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ORIGINAL ARTICLE

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Antiangiogenic effects of a protein kinase Cβ-selective small molecule

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Abstract *Background*: Protein kinase C frequently plays a central role in the intracellular signal transduction of growth factors and cytokines. *Methods*: The acyclic bisindolylmaleimide 317615·2HCl was identified as a potent selective inhibitor of protein kinase Cβ. The compound 317615·2HCl was tested in culture and in vivo in the rat corneal micropocket and in the SW2 small-cell lung carcinoma human tumor xenograft. *Results*: 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 μM, 72 h). When administered orally twice daily for 10 days, the compound 317615·2HCl markedly decreased the neoangiogenesis induced by VEGF or bFGF in the rat corneal micropocket assay. To assess antitumor efficacy, 317615·2HCl was administered orally twice daily to nude mice bearing SW2 xenograft tumors on days 14 through 30 after tumor implantation. The number of countable intratumoral vessels was decreased in a dose-dependent manner reaching as low as one-quarter the number in the control tumors. The decrease in intratumoral vessels was paralleled by increases in tumor growth delay. Treatment of the tumor-bearing animals with paclitaxel or carboplatin followed by treatment with 317615·2HCl resulted in a 2.5- to 3.0-fold increase in tumor growth delay compared with the standard chemotherapeutic agents alone. *Conclusions*: 317615·2HCl represents a new approach to antiangiogenic therapy in cancer-blocking multiple growth factor signaling pathways in endothelial cells with a single agent. 317615·HCl is in early clinical testing.

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Abbreviations *bFGF* basic fibroblast growth factor · *DMSO:* dimethyl sulfoxide · *EGF* epidermal growth factor · *EGTA* ethylene glycol-bis(ϵ-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid · *Flt-1* fms-like tyrosine kinase-1, VEGF receptor-1 · *HIF* hypoxia-inducible factor · *HUVEC* human umbilical vein endothelial cells · *KDR*/*Flk-1* kinase-insert domain-containing receptor, VEGF receptor-2 · *PKC* protein kinase C · *UCN-01* 7-hydroxy-staurosporine · *VEGF* vascular endothelial growth factor

Introduction

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 (angiogenesis) [50]. Since active angiogenesis is a critical component of the mass expansion of most solid tumors, this process is a valid target for therapy [47]. Angiogenesis during malignant growth is a complex process. 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 [19, 30, 47, 52, 59]. Endogenous stimulators of angiogenesis (angiogenic factors) include vascular endothelial growth factor (VEGF), transforming growth factors α and β, acidic and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), platelet-derived endothelial cell growth factor/thymidine phosphorylase, angiogenin, pleiotropin, hepatocyte growth factor, interleukins-1, -6 and -8, placental growth factor, E-selectin, tumor necrosis

factor-α, heparinase, angiopoietin, and hypoxia-inducible factor (HIF) [1, 20, 24, 30, 45, 48, 59, 60, 61, 70, 74].

Endogenous negative regulators or inhibitors of angiogenesis include thrombospondin, platelet factor IV, interferon-α, interferon-β, protamine, cartilage-derived inhibitors, angiostatin, endostatin, plasminogen activator inhibitor and the tissue inhibitor of metalloproteinases [5, 47, 50, 59]. Preclinical and clinical studies have shown that malignant cells in culture and tumors in vivo can and, most often, do express an array of angiogenesis stimulators and negative regulators. Clearly, 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 [23, 33, 40]. The combination of certain antiangiogenic agents with standard therapies appears to be synergistic [63].

The most clear-cut, direct-acting, most frequently found angiogenic factor in cancer patients is VEGF [18, 19, 47, 50, 52, 64]. VEGF expression has been associated with primary breast cancer, brain tumors, cervical neoplasias, lung cancer, stomach cancer, colon cancer and others. Furthermore, the upregulation of the VEGF receptors, Flt-1 and KDR, has been observed in tumor-associated endothelial cells in a variety of tumors including breast cancer, brain tumors, kidney tumors, bladder cancer, ovarian cancer and colon cancer. The signal transduction pathways of the KDR/Flk-1 and Flt-1 receptors include tyrosine phosphorylation, activation of phospholipase C, diacylglycerol generation, and inositol 1,4,5-triphosphate kinase with downstream activation of protein kinase C (PKC) and activation of the MAP kinase pathway [26, 44, 54, 73] or, possibly, translocation of PKC into the cell nucleus [12, 42].

PKC is a gene family consisting of at least 12 isoforms [10, 46, 73]. Based on differing substrate specificity, activator requirements and subcellular compartmentalization, it is hypothesized that activation of individual PKC isoforms preferentially elicits specific cellular responses [10, 46]. Treatment of adrenal cortex endothelial cells with VEGF results in PKC activation and elevated endothelial nitric oxide synthase (eNOS) expression. Inhibition of PKC with isoform-specific inhibitors abolishes VEGF-induced eNOS upregulation [57]. When PKC pathways are activated in human glioblastoma U373 cells by phorbol 12-myristate 13-acetate (PMA), VEGF mRNA expression is upregulated via a post-transcriptional mRNA stabilization mechanism [58]. PMA increases VEGF mRNA half-life from 0.8 to 3.6 h and this is blocked by PKC inhibitors (staurosporine or calphostin C) but not by protein kinase A or cyclic nucleotide-dependent protein kinase inhibitors.

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 protein phosphorylation activity of conventional and novel PKC isoforms via an interaction at the ATP binding site has been studied [2, 16, 31, 34, 75]. At concentrations predicted to selectively inhibit PKC completely, the compound abrogates VEGF-stimulated growth of bovine aortic endothelial cells [34]. Oral administration of the inhibitor decreases neovascularization in an ischemia-dependent model of in vivo retinal angiogenesis and blocks increases in retinal vascular permeability stimulated by the intravitreal instillation of VEGF [2, 16, 31].

Administration of LY333531 to animals bearing BNL-HCC hepatocellular carcinoma xenografts transfected with the VEGF gene under tetracycline control markedly decreases tumor growth subcutaneously and orthotopically and decreases VEGF overexpression in the tumors [75]. This PKCβ-selective inhibitor has also demonstrated antitumor activity alone and in combination with standard cancer therapies in the murine Lewis lung carcinoma and in several human tumor xenografts [65]. Recently, Kruger et al. [37] have shown that the PKC inhibitor, UCN-01, at concentrations lower than those necessary to inhibit cancer cell growth, inhibits proliferation of human endothelial cells in vitro, prevents microvessel out-growth from explant cultures of rat aortic rings and abrogates hypoxia-mediated transactivation of HIF-1-responsive promoters.

Based upon this background, a search was made for the optimal PKCβ inhibitor for use in oncology.

Materials and methods

Drugs

Paclitaxel and carboplatin were purchased from Sigma Chemical Co. (St. Louis, Mo.). The compound 317615·2HCl was prepared in Discovery Chemistry (Fig. 1).

Enzyme assays

PKC isozymes. Each of the human PKC enzymes was partially purified from Sf9 cells expressing the individual PKC enzymes [31, 34]. Briefly, the reaction mixture (250 µl) contained phosphatidylserine (120 µg/ml; Avanti Polar Lipids, Alabaster, Ala.), diacylglycerol (Avanti Polar Lipids) to maximally activate the enzyme in 20 m*M* HEPES buffer (Sigma), pH 7.5, 1 m*M* EGTA, 10 m*M* magnesium chloride (Sigma), 940 µ*M* calcium chloride (for the α, βI, βII and γ isozymes; Sigma), 10 µl DMSO or DMSO/compound and 30 µ*M* [γ-^{32}P]ATP (Dupont). Either histone type HL (Wor-

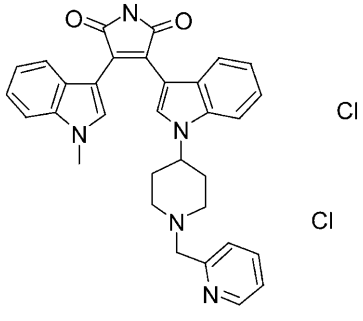

Fig. 1 Chemical structure of the acyclic bisindolylmaleimide 317615·2HCl

thington, Lakewood, N.J.) or mylein basic protein was used as substrate. The reaction was initiated by the addition of enzyme, incubated at 30°C for 10 min and stopped by the addition of 0.5 ml ice-cold trichloroacetic acid (Amresco, Solon, Ohio) followed by 100 ml of 1 mg/ml bovine serum albumin (Sigma). The precipitate was collected by vacuum filtration on glass filters and counted in a beta scintillation counter.

cAMP-dependent protein kinase (PKA). Briefly, the reaction mixture (250 µl) contained 20 m*M* HEPES buffer (Sigma), pH 7.5, 200 µg/ml Histone HL (Worthington), 10 m*M* magnesium chloride (Sigma), 10 µl DMSO or DMSO/compound and 30 µ*M* [γ-^{32}P]ATP (Dupont). The reaction was initiated by the addition of bovine heart cAMP-dependent kinase catalytic subunit (Sigma), incubated at 30°C for 10 min and stopped by the addition of 0.5 ml ice-cold trichloroacetic acid (Amresco) followed by 100 ml of 1 mg/ml bovine serum albumin (Sigma). The precipitate was collected by vacuum filtration on glass filters and counted in a beta scintillation counter.

Calcium calmodulin-dependent kinase (CaM). The calcium calmodulin-dependent protein kinase assay was as described previously [34, 36] using calcium calmodulin-dependent protein kinase (isolated from rat brain homogenates).

Casein protein kinase II (CK-II). The casein protein kinase II assay was as described previously [3, 34] using casein protein kinase II (isolated from rat brain homogenate).

Src protein tyrosine kinase (src-TK). The src-tyrosine kinase assay was as described previously (Oncogene Science, cat. no. PK02 and PK03, 1990; [34, 69]).

Cell culture studies

VEGF/HUVEC. Human umbilical vein endothelial cells (HUVEC; Biowhittaker/Clonetics, Walkersville, Md.) were maintained in endothelial cell growth medium (EGM) containing basal medium (EBM) with bovine brain extract, human EGF, hydrocortisone, gentamicin, amphotericin B and 2% fetal bovine serum. For the assay, HUVEC (5×10^3) in EBM (200 µl) with 0.5% fetal bovine serum were added to the wells of a 96-well cell culture plate and incubated at 37 °C for 24 h in humidified air containing 5% carbon dioxide. The 317615·2HCl was serially diluted in DMSO in concentrations from 0.0013 to 40 µ*M* and added to the wells in 20 µl. Finally, human VEGF (20 ng/ml in wells; R&D Systems, Minneapolis, Minn.) prepared from a stock solution of 100 µg/ml in phosphate-buffered normal saline containing 0.1% bovine serum albumin, was added to the wells. The HUVEC were incubated at 37 °C for 72 h in humidified air containing 5% carbon dioxide. WST-1 cell proliferation reagent (20 µl; Boehringer Mannheim, Indianapolis, Ind.) was added to the wells and the plates returned to the incubator for 1 h. The absorbance of each well at 440 nm was measured. The growth fraction was determined from the absorbance of treated wells with and without VEGF divided by the absorbance obtained from control wells set to zero and 1.0.

SW-2 small-cell lung carcinoma cells. The SW2 small-cell lung carcinoma cell line originated from the pleural fluid of a patient with small-cell lung cancer in 1979 [21, 22]. The SW2 cells were grown in RPMI-1640 medium supplemented with 0.2% sodium bicarbonate, 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, N.Y.). SW-2 cells in exponential growth were exposed to various concentrations of 317615·2HCl (0.0013 to 40 µ*M*) at 37°C for 72 h in air containing 5% carbon dioxide. After exposure to the agent, the cells were washed with 0.9% phosphate-buffered saline. SW2 cells were treated as single-cell suspensions and growth inhibition was determined using WST-1 cell proliferation reagent as described above. The results are expressed as the growth fraction of treated cells compared with that of control cultures.

Animal welfare assurances

All research involving animals was performed under protocols approved by the institutional Animal Care and Use Committee and in accordance with NIH guidelines in an Analytical Laboratory Accreditation Criteria Committee approved facility.

Corneal micropocket assay

Fisher 344 female rats (145–155 g; Taconic, Germantown, N.Y.) were anesthetized with acepromazine (2.5 mg/kg, i.p.) 20 min prior to initiation of 2–3% isoflurane/oxygen inhalation. The body temperature was maintained with a circulating hot water pad. The surgery was performed using an ophthalmic operating microscope (OMS.75 operating microscope; TopCon Corporation, Japan). A number 15 scalpel blade was used to make a vertical half-thickness linear corneal incision just lateral to the center of the eye. The tip of the scalpel blade was used to gently undermine the superior corneal layer of the cornea nearest to the limbus. A pocket was formed in the cornea using blunt dissection with corneal scissors (Roboz, Rockville, Md.). Nitrocellulose filters (0.45 µ*M*; Millipore, Bedford, Mass.) were cut into small disks using a 20 gauge needle punch. The disks were soaked in 2 µl human VEGF solution (0.82 µg/µl; R&D Systems) or human bFGF (0.20 µg/µl; R&D Systems) for 10 min on ice.

Using forceps, the disks impregnated with the angiogenic factor were inserted into the corneal pocket so that the disk was firmly covered with corneal epithelium. The animals were treated with 317615·2HCl (10 or 30 mg/kg) administered orally by gavage in phosphate-buffered saline twice per day on days 1 through 10 after implantation of the disks. The eyes were photographed on days 7 and 14 after implantation of the disks. For photography, the animals were treated with atropine sulfate (AmTech Group, Phoenix Scientific, St. Joseph, Mo.) topically for mydriasis and anesthetized with 2–3% isoflurane/oxygen. The eyes were photographed using the ophthalmic microscope and the images were saved using Image Pro-Plus software. The images were analyzed by converting the area of interest to a high contrast black and white reversed image and counting the bright pixels as a determination of the vascular area. The data are the images from at least six eyes.

Intratumoral vessel counting

Male nude mice (Charles River Laboratories, Wilmington, Mass.) at 7 to 8 weeks of age were exposed to 4.5 G of total body radiation delivered using a GammaCell 40 irradiator (Nordion, Ottawa, Ontario). Human SW2 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.) in a hind-leg of the animals. The animals were treated with 317615·2HCl (3, 10 or 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 submerged in Tissue-Tec (OCT 4583 compound; Sakura Finetek USA, Torrance, Calif.) on dry ice. The tissue blocks were stored at –80°C until processing. The tissues cut into 6 mm sections with a cryostat were fixed in cold acetone, dried and stored at –80 °C until staining. Sections were rinsed with phosphate-buffered 0.9% saline three times and blocked with peroxidase blocking reagent and protein block (DAKO, Carpinteria, Calif.). Sections were then stained with 5 µg/ml of anti-CD31 (Pharmingen, San Diego, Calif.) or anti-Factor VIII (DAKO) at room temperature for 30 min. Sections were rinsed and incubated with 2.5 mg/ml of biotin-conjugated goat anti-rat antibody (Pharmingen) for 10 min and then peroxidase-conjugated streptavidin (DAKO) at a dilution of 1:500 for 10 min. Sections were developed with DAKO AEC substrate-chromogen system for 20 minutes at room temperature and counterstained with Mayer's hematoxylin (DAKO). The quantification of blood vessels was performed as described previously [38]. The most vascular area of 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 for ten high-power fields.

Tumor growth delay experiments

Male nude mice were purchased from Charles River Laboratories (Wilmington, Mass.) at 5 to 6 weeks of age. When the animals were 7 to 8 weeks of age they were exposed to 4.5 G of total body radiation delivered using a GammaCell 40 irradiator (Nordion, Ottawa, Ontario). Human SW2 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.) in a hind-leg of the animals. Untreated SW2 tumors grew to 500 mm^3 in 25.4 ± 4.2 days. Treatments were initiated on day 7 after tumor cell implantation when the tumors were approximately 50 to 100 mm^3 in volume. Animals were treated with 317615·2HCl (3, 10 or 30 mg/kg) orally by gavage twice per day on days 14 through 30 alone or along with paclitaxel (24 mg/kg) by intravenous injection on days 7, 9, 11 and 13 or carboplatin (50 mg/kg) by intraperitoneal injection on day 7 after tumor cell implantation.

The progress of each tumor was measured three times 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 for the untreated control tumors. Each treatment group included five animals. TGD times (days) are the means ± SE for the treatment group compared with those for the control group [35].

Results

The acyclic bisindolylmaleimide 317615·2HCl is a potent and selective inhibitor of PKCβ (Fig. 1). When compared in a panel of kinases with the well-known kinase inhibitor staurosporine, 317615·2HCl demonstrated marked selectivity for inhibition of the PKCβ isoforms versus other PKC isoforms as well as protein kinase A, calcium calmodulin kinase, casein kinase src-tyrosine kinase and rat brain PKC (Table 1).

In monolayer culture, HUVEC in basal medium were stimulated to proliferate by exposure to human VEGF (20 ng/ml). When various concentrations of 317615·2HCl were added to the cultures for 72 h, the proliferation of the VEGF-stimulated HUVEC was profoundly inhibited by 600 n*M* of the compound (Fig. 2). The IC_{50} for the assay was 150 n*M* of 317615·2HCl. In a similar experiment, when human SW2 small-cell lung carcinoma cells were exposed to various concentrations of 317615·2HCl for 72 h, a potency differential in the effect of the compound on the malignant cells versus the HUVEC was apparent. The IC_{50} for 317615·2HCl in the SW2 cells was 3.5 μ*M*. Thus there was a 23-fold selectivity in the concentration of 317615·2HCl for the growth inhibition of HUVEC compared to human SW-2 small-cell lung carcinoma cells in culture.

The cornea is normally an avascular tissue. Surgical implantation of a small filter disc impregnated with VEGF into the cornea of a rat will result in robust neoangiogenesis that is quantifiable in 7 to 10 days. Administration of 317615·2HCl orally twice per day on days 1 through 10 after implantation of VEGF-impregnated filters resulted in markedly decreased vascular growth in the cornea of Fisher 344 female rats. A dose of 10 mg/kg of 317615·2HCl decreased vascular growth to about one-half that of the VEGF-stimulated controls, while a dose of 30 mg/kg of 317615·2HCl decreased vascular growth to the level of the unstimulated surgical control (Figs. 3 and 4).

bFGF is another major angiogenic factor in tumors and is known to be a substrate for phosphorylation by PKC. Surgical implantation of a small filter disc

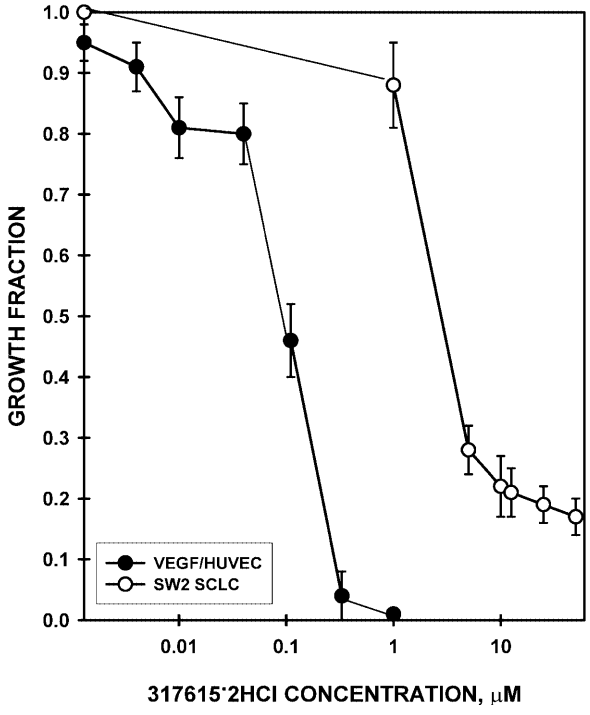

Fig. 2 Concentration-dependent growth inhibition of HUVEC and human SW2 small-cell lung carcinoma cells after 72 h exposure to various concentrations of 317615·2HCl as determined by the WST-1 assay. Points are the means of three determinations (*bars* SEM)

Table 1 PKC isozyme and other kinase IC_{50} values (μ*M*) for LY317615·2HCl and staurosporine

Compound	PKC isozyme								PKA	CA calmodulin	Casein kinase	src-TK	Rat brain PKC
	α	βI	βII	γ	δ	ϵ	ζ	η					
LY317615·2HCl	0.8	0.03	0.03	2	1	0.3	8	0.4	> 100	10	> 100	> 100	1
Staurosporine	0.045	0.023	0.019	0.11	0.028	0.018	> 1.5	0.005	0.1	0.004	14	0.001	0.19

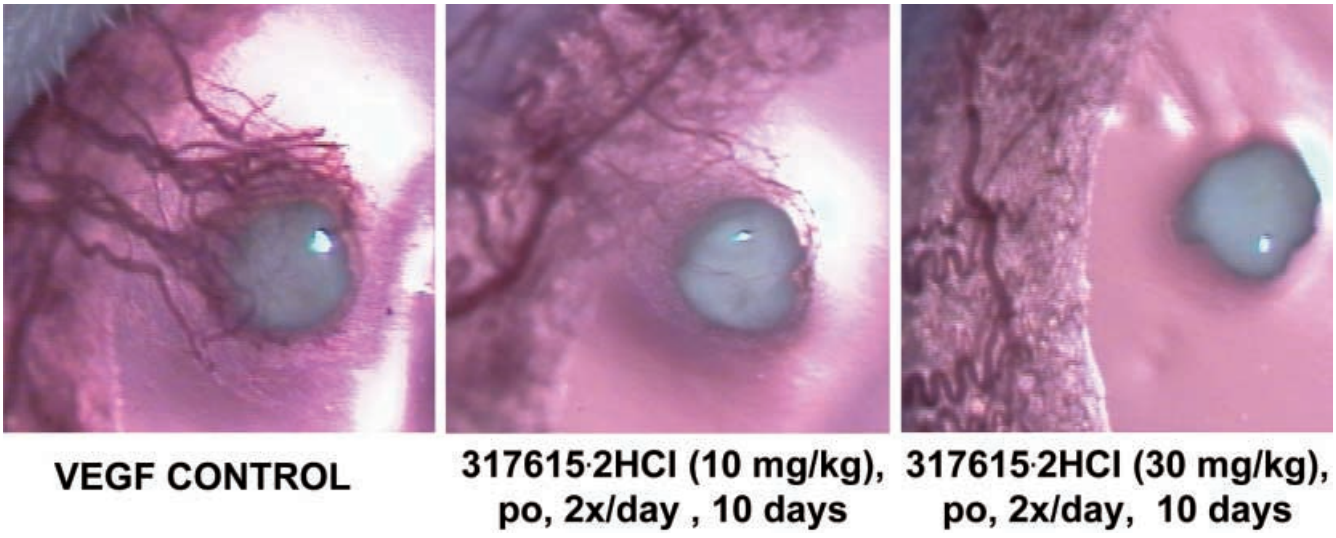

Fig. 3 Photographic image of VEGF-induced corneal neovascularization in a rat eye taken at 10 days after implantation of the stimulus after no treatment or after treatment of the animals with 317615.2HCl (10 or 30 mg/kg) orally twice per day for 10 days. Images are representative of each treatment group

impregnated with bFGF into the cornea of Fisher 344 female rats resulted in robust neoangiogenesis that was quantifiable in 7 to 10 days. Administration of 317615·2HCl (30 mg/kg) orally twice per day on days 1 through 10 after implantation of bFGF resulted in decreased vascular growth to a level of 26% of that of the bFGF control (Fig. 5).

Nude mice bearing human SW2 small-cell lung carcinoma growing as a subcutaneous xenograft on the thigh were treated with 317615·2HCl 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 Factor VIII or CD31. The number of intratumoral vessels in the samples was quantified by counting stained regions in ten high-power microscope fields (×200). There was a 317615·2HCl dose-dependent decrease in the number of countable intratumoral vessels in the human SW2 xenograft tumors. The number of intratumoral vessels stained by Factor VIII was decreased to one-half that of the controls in animals treated with 317615·2HCl (30 mg/kg) and the number of vessels stained by CD31 was decreased to one-quarter that of the controls in animals treated with 317615·2HCl (30 mg/kg) (Fig. 6).

A sequential treatment regimen was used to examine the efficacy of 317615·2HCl in the SW2 small-cell lung cancer xenograft. The compound 317615·2HCl was effective in this tumor model. Administration of 317615·2HCl alone on days 14 through 30 after tumor implantation over a dosage range from 3 to 30 mg/kg produced TGDs between 7.4 and 9.7 days in the SW2 small-cell lung cancer (Fig. 7). The SW2 tumor is quite responsive to paclitaxel, and treatment with that drug alone produced a 25-day TGD of the SW2 tumor. Treatment with paclitaxel followed by 317615·2HCl (30 mg/kg) resulted in a TGD of over 60 days, a 2.5-fold increase in the duration of tumor response. The SW2 small-cell lung cancer was less responsive to carboplatin,

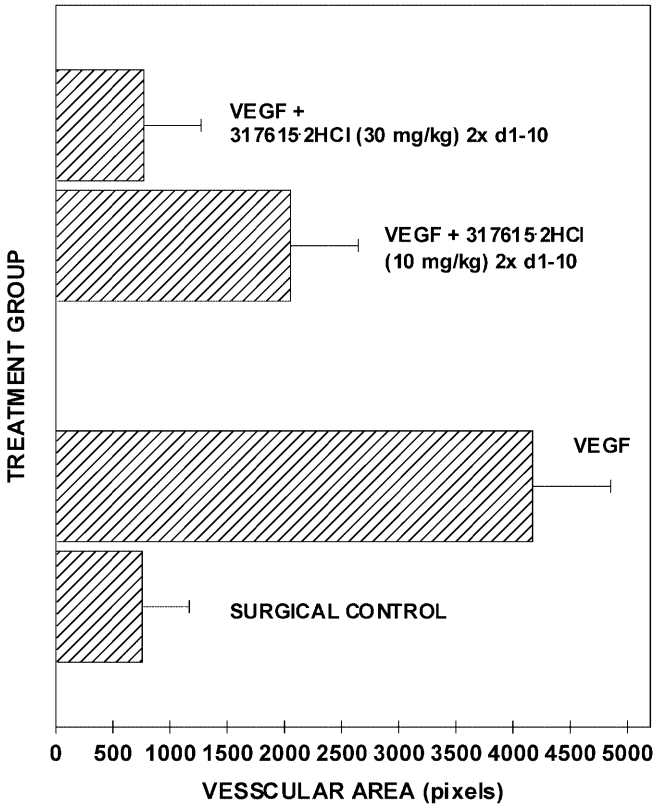

Fig. 4 Vascular area determined by image analysis and described in terms of pixel numbers for Fisher 344 female rats implanted with a small filter disc (inside diameter of a 20 G needle) impregnated with VEGF (except the surgical control). Animals were untreated or treated with 317615·2HCl (10 or 30 mg/kg) administered orally twice per day on days 1–10. Data are the means of four to six determinations from photographs on day 14 (*bars* SEM)

which produced a TGD of 4.5 days in that tumor. Sequential treatment with carboplatin followed by 317615·2HCl resulted in a TGD of 13.1 days.

Discussion

PKC is present in all cells. Because of this ubiquitous nature, there was serious concern about selectivity of PKC inhibitors for target cells versus critical normal cells for many years [10, 49]. The elucidation of the existence of multiple isoforms of PKC with different functional specificity and cellular context and the clearly established ability of medicinal chemistry to selectively target ATP-binding sites have relieved these concerns. Early synthetic protein kinase ATP-binding site-directed inhibitors such as staurosporine, GF109203X, Go9678, Ro31-8330, Ro31-8425, K252-a and UCN-01 have modest selectivity for PKC compared with other kinases [4, 11, 17, 27, 28, 29, 31, 32, 38, 43, 62, 71]. Inhibitors have now been developed with enhanced selectivity for PKC relative to other kinases and with selectivity for PKC isoforms.

The potential for modulating PKC in the treatment of cancer has been recognized for some time [6, 13]. PKC plays a key role in cell replication and differentiation and is involved in PKC-mediated induction of proto-oncogenes [41]. PKC is activated by a variety of mitogenic signals mediated through receptor tyrosine kinases including VEGF, platelet-derived growth factor and EGF, cytokine receptors and G-protein-coupled receptors. The spectrum of PKC isozymes expressed by various malignant cells is just now being elucidated [7, 8, 9, 14, 25, 51, 53, 76]. The ovarian carcinoma 2008 cell line and the cisplatin-resistant subline 2008/C13 express PKCα, PKCϵ and PKCζ but do not express PKCβ or PKCγ [72]. Human MCF-7 breast carcinoma cells express PKCα, PKCδ, PKCϵ, PKCλ, PKCμ, PKCι and PKCζ [9]. Human cervical carcinoma cells express PKCα, PKCϵ and PKCζ [8]. Five human neuroblastoma cell lines express PKCα, PKCβII, PKCδ and PKCϵ but not PKCι or PKCτ and only one line, LAN-2, expresses PKCγ [77]. The parental Mes-sa human uterine sarcoma cell lines and two paclitaxel-resistant sublines express PKCα, PKCγ, PKCι, PKCλ and PKCμ [15]. Zhu et al. [78] recently reported PKC isozyme profiling for the human AGS gastric carcinoma cell line. The PKC isoforms α, β1, β2, γ, ζ, ϵ, ι, μ, η and δ are all expressed in the AGS cells at levels quantifiable by Western blot analysis, with α, β1, β2, η and ζ being

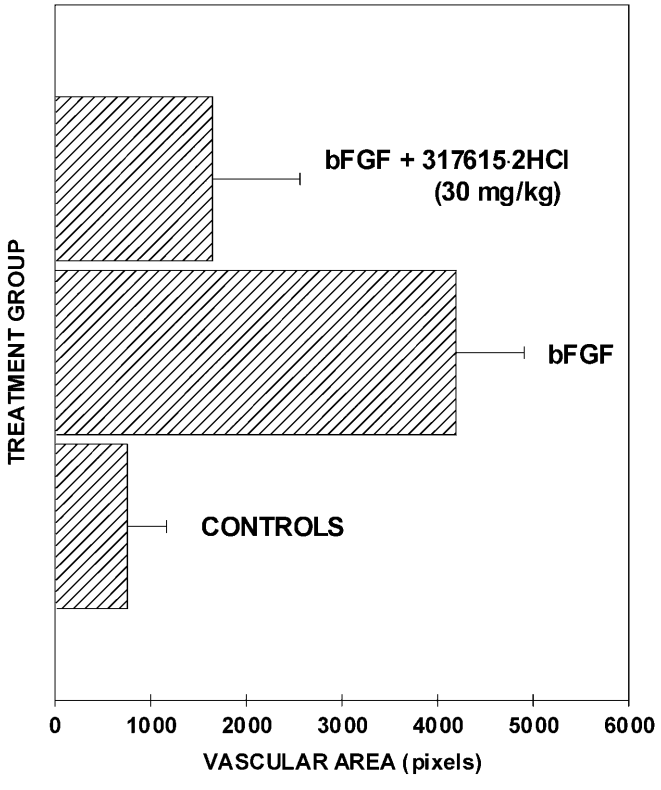

Fig. 5 Vascular area determined by image analysis and described in terms of pixel numbers for Fisher 344 female rats implanted with a small filter disc (inside diameter of a 20 G needle) impregnated with bFGF (except the surgical control). Animals were untreated or treated with 317615·2HCl (10 or 30 mg/kg) administered orally twice per day on days 1–10. Data are the means of four to six determinations from photographs on day 14 (*bars* SEM)

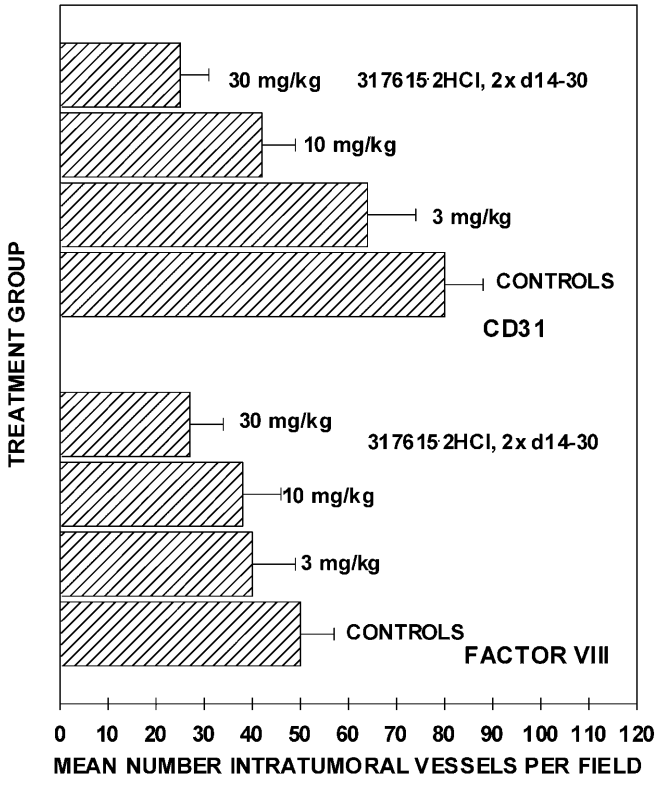

Fig. 6 Countable intratumoral vessels in human SW2 small-cell lung carcinoma xenograft tumors after treatment of the tumor-bearing animals with 317615·2HCl (3, 10 or 30 mg/kg) orally twice per day on days 14 through 30 after tumor implantation. Tumors were immunohistochemically stained for Factor VIII or CD31. Intratumoral vessels were counted manually. Data are the means of ten determinations (*bars* SEM)

the predominant isoforms. The isoforms λ and θ could not be detected.

From these early results, it appears that PKCβ is not frequently expressed in malignant cells. These findings are consistent with the observation that the compound 317615·2HCl, which has selectivity for inhibition of the β-isoforms of PKC, is a much more potent growth inhibitor of HUVEC than of the human SW-2 malignant cell line. Similarly, cellular growth inhibition of human T98G glioblastoma, human HS746 T gastric cancer, human Hep3B hepatocellular carcinoma and human Calu-6 non-small-cell lung cancer cells occurs with IC_{50} values between 26 μM and >250 μM [66, 67, 68]. Therefore, it is unlikely that inhibition of this enzyme in many malignant cells will contribute single-agent anticancer activity.

The signal transduction from extracellular protein growth factors occurs by a variety of mechanisms that share many common features. Activation of specific receptor kinases do not activate unique intracellular kinases which then result in a linear signaling pathway; rather multiple signaling cascades can be activated producing combinatorial effects that allow more refined regulation of the biological outcome [39]. The intracellular signal transduction pathways for VEGF and bFGF in endothelial cells have not been fully elucidated, but it is likely that PKC is an important pathway component

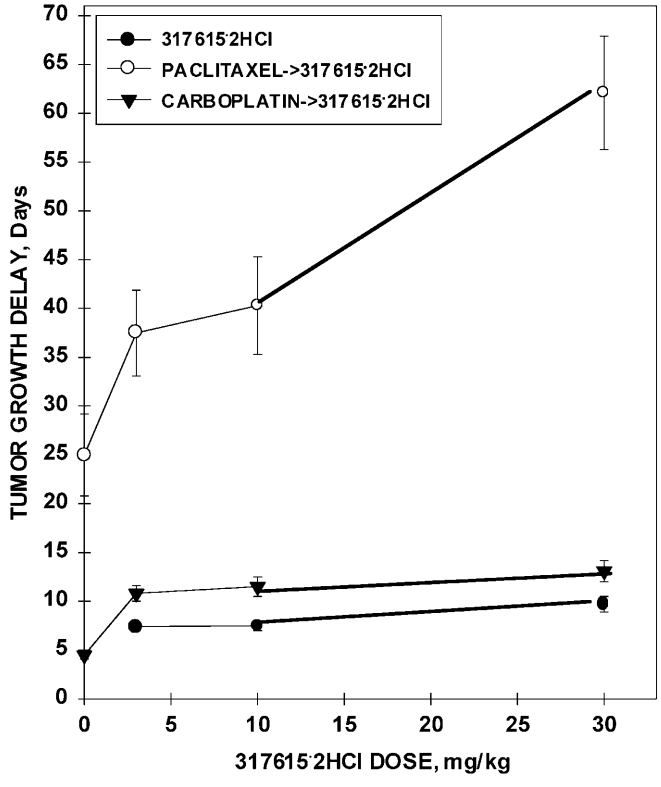

Fig. 7 Growth delay of the human SW2 small-cell lung carcinoma after treatment with 317615·2HCl (3, 10 or 30 mg/kg) orally twice per day on days 14 through 30 alone or along with paclitaxel (24 mg/kg, i.v.) on days 7, 9, 11 and 13, or carboplatin (50 mg/kg, i.p.) on day 7. Points are the means from five animals (*bars* SEM)

for both mitogens. Neoangiogenesis in the eyes of rats bearing corneal micropocket implants of either VEGF or bFGF was inhibited by treatment of the animals with 317615·2HCl orally twice per day. The human SW-2 small-cell lung carcinoma growing in nude mice secretes VEGF and bFGF that is measurable in the plasma of the animals. Treatment of SW-2-bearing mice with 317615·2HCl orally twice per day resulted in a countable decrease in intratumoral vessels and a corresponding slowing of tumor growth. These studies support the notion that the primary site of action of 317615·2HCl is the endothelium.

In cell culture, Schwartz et al. [56] have shown that exposure to concentrations of the non-specific protein kinase inhibitor, staurosporine, or the PKC inhibitor, safingol, blocks the invasion of the human SK-GT-1 and SK-GT-5 gastric carcinoma cells in the Boyden chamber assay. Exposure of human SK-GT-5 and MKN-74 gastric cancer cells to safingol increases the cytotoxicity of mitomycin C toward the cells by promoting drug-induced apoptosis [55]. In vivo, sequential treatment of mice bearing SW-2 human small-cell carcinoma with cytotoxic chemotherapy, paclitaxel or carboplatin, followed by 317615·2HCl resulted in markedly increased tumor response. This treatment schema was designed to detect tumor effects through the antiangiogenic mechanism and therefore specifically did not treat with the PKCβ inhibitor simultaneously with the chemotherapy. In preclinical models of other tumor types such as T98G glioblastoma multiforme, decreases in countable intratumoral vessels were decreased to one-half to one-third of the number in the control tumors and survival times of animals bearing intracranial tumors treated with 317615·2HCl along with BCNU were doubled [66]. On the other hand, in the HS746 T gastric cancer, the decrease in countable intratumoral vessels upon 317615·2HCl treatment was not significant, but the TGD produced by 5-fluorouracil in combination with 317615.2HCl was fourfold greater than that produced by 5-fluorouracil alone [67]. Therefore, the effects on countable intratumoral vessels do not always predict tumors where the greatest therapeutic benefit may result from a combination therapy regimen including 317615·2HCl [66, 67, 68].

Based upon these results, we conclude that 317615·2HCl acts as an antiangiogenic agent by selective inhibition of PKCβ with the ability to block multiple angiogenic factors and that the endothelial cell is most likely the important cellular target for this compound in vivo.

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