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ORIGINAL ARTICLE

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Antiangiogenic and antitumor effects of a protein kinase Cβ inhibitor in murine Lewis lung carcinoma and human Calu-6 non-small-cell lung carcinoma xenografts

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Abstract In cell culture, the compound 317615·2HCl, a potent inhibitor of VEGF-stimulated HUVEC proliferation, was not very effective against Calu-6 non-small-cell lung carcinoma cells (IC_{50} 26 μ*M*). Exposure to combinations of paclitaxel or carboplatin and 317615·2HCl with Calu-6 cells in culture resulted in cell survival that reflected less-than-additivity to additivity of the two agents. Administration of 317615·2HCl orally twice daily to nude mice bearing subcutaneous Calu-6 tumors resulted in a decreased number of intratumoral vessels as determined by CD31 and CD105 staining to 50% of the number in control tumors. 317615·2HCl showed antitumor activity against the Lewis lung carcinoma and increased the tumor growth delay produced by paclitaxel by 5-fold, that produced by gemcitabine by 2-fold and that produced by carboplatin by 1.7-fold. There was a decrease in the number of lung metastases in the Lewis lung carcinoma that paralleled the increased response of the primary tumor with each treatment combination. Administration of 317615·2HCl also increased the tumor growth delay produced by fractionated radiation therapy in the Lewis lung tumor. Treatment with 317615·2HCl was an effective therapy in the Calu-6 non-small-cell lung carcinoma xenograft when the compound was administered early (days 4–18) or later (days 14–30). Combination treatment regimens in which 317615·2HCl was administered along with or sequentially with paclitaxel or carboplatin were much more effective than the chemotherapeutic agents administered alone. 317615·2HCl is in early clinical testing.

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Introduction

Non-small-cell lung cancer is the leading cause of cancer-related death in most industrialized countries [6, 7, 28, 29]. Treatment with radiation therapy provides symptomatic care and survivals averaging only 9 to 10 months. Chemotherapy, particularly cisplatin-based chemotherapy, followed by radiation therapy improves 5-year survival by three- to fourfold. Paclitaxel, docetaxel, vinorelbine, gemcitabine, topotecan and irinotecan have demonstrated single-agent activity in lung cancer [2, 6, 7, 8, 9, 10, 23, 31, 32, 40]. In clinical trials comparing the activity of paclitaxel with either cisplatin or carboplatin with etoposide/cisplatin have generally found that combinations including paclitaxel produce significantly better response rates [2]. In combination regimens, docetaxel/gemcitabine has been compared with docetaxel/cisplatin and has shown comparable efficacy and toxicity [23, 32, 40]. Combinations of chemotherapy and radiation therapy have shown benefit in advanced disease [9, 10, 31]. Lung cancer remains an important disease for the clinical examination of new treatments [6, 7, 8, 28, 29, 32].

Most solid tumors increase in mass through the proliferation of malignant cells and stromal cells including endothelial cells leading to the formation of a tumor vasculature [46]. Since active angiogenesis is a critical component of the mass expansion of most solid tumors, this process is a valid target for therapy [43]. Elucidation of the process has involved recognition of angiogenic stimuli such as hypoxia and nutrient deprivation, recognition of angiogenic factors produced by malignant cells, fibroblasts and tumor-infiltrating leukocytes, and recognition that there may be a concomitant decrease in negative angiogenic regulators by the same three cell populations within the tumor for

angiogenesis to occur [17, 24, 43, 45, 50]. Angiogenesis is a highly complex and closely regulated process and it is not surprising that vasculature in malignant masses is often poorly formed, irregular, lacking complete structure and inadequate to feed the tissue [18, 26, 37]. The combination of certain antiangiogenic agents with standard therapies appears to be synergistic [52].

The most clear-cut, direct-acting, most frequently found angiogenic factor in cancer patients is vascular endothelial growth factor (VEGF) [12, 17, 43, 46, 47, 54]. The signal transduction pathways of the KDR/Flk-1 and Flt-1 receptors include tyrosine phosphorylation, activation of PLCγ, diacylglycerol generation, and PI-3 kinase with downstream activation of protein kinase C (PKC) and activation of the MAP kinase pathway [22, 39, 48, 61] or, possibly, translocation of PKC into the cell nucleus [5, 38].

Protein kinase C is a gene family consisting of at least 12 isoforms [3, 41, 42]. Based on differing substrate specificity, activator requirements and subcellular compartmentalization, it is hypothesized that activation of individual protein kinase C isoforms preferentially elicit specific cellular responses [27, 42, 56]. To assess the contribution of PKC activation to VEGF signal transduction leading to neovascularization and enhanced vascular permeability, the effects of a PKCβ selective inhibitor which disrupts the phosphotransferase activity of conventional and novel PKC isoforms via an interaction at the ATP binding site have been studied [1, 11, 25, 27, 62]. Activation of specific receptor kinases do not activate unique intracellular kinases which then results in a linear signaling pathway; rather multiple signaling cascades can be activated producing combinatorial effects that allow more refined regulation of the biological outcome [36]. The intracellular signal transduction pathways for VEGF and bFGF in endothelial cells have not been fully elucidated; however, it is likely that protein kinase C is an important pathway component for both mitogens. Neoangiogenesis in the eyes of rats bearing corneal micropocket implants of either VEGF or bFGF is inhibited by treatment of the animals with 317615·2HCl orally twice per day [57]. Treatment of small-cell lung carcinoma SW-2-bearing mice with 317615·2HCl orally twice per day results in a countable decrease in intratumoral vessels and a corresponding slowing of tumor growth [57].

The current study was undertaken to examine the effect of the small molecule protein kinase Cβ inhibitor 317615·2HCl on intratumoral vessel development and response of subcutaneous human Calu-6 non-small-cell lung carcinoma and murine Lewis lung carcinoma to treatment with chemotherapy (paclitaxel, carboplatin and gemcitabine) and radiation therapy.

Materials and methods

Drugs

Paclitaxel and carboplatin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Gemcitabine was obtained from the Lilly Gemzar Product team. The compound 317615·2HCl was prepared by Discovery Chemistry, Lilly Research Laboratories. The compound 317615·2HCl was prepared in normal saline for oral delivery in a dose volume of 10 ml/kg.

Tumor lines

The murine Lewis lung tumor was carried in male C57BL mice (Taconic Farms, Germantown, N.Y.) [53, 55]. The Calu-6 lung adenocarcinoma was from a 61-year-old female previously treated with radiation therapy [13, 14]. The tumor line is hypotriploid with a modal chromosome number of 59.

Cell survival analysis

The Calu-6 cells were grown in RPMI-1640 medium supplemented 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, N.Y.). Cells grown in 25-cm^2 flasks to about 70% confluence were exposed to various concentrations of 317615·2HCl (0, 1, 5, 10, 50, 100 or 250 μM) for 24 h, carboplatin (0, 1, 5, 10, 50, 100 or 250 μM) for 1 h or paclitaxel (0, 0.05, 0.1, 0.5, 1, 5 or 10 μM), or were exposed to 317615·2HCl (10 or 100 μM) for 24 h along with various concentrations of carboplatin during the third hour or simultaneously with paclitaxel in 2% fetal bovine serum. After exposure to the agent or combination of agents, the cells were washed with 0.9% phosphate-buffered saline and suspended by exposure to 0.25% trypsin/0.1% EDTA. The cells were plated in duplicate at three or more dilutions for colony formation. After 10 to 14 days, the colonies were visualized by staining with crystal violet in methanol. Colonies of 50 cells or more were counted. The results are expressed as the surviving fraction of treated cells compared with control cultures.

Intratumoral vessel counting

Female nude mice (Charles River Laboratories, Wilmington, Mass.) 7 to 8 weeks of age were exposed to 4.5 G total body radiation delivered using a GammaCell 40 irradiator (Nordion, Ottawa, Ontario). Human Calu-6 non-small-cell lung carcinoma cells (5×10^6) prepared from a brei of several donor tumors were implanted 24 h later subcutaneously in a 1:1 mixture of RPMI tissue culture medium and Matrigel (Collaborative Biomedical Products, Bedford, Mass.) into a hind-leg of the animals. The animals were treated with 317615·2HCl (30 mg/kg) orally by gavage twice per day on days 14 through 30 after tumor implantation. On day 35, the tumors (about 300 mm^3) were excised and fixed in fresh 4% paraformaldehyde then processed by dehydration, infiltration with paraffin and embedding. Sections (5 μm) of tissue were then mounted on electrostatically charged slides (ProbeOn Plus, Fisher Scientific), rehydrated, quenched of endogenous peroxidase and blocked in normal serum. The sections were then incubated with 5 μg/ml of rat anti-mouse CD31 or anti-mouse CD105 antibody (Pharmingen, San Diego, Calif.) at room temperature for 30 min, followed by the biotinylated rabbit anti-rat (mouse adsorbed) secondary antibody (Vector) for 10 min and visualization with avidin-biotin complex (LSAB, DAKO, Carpinteria, Calif.) using diaminobenzidine as the chromagen. Sections were counterstained with DAKO Mayer's hematoxylin (DAKO, Carpinteria, Calif.). Blood vessels were quantified as described previously [30]. The most vascular area of the tumors was identified in a low-power (×100) field and vessels were counted in ten high-power fields (×200). The data are presented as the means ± SEM from ten high-power fields.

Tumor growth delay experiments

Male C57Bl mice were purchased from Taconic Farms (Germantown, N.Y.). Female nude mice were purchased from Charles River Laboratories (Wilmington, Mass.) at 5 to 6 weeks of age. For the experiments, 2×10^6 murine Lewis lung carcinoma tumor cells

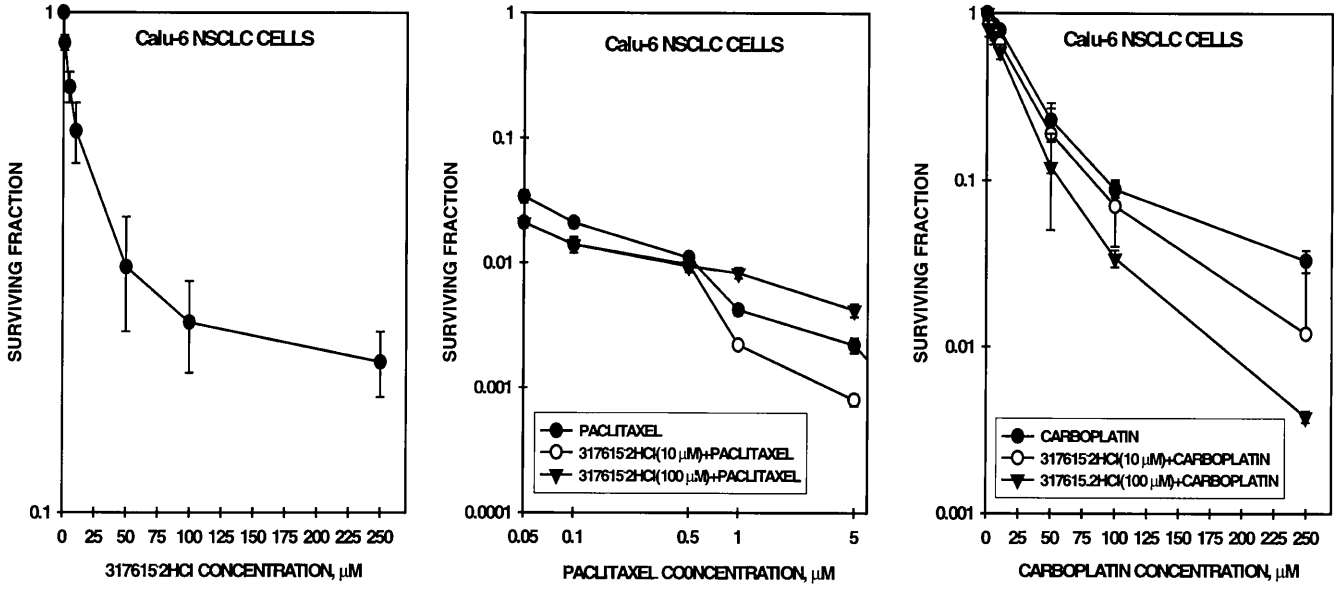

Fig. 1 Survival of human Calu-6 non-small-cell lung carcinoma cells after exposure to various concentrations of 317615·2HCl for 24 h, various concentrations of carboplatin for 1 h, paclitaxel for 24 h, or 317615·2HCl (10 or 100 μM, 24 h) along with various concentrations of carboplatin during the third hour or simultaneously with paclitaxel. Points are the means of two determinations (*bars* SEM)

prepared from a brei of several stock tumors were implanted subcutaneously into the legs of male C57Bl mice at 8 to 10 weeks of age on day 0. When the female nude mice were 7 to 8 weeks of age, they were exposed to 4.5 G total body radiation delivered using a GammaCell 40 irradiator (Nordion, Ottawa, Ontario). Human Calu-6 non-small-cell lung carcinoma cells (5×10^6) prepared from a brei of several donor tumors were implanted 24 h later subcutaneously in a 1:1 mixture of RPMI tissue culture medium and Matrigel (Collaborative Biomedical Products, Bedford, Mass.). Untreated Lewis lung tumors grew to 500 mm^3 in 10.4 ± 0.9 days and untreated Calu-6 tumors grew to 500 mm^3 in 19.0 ± 3.4 days. Animals were treated with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18 or on days 14 through 30 alone or along with paclitaxel (24 mg/kg, i.v.) on days 7, 9, 11 and 13, along with carboplatin (50 mg/kg, i.p.) on day 7 or along with gemcitabine (60 mg/kg, i.p.) on days 7, 10 and 13. For animals bearing the Lewis lung carcinoma, fractionated radiation therapy was delivered locally to the tumor-bearing limb as 2-, 3-, or 4-G fractions once daily on days 7 through 11 using a GammaCell 40 (Nordion, Ottawa, Canada).

The progress of each tumor was measured twice per week until it reached a volume of 4000 mm^3. Tumor growth delay (TGD) was calculated as the time taken by each individual tumor to reach 1000 mm^3 compared with the time in the untreated controls. Each treatment group included five animals. TGD (days) are the means $\pm$ SE for the treatment group compared with those for the control group [29, 56]. Lungs were collected from three animals per group on day 20. The lungs were fixed in Bouin's solution and the number of metastases on the external surface of each lung was counted manually. The radiation dose modifying factor was determined by comparison of the slopes of the radiation tumor response curves in the presence and absence of 317615·2HCl.

Results

The compound 317615·2HCl was not very cytotoxic toward human Calu-6 non-small-cell lung cancer cells in monolayer culture with an IC_{50} of about 26 μM for a 24-h exposure (Fig. 1). Exposure of Calu-6 cells to paclitaxel for 24 h resulted in an IC_{99} of about 0.5 μM. Exposure of Calu-6 cells to 317615·2HCl at 10 μM or 100 μM for 24 h along with paclitaxel resulted in IC_{99} values for both combinations of 0.5 μM, indicating essentially no positive effect of 317615·2HCl on the cytotoxicity of paclitaxel toward these cells. Exposure of Calu-6 cells to carboplatin for 1 h resulted in an IC_{90} of 92 μM. Exposure of Calu-6 cells to 317615·2HCl at 10 μM or 100 μM for 24 h along with carboplatin during the third hour resulted in IC_{90} values of the two combinations of 80 μM and 60 μM, respectively, indicating primarily additivity of the agents.

Nude mice bearing human Calu-6 non-small-cell lung carcinoma growing as a subcutaneous xenograft on the thigh were treated with 317615·2HCl (30 mg/kg) orally twice daily on days 14 through 30 after tumor cell implantation. On day 31, tumors were collected, preserved in 10% phosphate-buffered formalin and 5-mm thick sections were immunohistochemically stained for expression of endothelial specific markers, either CD31 or CD105. The number of intratumoral vessels in the samples was determined by counting stained regions in ten high-power microscope fields (×200). There was a 317615·2HCl-dependent decrease in the number of countable intratumoral vessels in the human Calu-6 tumors. In the animals treated with 317615·2HCl (30 mg/kg), both the number of intratumoral vessels stained by CD31 and the number stained by CD105 were one-half of the number in the controls (Fig. 2).

The Lewis lung carcinoma is a relatively rapidly growing murine non-small-cell lung carcinoma that is widely used for the study of antiangiogenic agents. This tumor is grown in the syngeneic host (C57Bl male mice) and avidly metastasizes to the lungs from a primary tumor implanted subcutaneously on the thigh. The compound 317615·2HCl (10 and 30 mg/kg) was effective against the primary Lewis lung carcinoma producing 4

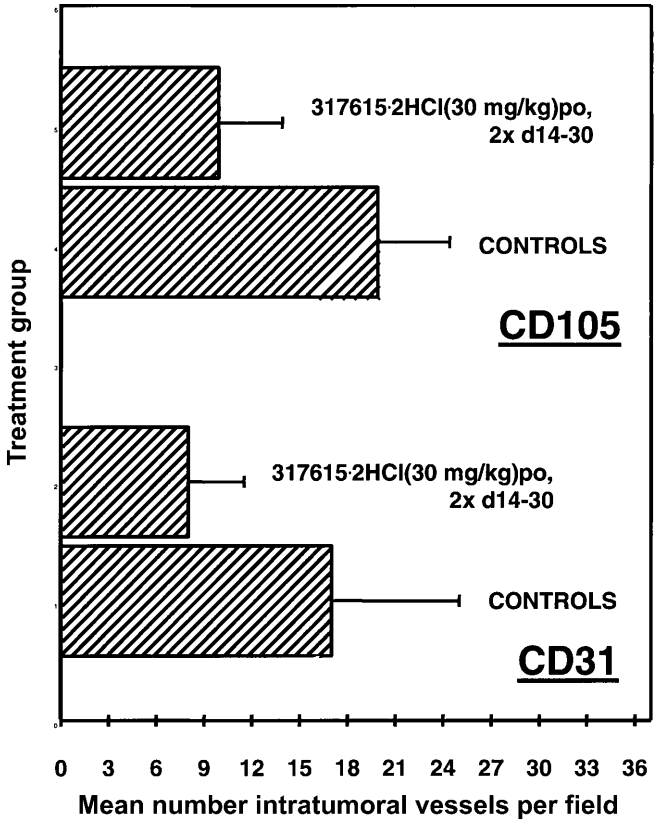

Fig. 2 Countable intratumoral vessels in human Calu-6 non-small-cell lung carcinoma xenograft tumors after treatment of the tumor-bearing animals with 317615·2HCl (30 mg/kg) orally twice per day on days 14 through 30 after tumor implantation. Tumors were immunohistochemically stained for Factor VIII or CD31. Intratumoral vessels were counted manually. Data are the means of ten determinations (*bars* SEM)

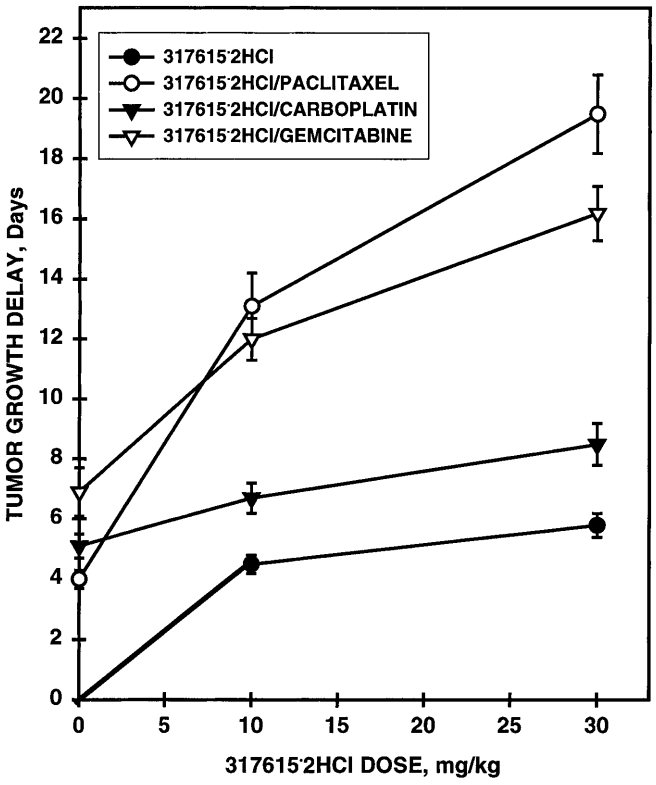

Fig. 3 Growth delay of the murine Lewis lung carcinoma after treatment of tumor-bearing mice with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18 alone or along with paclitaxel (24 mg/kg, i.v.) days 7, 9, 11 and 13, carboplatin (50 mg/kg, i.p.) day7 or gemcitabine (60 mg/kg, i.p.) days 7, 10 and 13. Points are the means from five animals (*bars* SEM)

to 6 days TGD (Fig. 3). The compound markedly increased the efficacy of paclitaxel. The TGD of paclitaxel was 5-fold greater when combined with the higher dose of 317615·2HCl. The TGD of carboplatin was increased 1.7-fold and the TGD of gemcitabine was increased 2-fold when administered along with the higher dose of 317615·2HCl.

The Lewis lung carcinoma syngeneic model system allows the opportunity to determine the effect of treatments against systemic disease. The compound 317625·2HCl was effective in decreasing the number of lung metastases as a single agent. Treatment with 317615·2HCl decreased Lewis lung tumor lung metastases from a mean of 30 to 18 at the higher dose, and was even more effective when used in combination with a chemotherapeutic agent (Fig. 4). The higher dose of 317615·2HCl decreased the mean number of lung metastases to 8.5 when combined with paclitaxel, to 4.5 when combined with carboplatin and to 6.5 when combined with gemcitabine compared with 30 lung metastases in the controls.

Mice bearing subcutaneously implanted Lewis lung carcinoma tumors were treated with fractionated radiation therapy (2, 3 or 4 G ×5) locally to the tumor-bearing limb either alone or along with administration of 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18 after tumor implantation. Fractionated radiation therapy produced a dose-dependent TGD (Fig. 5). Administration of 317615·2HCl markedly augmented the antitumor effect of the radiation therapy at both doses. Especially noteworthy was an increase in TGD from 3.2 days to 8 to 10 days by administration of 317615·2HCl at the radiation dose of 2 G (which is in the clinical radiation dosage range). The compound 317615·2HCl produced measurable radiation dose modification by altering the slope of the radiation dose-response curve, indicating an interaction between the therapies. The radiation dose-modifying factors (DMFs) were 1.5 and 1.6 for 317615·2HCl at doses of 10 and 30 mg/kg, respectively.

In female nude mice bearing the Calu-6 non-small-cell lung carcinoma xenograft, when initiated early in the disease (day 4) and continued for 2 weeks (day 18), 317615·2HCl showed a dose-dependency producing a TGD of 5 days at 10 mg/kg and 10 days at 30 mg/kg (Fig. 6). The combination of the 317615·2HCl (at 30 mg/kg) with paclitaxel on days 7 through 13 resulted in a 2.6-fold increase in TGD.

A very similar response pattern was observed when treatment of the Calu-6 non-small-cell lung carcinoma

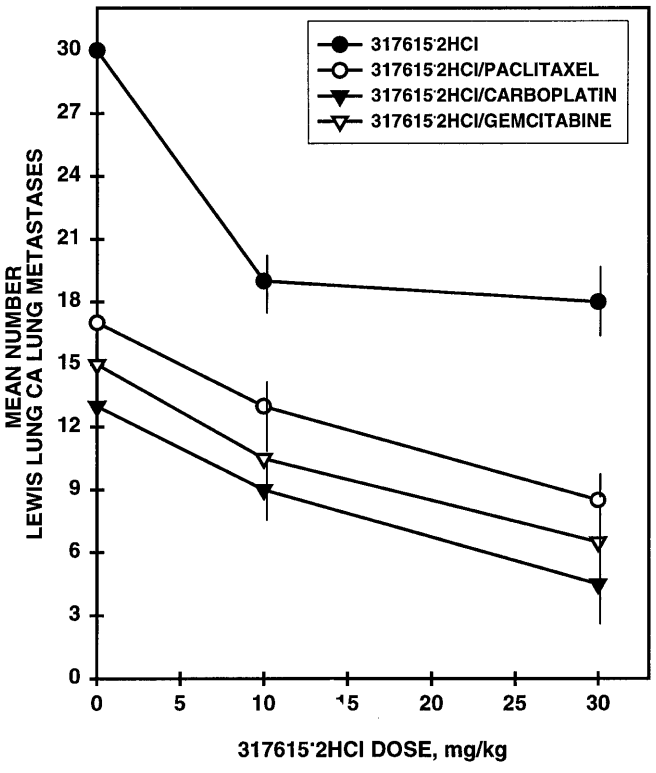

Fig. 4 Mean number of lung metastases from subcutaneously implanted Lewis lung carcinoma in control animals and Lewis lung tumor-bearing animals treated with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18 alone or along with paclitaxel (24 mg/kg, i.v.) days 7, 9, 11 and 13, carboplatin (50 mg/kg, i.p.) day 7 or gemcitabine (60 mg/kg, i.p.) days 7, 10 and 13 (*bars* SEM)

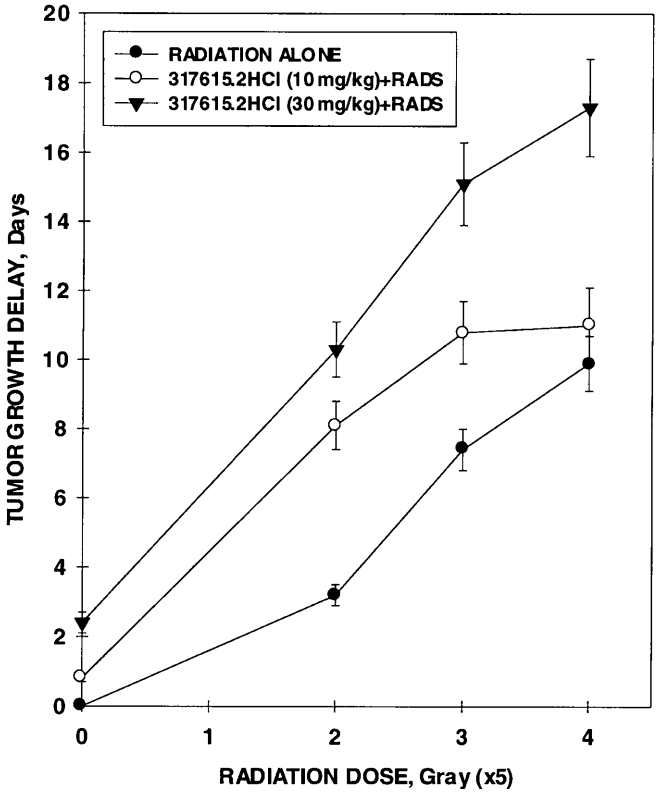

Fig. 5 Growth delay of the murine Lewis lung carcinoma after treatment with fractionated radiation therapy locally to the tumor-bearing limb alone or along with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18. Points are the means from five animals (*bars* SEM)

xenografts with 317615·2HCl was delayed until day 14 after tumor implantation and maintained until day 30. Over the dosage range tested (3 to 30 mg/kg) the TGD produced by 317615·2HCl alone increased from 4.4 days to 8.8 days (Fig. 7). The combination of 317615·2HCl and paclitaxel was again very effective, although the effect with paclitaxel and 317615·2HCl (10 mg/kg) was unexpectedly low. Sequential treatment with paclitaxel followed by 317615·2HCl (30 mg/kg) resulted in a nearly 4-fold increase in TGD compared with paclitaxel alone. A sequential regimen was also very effective when the chemotherapeutic agent was carboplatin. Treatment with carboplatin alone produced a TGD of 5.3 days, but when the carboplatin was followed by 317615·2HCl (30 mg/kg) the TGD increased to 21.5 days, a 4-fold increase, which was a more pronounced effect than was seen with carboplatin in the Lewis lung tumor.

Discussion

Angiogenesis is required for tumor growth beyond 1 to 2 mm in diameter and plays an important role in the metastatic spread of malignant disease. Both retrospective and prospective studies in non-small-cell lung cancer have shown that angiogenesis, as assessed by microvessel counting, is an important prognostic factor in operable non-small-cell lung cancer, with high microvessel counts being associated with disease spread and poor survival [15, 19, 44, 49]. Expression of VEGF correlates with high microvessel counts and a poor prognosis in non-small-cell lung cancer [16, 20, 45, 58, 59]. Immunoreactivity for platelet-derived endothelial cell growth factor (thymidine phosphorylase) in tumor cells but not in stromal cells correlates with angiogenesis and prognosis in non-small-cell lung cancer, high expression being associated with angiogenesis and with a poor outcome [21, 35, 60]. Significant positive correlations have also been found between mRNA expression of angiopoietin-1 and Tie2 and angiogenesis in non-small-cell lung cancer [51]. In a recent study of 223 patients with operable non-small-cell ling cancer, coexpression of VEGF and platelet-derived endothelial cell growth factor was not associated with a higher microvessel count than in tumors expressing only VEGF or platelet-derived endothelial cell growth factor [44]. A univariate analysis has shown tumor size, nodal status, microvessel density and VEGF and platelet-derived endothelial growth factor expression to be significant prognostic factors. In multivariate analysis, tumor size and microvessel density remain significant [44].

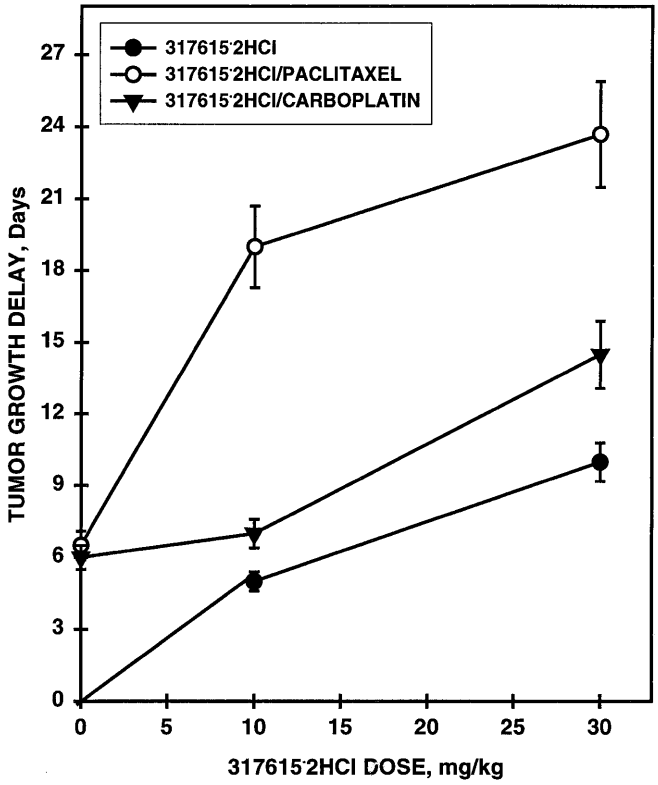

Fig. 6 Growth delay of the human Calu-6 non-small-cell lung carcinoma after treatment with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 4 through 18 alone or along with paclitaxel (24 mg/kg, i.v.) days 7, 9, 11 and 13 or carboplatin (50 mg/kg, i.p.) day 7. Points are the means from five animals (*bars* SEM)

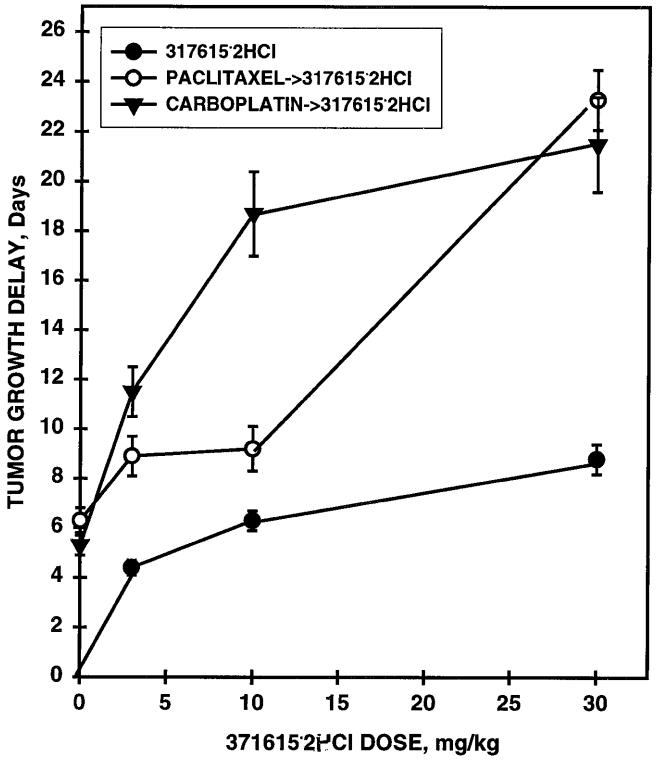

Fig. 7 Growth delay of the human Calu-6 non-small-cell lung carcinoma after treatment with 317615·2HCl (10 or 30 mg/kg) orally twice per day on days 14 through 30 alone or along with paclitaxel (24 mg/kg, i.v.) days 7, 9, 11 and 13 or carboplatin (50 mg/kg, i.p.) day 7. Points are the means from five animals (*bars* SEM)

In cell culture, 317615·2HCl was a more potent inhibitor of VEGF-stimulated HUVEC proliferation (IC_{50} 150 n*M*, 72 h) than of human SW2 small-cell lung carcinoma cell proliferation (IC_{50} 3.5 μ*M*, 72 h) [57]. In the current cell culture studies, 317615·2HCl was not very cytotoxic toward Calu-6 cells and did not appear to interact actively with paclitaxel or carboplatin when the cells in culture were exposed to the compounds in combination. To account for the increased efficacy observed with the treatment combinations in vivo, therefore, it appears most likely that the primary effect of 317615·2HCl is on the endothelial cell component of the tumor leading to an antiangiogenic effect. In vivo in the Calu-6 tumor, treatment of the animals with 317615·2HCl for 16 days resulted in a decrease in the number of countable intratumoral vessels to 50% of normal and corresponded to a TGD of about 9 days. The combination of 317615·2HCl with other chemotherapeutic agents including paclitaxel, gemcitabine, carboplatin (in one selected case) or fractionated radiation therapy, led to a much improved primary tumor response that was paralleled by the systemic disease in the Lewis lung tumor.

There has recently been renewed interest in the potential antiangiogenic effects of cytotoxic anticancer agents [4, 33, 34]. Paclitaxel is a cytotoxic agent that has been recognized to be effective against endothelial cells. While the scheduling of the therapeutic regimens used in the current studies was the traditional high episodic dosing used to kill malignant cells and were not designed to highlight the potential dual antiangiogenic activity of 317615·2HCl and paclitaxel. It is very likely that the enhancement in tumor response observed with the combination regimens reflected an increased response of both endothelial cells and malignant cells. It would be interesting to know whether the combination of 317615·2HCl and paclitaxel administered in a more continuous low-dose regimen could further increase tumor response and decrease the number of intratumoral vessels. As has been observed with other antiangiogenic therapeutic agents, there did not appear to be a selective effect on metastatic disease. Instead, the lung metastasis from the Lewis lung carcinoma appeared to parallel the primary tumor in response to the single agents and the 317615·2HCl combination regimens.

The compound 317615·2HCl is in early phase I clinical testing.

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