6 viable HEY cells, as determined by trypan blue staining, resuspended in 200 μL of phosphate-buffered saline. After 7 days, when established tumors of approximately 0.2–0.3 cm^3 in diameter were detectable, mice were randomized in groups (n = 10) to receive different treatments. One group was treated intraperitoneally for 21 days with ABT-627 at 2 mg/kg per day. Paclitaxel was used as reference compound (20 mg/kg per dose given i.v. three times a day every 4 days).

In order to determine the effects of combined treatment, three different experiments with a total of 60 mice for each experiment were performed. In each experiment, each group consisted of 10 mice. One group was treated intraperitoneally for 21 days with ABT-627 (2 mg/kg per day) dissolved in $NaHCO_3$ 0.25 N; one group was treated intravenously with paclitaxel (20 mg/kg per dose, three times a day every 4 days); one group was treated intravenously with paclitaxel in combination with ABT-627 (given i.p. on days 1–21); one group with cisplatinum as a single agent (5 mg/kg i.p. on day 1); and one group with cisplatinum in combination with ABT-627. Control mice were injected in the same way with 200 μL of drug vehicle. One experiment was terminated after 40 days to allow harvesting of tumor xenografts for immunohistochemical and Western blot analysis. Tumor size was measured with calipers and was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2.

Statistical Analysis

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

RESULTS

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 HEY cells, which overexpress ET_A receptor and secrete high levels of ET-1,[20] were grown as subcutaneous tumors in nude mice. Seven days later, when well established HEY xenografts were palpable with a tumor size of ~0.25 cm^3, mice were randomized into treatment and vehicle control groups of 10 animals each. The treated mice were injected intraperitoneally for 21 days with ABT-627 (2 mg/kg per day). Treatment with ABT-627 produced a 65% inhibition of HEY tumor growth on day 40 after tumor injection ($P < 0.001$ compared with control). ABT-627 treatment was well tolerated because no detectable signs of acute or delayed toxicity were observed. Tumor growth suppression by treatment with 2 mg/kg per day ABT-627 was comparable to that achieved by treatment with paclitaxel (20 mg/kg i.v. three times a day every 4 days). Comparison

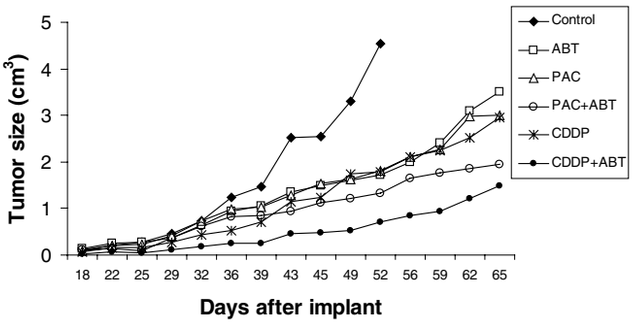

FIGURE 1. Antitumor activity of ABT-627 treatment in combination with paclitaxel or cisplatinum on established HEY human ovarian carcinoma xenografts. Mice were injected subcutaneously in the dorsal flank with 1.5×10^6 HEY cells. After 7 days the mice were treated intraperitoneally for 21 days with vehicle (control), or with ABT-627 (ABT) (2 mg/kg per day) alone, or with paclitaxel (PAC) alone (20 mg/kg i.v. three times a day every 4 days), or ABT-627 in combination with paclitaxel (PAC+ABT), or with cisplatinum (CDDP) (5 mg/kg i.p. on day 1 of treatment) alone, or cisplatinum in combination with ABT-627 (CDDP+ABT). Three different experiments with a total of 60 mice (10 mice per group) were performed. Data represent the averages. Comparison of time-course of tumor growth curves by two-way analysis of variance with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P \leq 0.0001$).

of time-course of tumor growth curves by two-way analysis of variance, with group and time as variables, showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.0001$) (Fig. 1). Furthermore, the tumor growth inhibition obtained with ABT-627 persisted for up to 4 weeks following termination of treatment.

For combined treatment, the 2 mg/kg per day dose of ABT-627, which corresponds to that used in human clinical trials,[21,22] was given intraperitoneally for 21 days in combination with three intravenous administrations of paclitaxel (20 mg/kg) given three times every 4 days (on day 1, day 5 and day 9) and with cisplatinum (given i.p. 5 mg/kg on day 1). Although cisplatinum as a single agent (5 mg/kg i.p. on day 1) markedly inhibited HEY tumors, atrasentan was very effective in potentiating this effect, with partial or complete tumor regression. As shown in Figure 1, more marked tumor growth inhibition (90% of controls) was elicited by combined treatment with ABT-627 and paclitaxel and/or cisplatinum ($P < 0.0001$). HEY tumor xenografts freshly excised on day 40 after tumor cell injection were analyzed for tumor growth inhibition and immunohistochemical analysis. The combined treatment was highly effective, with no histological evidence of HEY tumors in 4 of 10 mice for paclitaxel and ABT-627 and in 5 of 10 mice for the combined treatment of ABT-627 and cisplatinum. The dual treatments 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 determined the duration for which bioactivity persisted with combined paclitaxel and/or cisplatinum and ABT-627 treatment by maintaining the treated animal cohort for up to 66 days. As compared with control tumor xenografts, the growth delay in established tumors persisted for up to 4 weeks following termination of treatment with ABT-627 combined with paclitaxel.

DISCUSSION

The ET_A receptor autocrine pathway contributes to ovarian cancer progression by inducing cell proliferation, survival, angiogenesis and metastatic spread.[6,11,13,15] These results suggest that pharmacological inhibition of the ET_A receptor signaling pathway may improve cancer treatment.[1] A promising approach in this context has been the development of small molecules capable of inhibiting binding of the endogenous ligand to the ET_A receptor. Among these novel compounds, ABT-627 is a potent and orally bioavailable antagonist that reverses or blocks the ET-1-mediated effects in vitro and in vivo.[1,16]

In view of this, the therapeutic potential of ABT-627 was evaluated by analyzing its activity on tumor growth inhibition in ovarian carcinoma cells with a functional ET_A receptor-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.[23] The extent of tumor inhibition was similar to that obtained using the cytotoxic drug paclitaxel. Of major interest was the enhancement of this antitumor activity that was observed when mice were treated with ABT-627 in combination with paclitaxel or with cisplatinum. The combination treatment produced complete clinical and cytological tumor regression in 40% and in 50% in mice treated with paclitaxel or cisplatinum plus ABT-627, respectively. Furthermore, in the remaining animals these schedules caused almost complete suppression of tumor growth.

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 microvessel density. The selective ET_A receptor antagonist therefore appears to exert an antitumor effect in vivo that may be attributable to direct blockade of the ET_A receptor-dependent mitogenic pathway, and at least in part to inhibition of VEGF secretion, which sustains the proliferation of tumor blood vessels.[23,24]

Tumors of ABT-627-treated animals exhibited also a significant decrease in MMP-2 expression[23] and in phosphorylation/inactivation of connexin 43,[14] which are critical mediators of invasiveness in ovarian carcinoma. Inhibition of MMP-2 activity and restoration of connexin-43-based gap junction plaques are likely to represent relevant mechanisms by which ABT-627 could inhibit tumor invasiveness.

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 (terminal deoxynucleotidyl transferase-mediated dUTP biotin nickend labeling). Endothelin-1 acts through the ET_A receptor as a survival factor by protecting the ovarian carcinoma cell lines HEY and OVCA 433 cells against paclitaxel-induced apoptosis via activation of anti-apoptotic signaling pathways.[15] Because impairment of apoptotic pathways is a major molecular mechanism leading to chemoresistance,[25] ET_A receptor blockade by ABT-627 leading to sensitization of tumor cells to paclitaxel-induced or cisplatinum-induced apoptosis could produce an additive therapeutic effect. This indeed appears to occur in vivo following combined treatment with ABT-627 and paclitaxel or cisplatinum, because the inhibition of tumor growth achieved with this protocol correlates with the highest tumor apoptotic index.

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, in patients with ovarian tumors and potentially in other epithelial tumors that overexpress functional ET_A receptor, such as cervical carcinoma.[26]

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Dr Perry Nisen and Dr Azmi Nabulsi of Abbott Laboratories for kindly providing the ABT-627 (Global Oncology Development), Marco Varmi and Giacomo Elia for help with the animal studies and Maria Vincenza Sarcone for excellent secretarial assistance. This work was supported by grants from the Associazione Italiana Ricerca sul Cancro, Ministero della Salute and CNR-MIUR. FS is the recipient of a fellowship from Fondazione Italiana Ricerca sul Cancro.

REFERENCES

1. Nelson J, Bagnato A, Battistini B, et al. The endothelin axis: emerging role in cancer. *Nat Rev Cancer.* 2003;3:110–116.
2. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. *Pharmacol Rev.* 1994;46:325–415.
3. Bagnato A, Catt KJ. Endothelins as autocrine regulators of tumor cell growth. *Trends Endocrinol Metab.* 1998;9:378–383.
4. Nelson JB, Hedican SP, George DJ, et al. Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. *Nat Med.* 1995;1:994–999.
5. Gohji K, Kitazawa S, Tamada H, et al. Expression of endothelin receptor A associated with prostate cancer progression. *J Urol.* 2001; 165:1033–1036
6. Bagnato A, Salani D, Di Castro V, et al. Expression of endothelin-1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res.* 1999;59:720–727.
7. Venuti A, Salani D, Manni V, et al. Expression of endothelin-1 and endothelin A receptor in HPV-associated cervical carcinoma: new potential targets for anticancer therapy. *FASEB J.* 2000;14:2277–2283.
8. Bagnato A, Tecce R, Moretti C, et al. Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. *Clin Cancer Res.* 1995;1:1059–1066.
9. Bagnato A, Tecce R, Di Castro V, et al. Activation of mitogenic signaling by endothelin-1 in ovarian carcinoma cells. *Cancer Res.* 1997; 57:1306–1311.
10. Vacca F, Bagnato A, Catt KJ, et al. Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res.* 2000;60:5310–5317.
11. Salani D, Di Castro V, Nicotra MR, et al. Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol.* 2000;157: 1537–1547.
12. Spinella S, Rosanò L, Di Castro V, et al. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1α in ovarian carcinoma cells. *J Biol Chem.* 2002;277:27850–27855.
13. Rosanò L, Varmi M, Salani D, et al. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. *Cancer Res.* 2001;61:8340–8346.
14. Spinella S, Rosanò L, Di Castro V, et al. Endothelin-1 decreases gap-junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. *J Biol Chem.* 2003;278: 41294–41301.
15. Del Bufalo D, Di Castro V, Biroccio A, et al. Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for AKT activation. *Mol Pharmacol.* 2002;61:524–532.
16. Verhaar MC, Grahn AY, Van Weerdt AW, et al. Pharmacokinetics and pharmacodynamic effects of ABT-627, an oral ETA selective endothelin antagonist, in humans. *Br J Clin Pharmacol.* 2000;49:562–573.
17. Ozols RF. Paclitaxel plus carboplatin in the treatment of ovarian cancer. *Semin Oncol.* 1999;26:84–89.
18. Greenlee RT, Murray T, Bolden S, et al. Cancer statistics, 2000. *CA Cancer J Clin.* 2000;50:7–33.
19. Buick RN, Pullano R, Trent JM. Comparative properties of five human ovarian adenocarcinoma cell lines. *Cancer Res.* 1985;45:3668–3676.
20. Salani D, Rosanò L, Di Castro V, et al. ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. *Clin Sci.* 2002;103:318S–321S.
21. Carducci MA, Nelson JB, Bowling MK, et al. Atrasentan, an endothelin-receptor antagonist for refractory adenocarcinomas: safety and pharmacokinetics. *J Clin Oncol.* 2002;20:2171–2180.
22. Carducci MA, Padley RJ, Breul J, et al. Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J Clin Oncol.* 2003;21:679–689.
23. Rosanò L, Spinella F, Salani D, et al. Therapeutic targeting of the endothelin A receptor in human ovarian carcinoma. *Cancer Res.* 2003; 63:2447–2453.
24. Bagnato A, Spinella F. Emerging role of endothelin-1 in tumor angiogenesis. *Trends Endocrinol Metab.* 2002;14:44–50.
25. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell.* 1994;78:539–542.
26. Bagnato A, Cirilli A, Salani D, et al. Growth inhibition of cervix carcinoma cells in vivo by endothelin A receptor blockade. *Cancer Res.* 2002;62:6381–6384.

