Selective Cytokine Inhibitory Drugs with Enhanced Antiangiogenic Activity Control Tumor Growth through Vascular Inhibition

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Abstract

Selective cytokine inhibitory drugs (SelCIDs) are a novel class of phosphodiesterase 4 inhibitors discovered during a thalidomide analog discovery program. These analogs were evaluated for their ability to inhibit tumor angiogenesis, vascularity, and growth. Two analogs (CC-7034 and CC-9088) were identified that had enhanced antiangiogenic activity in Matrigel assays compared with parental thalidomide. These analogs also inhibited the growth of established K1735 and RENCA murine tumors. Tumors whose growth was suppressed by SelCID treatment exhibited decreased vessel density together with increased tumor cell hypoxia and death. The decrease in vascularity produced by SelCID treatment is attributed to a selective loss of vessels devoid of pericyte coverage, suggesting that these agents target immature tumor vessels. That tumor cell death was localized to relatively avascular or hypoxic areas, coupled with the fact that none of the analogs was cytotoxic in vitro against the tumor cells, demonstrates that these analogs are novel antivascular agents with potent antitumor activity.

Introduction

Thalidomide is a glutamic acid derivative initially marketed in the late 1950s as a sedative-hypnotic. Discovery of its teratogenicity led to its removal from the drug market in the early 1960s. More recently, thalidomide has been shown to be of clinical benefit in a number of pathological conditions including erythema nodosum leprosum, rheumatoid arthritis, HIV-associated oral ulcers, and chronic graft-versus-host disease (1–6). These activities appear to be related to its ability to inhibit tumor necrosis factor-α production in vitro and in vivo (7–9). In addition, thalidomide has been shown to have activity against a number of human malignancies, particularly multiple myeloma (10, 11). Thalidomide is now often used in several lines of therapy in the treatment of multiple myeloma. Although the precise mechanism of its antitumor activity remains to be established, multiple activities including inhibition of angiogenesis have been speculated to have a role. Thalidomide has been shown in a number of studies to be a potent inhibitor of angiogenesis in experimental models of neovascularization (12–14). Additionally, in multiple myeloma patients treated with thalidomide, decreases in bone marrow vascularity have been observed (11, 15). Structural analogues of thalidomide have been shown to inhibit tumor angiogenesis, vascularity, and growth. Two analogs (CC-7034 and CC-9088) were identified that had enhanced antiangiogenic activity in Matrigel assays compared with thalidomide. These agents were demonstrated to decrease the vascularity and inhibit the growth of two murine tumor types (K1735 melanoma and RENCA renal cell carcinoma). The decrease in tumor vascularity was attributable to a preferential loss of immature tumor vessels. Successful treatment was associated with an increase in tumor cell hypoxia, and tumor cell apoptosis produced by treatment was localized to hypoxic regions. Together, these studies indicate that SelCIDs represent a novel group of angiogenesis inhibitors with potential as cancer therapeutic agents.

Materials and Methods

Mice and Cell Lines. BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), whereas C3H/HeN mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were females, 6–8 weeks of age, maintained in microisolator cages under sterile conditions. The K1735 (21) murine melanoma and the RENCA (22) renal cell adenocarcinoma cell lines (syngeneic with C3H/HeN and BALB/c, respectively) were maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin.

SelCIDs. The synthesis of SelCIDs has been described previously based on the replacement of the thalidomide glutarimide ring and substitutions on the phthaloyl ring (17). CC-7034 is 3-(4-acylaminolino-1,3-dioxo-1,3-dihydroisindol-2-yl)-3-(3-ethoxy-4-methoxy-phenyl)-N-hydroxy-propionamide and CC-9088 is 2-dimethylamino-4-{[1-(3-ethox-y-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3-dioxo-1H-isindol-4-yl}-acetamide hydrochloride. The SelCID class of analogues is distinguished by the presence of a 3,4-dialkoxylphenyl moiety similar to some other classes of PDE4 inhibitors. For in vivo administration, the water-soluble analog CC-9088 was dissolved in 5% dextrose in water, whereas the other analogs were suspended in saline containing 0.01% Tween 80 and sonicated before use.

In Vivo Studies. An in vivo Matrigel assay for angiogenesis was performed essentially as described (23) using SCK tumor cells as the angiogenesis stimulant. Treated mice received thalidomide or one of the SelCID analogues i.p. (100 mg/kg) on days 1–5, and neovascularization was measured on day 6. To quantitate angiogenesis, Matrigel hemoglobin content was determined by the Drabkin method (Sigma). For tumor growth studies, 105 tumor cells were
injected s.c. into syngeneic mice. When established tumors reached a diameter of 1 mm, generally 2–3 weeks after cell inoculation, the SelCID analogs or vehicle control was administered i.p. Tumors were measured bidirectionally, and tumor volume was calculated by approximating the volume of a spheroid \([0.52 \times (\text{width})^2 \times \text{length}]\). For hypoxia studies, tumor-bearing mice were injected i.v. with 10 mM EF5 (provided by C. Koch, University of Pennsylvania, PA) in 0.9% NaCl 3 h before tumor excision. Mice were euthanized according to guidelines established by the Institutional Animal Care and Use Committee.

**In Vitro Assay for Cytotoxicity.** A colorimetric tetrazolium salt cleavage assay for cell viability was performed according to the manufacturer’s instructions (XTT kit; Intergen, Purchase, NY). Briefly, 1 ≈ 10^3 tumor cells were seeded in growth medium and incubated overnight. The next day, the thalidomide or the SelCID analogs were added at the concentrations indicated for 48 h. Equivalent molar concentrations of paclitaxel (Sigma) were used as a positive control. XTT solution was added at a final concentration of 0.5 mg/ml for the final 5 h. A microtiter ELISA reader was used to measure absorbance (450 nm) of formazan products.

**Confocal Microscopy of Tumor Vasculature.** Tumor-bearing mice were injected with 150 μl of 2 mg/ml FITC-conjugated Lycopersicon esculentum lectin (Vector Labs, Burlingame, CA) i.v. 15 min before tumor excision. Tumors were sectioned and imaged essentially as described previously (24). Images were viewed by ×10 objective lens (1004.5 μm × 1004.5 μm), and image stacks were acquired at 2-μm intervals over 100-μm depth.

**Automated Analysis of Confocal Vascular Images.** Quantitative determination of vessel size and density from confocal image stacks was performed using a previously described automated vessel seed point selection process (25). Briefly, each image stack is projected onto the xy plane using maximum projection, and a grid spaced 15 pixels apart was superimposed on the resulting two-dimensional image, denoted \(I_{xy}\). The gray-level values on each gridline are low-pass filtered using a one-dimensional kernel of the form \([0.25, 0.5, 0.25]\). Seed point candidates are identified as local intensity maxima on each line using a one-dimensional neighborhood of 15 pixels with intensities larger than \(\gamma_{v} + \sigma_{v}\), where \(\gamma_{v}\) is the median pixel intensity of \(I_{xy}\) and \(\sigma_{v}\) is the SD. Each seed point \(P(x, y)\) is then projected back into the original three-dimensional volume, where the plane \(Z = z^*\), resulting in maximum intensity in a local neighborhood around \(P(x, y, z^*)\) is assumed to be the true three-dimensional location of the seed point. A set of three-dimensional directional edge detector kernels is then applied, and only those seed points associated with vessel boundaries are included in the vessel density and diameter calculations.

**Immunohistochemistry.** Thin sections (10 μm) from cryopreserved tumors were stained for endothelial cells using a rat anti-mouse CD31 monoclonal antibody (PharMingen, San Diego, CA) and for pericytes using a mouse anti-α-smooth muscle actin antibody (Dako, Carpintieria, CA) as described previously (26). Staining of frozen tumor sections for the hypoxia marker EF5 was performed essentially as described (27) using a cyanine-3 conjugated anti-EF5 monoclonal antibody provided by C. Koch. Apoptotic cells were detected using the Apoptag kit (Intergen). All histological specimens were viewed under a Microphot-FX light microscope (Nikon, Melville, NY) equipped with a krypton-argon laser and optical filters for visualization of FITC, Texas Red, and cyanine-3 fluorescence. Images were acquired by the Photometrics Coolsnap digital camera and image acquisition software (Roper Scientific, Trenton, NJ).

**Image Analysis.** For microvessel density measurements, slides were scanned at low power (×40) to identify areas of highest vascularity. Ten to twenty high-powered (×200) fields were then selected randomly within these areas, and microvessel densities were calculated based on the number of CD31-positive structures. In addition, vessel lumen cross-sectional areas were determined for all counted vessels automatically (ImageTool, University of Texas, San Antonio, TX) based on spatial calibration parameters established with a slide micrometer. Microvessel counting was performed by multiple blinded observers in conjunction with a pathologist. A pericyte-positive vessel was defined as a CD31-positive vessel surrounded by at least one cell staining positive for α-smooth muscle actin. Two to three sections were examined per tumor, and at least five tumors were examined per treatment group.

**Statistical Analysis.** Assessment of statistical significance was performed either by Student’s t test (for normally distributed data sets) or Mann-Whitney U test (for non-normally distributed data sets). All statistical analyses were performed using Instat software for the Macintosh version 2.0 (Graphpad Software, Philadelphia, PA).

**Results**

**SelCIDs Inhibit Tumor Cell-induced Angiogenesis.** We screened a series of SelCIDs for their ability to inhibit tumor cell angiogenesis using an in vivo Matrigel neovascularization assay (Fig. 1). Systemic administration of thalidomide itself reduced SCK cell-induced Matrigel angiogenesis by 33.7 ± 25.7% compared with untreated mice (P < 0.05). Four analogs were screened for activity. Although two of them showed minimal activity at the doses tested (data not shown), treatment with the two other SelCIDs was associated with statistically significant (P < 0.05, Student’s t test) reduction in Matrigel neovascularization, CC-7034 (77.2 ± 7.4%) and CC-9088 (65.0 ± 15.8%). On the basis of these results, these compounds were then evaluated for antitumor activity.

**SelCID Effects on Tumor Growth.** The maximum tolerated doses of CC-7034 and CC-9088 were determined in mice of two strains, C3H/HeN and BALB/c, that serve as hosts for K1735 melanomas and RENCA renal cell carcinomas, respectively. Various doses of SelCID (100, 250, and 500 mg/kg/day) were given to mice daily for 7 days. Mice of both strains tolerated CC-7034 at the highest dose, except that all developed ruffled fur. There were no other physical, behavioral, or weight loss signs of toxicity, except for one BALB/c mouse that was found dead after the last dose. CC-9088 at 500 mg/kg/day was toxic for mice of both strains but was well tolerated at 250 mg/kg/day except for the development of ruffled fur in BALB/c mice. On the basis of these results, we used CC-7034 at doses up to 500 mg/kg/day and CC-9088 at doses up to 250 mg/kg/day for both BALB/c and C3H/HeN mice.

**Hemoglobin content (% of control)**

![Fig. 1. Inhibition of tumor cell-induced angiogenesis by thalidomide and its analogs.](image-url) Thalidomide and SelCID analogs were tested for their ability to inhibit angiogenesis in a Matrigel in vivo neovascularization assay. SCK mammary carcinoma cells provided the angiogenic stimulus, and angiogenesis was assessed by measuring Matrigel hemoglobin content. n = 5 mice per group; bars, SD.
We next assessed the ability of the thalidomide analogs to inhibit growth of two murine tumor types, K1735 melanomas and RENCA renal cell carcinomas. K1735 or RENCA tumor cells (10^6) were implanted s.c. in C3H/HeN or BALB/c mice, respectively, and daily administration of these SelCIDs or PBS/Tween 80 vehicle was initiated once tumors were established. CC-7034 at 100 mg/kg/day consistently slowed RENCA tumor growth (Fig. 2A), and by day 33, CC-7034-treated tumors were on average only 17% the size of vehicle-treated tumors (85 ± 78 mm^3 versus 499 ± 123 mm^3). CC-9088 at the same dosage did not inhibit growth of RENCA tumors. However, increasing CC-9088 to 250 mg/kg/day resulted in significant suppression of RENCA tumor growth (Fig. 2C). A different pattern of antitumor activity was observed in K1735 tumors. Here, CC-9088 at 100 mg/kg/day, the lowest dose tested, had a pronounced effect on K1735 growth, with average tumor volumes (43 ± 40 mm^3) <10% that of vehicle-treated tumors (439 ± 112 mm^3) after 2 weeks of treatment and growth retardation continuing after >4 weeks of treatment. Increasing the dose of CC-9088 did not significantly enhance activity (data not shown). CC-7034 demonstrated dose-dependent antitumor activity,
with maximum growth suppression occurring at the highest dose of 500 mg/kg/day (Fig. 2, B and D).

To determine whether the antitumor effect of these SelCIDs was attributable to a direct cytotoxic effect on the tumor cells, the latter were exposed to the thalidomide analogues in vitro, and cell viability was assessed by XTT assay (Fig. 2, E–G). Tumor cells were exposed to SelCIDs for 48 h at concentrations from 0.01 to 1.0 μM, the range over which thalidomide and its analogs have been shown to inhibit tumor necrosis factor-α production in vitro (18). This concentration range should also be a reasonable approximation of in vivo exposure, because intraperitoneal administration of SelCIDs to mice has been associated with a plasma Cmax of 1.65 μM (data not shown). No significant effect on RENCA or K1735 tumor cell viability was observed with either analog. In contrast, 0.01 μM of the cytotoxic agent, paclitaxel, was sufficient to reduce RENCA and K1735 cell viability dramatically (by 59.8 and 41.9%, respectively; Fig. 2, E and F). Additionally, neither thalidomide nor any of the SelCIDs were directly toxic to SCK cells in vitro (Fig. 2G), indicating that the antiangiogenic activity exhibited in the Matrigel neovascularization assay was not attributable to a tumor cell cytotoxic effect. Because none of the analogues exhibited significant tumor cell cytotoxicity, we turned our attention to their effect on tumor vasculature.

Effects of CC-7034 and CC-9088 on Tumor Vasculature. We initially assessed tumor vascularity by injecting tumor-bearing mice i.v. with fluorescein-tagged *Lycopersicon esculentum* (tomato) lectin (28) to outline tumor vessels bearing blood flow; we imaged the vasculature by confocal microscopy (Ref. 24; Fig. 3, A–D). The vessel image stacks were analyzed by applying an algorithm described previously (25) to determine vessel density and size in three dimensions. For this analysis, we examined tumors treated at the lowest SelCID dose (100 mg/kg/dose) necessary to control tumor growth, which were K1735 tumors treated with CC-9088 and RENCA tumors treated with CC-7034. Analysis of the RENCA confocal images (Fig. 3E) revealed that CC-7034 treatment led to a 38% reduction in overall tumor vascular density (3840 ± 598 vessels/mm³ vehicle versus 2388 ± 573 vessels/mm³ CC-7034), which is statistically significant (P < 0.05, Mann-Whitney U test). Similarly in K1735 tumors, CC-9088 treatment led to a 30% reduction in tumor vascularity (5115 ± 757 vessels/mm³ vehicle versus 3575 ± 465 vessels/mm³ treated, P < 0.01; Fig. 3G). We also assessed tumor vessel size by this method (Fig. 3, F and H). A significant increase in mean vessel diameter compared with vehicle-treated tumors was observed in K1735 tumors treated with CC-9088 (54% increase in diameter; P < 0.01), along with RENCA tumors treated with CC-7034 (42% increase; P < 0.005). Thus, successful control of tumor growth with SelCID therapy is associated with decreases in tumor vascularity and increases in tumor vessel size.

SelCID Treatment Leads to Selective Regression of Immature Tumor Blood Vessels. Previous studies of angiogenesis inhibitors have shown that some specifically target smaller, less mature blood vessels (29); therefore, we asked whether there was selectivity in the tumor vessels targeted by our SelCIDs. We stained histological sections from K1735 and RENCA tumors treated and untreated with SelCIDs for the endothelial cell marker CD31 and the periendothelial cell marker SMA (Fig. 4, A–F). In both K1735 and RENCA tumors, treatment with CC-7034 and CC-9088 at doses sufficient to inhibit tumor growth was associated with a decrease in microvessel density as well as an increase in the percentage of tumor vessels that were pericyte covered (Table 1). We also quantified the density of single endothelial cells, which we showed previously to be an indicator of angiogenic endothelial sprouts (26). In the SelCID-treated tumors, single endothelial cells represent a lower percentage of total tumor vessels. Taken together, these data indicate that tumor treatment with CC-7034 and CC-9088 leads to a preferential decrease in immature tumor vessels.

Tumor Cell Death During SelCID Treatment Localizes to Regions of Tumor Hypoxia. Histological analysis of tumors effectively treated with CC-7034 and CC-9088 revealed large areas of tumor cell death in regions distant from the nearest apparent blood vessel (Fig. 4, A–F). In fact, the interface between dead and viable tumor areas was regularly 5–10 cell diameters from the nearest vessel, a distance at which oxygen delivery has been shown to be limiting (30). Such a pattern would suggest that the large amount of tumor cell death induced by SelCID treatment is a product of its antivascular effect, with tumor cell death occurring selectively in areas without adequate access to perfusion.

To assess tumor ischemia, we used the nitroimidazole EF5 to indicate tumor cell hypoxia (27). K1735 tumors were examined because these tumors are known to have little to no spontaneous hypoxia at baseline, so that development of significant hypoxia is attributable to treatment. SelCID-treated K1735 tumors immunostained for both EF5 and CD31 demonstrated patchy areas of tumor hypoxia in regions distant from the nearest blood vessel (Fig. 4, H–I). In contrast, vehicle-treated tumors contained almost no areas of EF5 staining (Fig. 4G). When we stained with TUNEL in addition to EF5, we found that tumor cell apoptosis, virtually absent in vehicle-treated K1735 tumors, was dramatically increased with SelCID treatment and localized predominantly within EF5-staining tumor areas (Fig. 4, J–O). Together, these results indicate that SelCID therapy of K1735 and RENCA tumors produces tumor cell death that is a consequence of the ischemia induced by angiogenesis inhibition.
Discussion

We have screened a series of SelCID structural thalidomide analogs for antivascular activity. Two of the analogs (CC-7034 and CC-9088) exhibited an ability to inhibit angiogenesis in an in vivo Matrigel neo-vascularization assay that was superior to the parental thalidomide compound. Both compounds were able to control the growth of two different murine tumor models, K1735 melanoma and RENCA renal cell carcinoma. Their effects on functional tumor vasculature were assessed by confocal imaging of lectin-stained blood vessels in thick tumor sections. This provided three-dimensional imaging that eliminates sectioning artifacts and improves sampling by imaging a much larger volume of tumor tissue. Only functional vessels are imaged because the labeling dye is given i.v. To extract quantitative information from these images, we applied a rapid seed-point analysis algorithm to calculate the density and luminal diameter of perfused blood vessels. We also immunostained thin histological sections for CD31, SMA, EF5, and TUNEL to relate vascular changes to potential microenvironmental and tumor cell consequences. Together, these methods revealed that successfully treated tumors exhibited a decrease in vessel density that was attributable to a preferential reduction in blood vessels devoid of pericytes as well as a loss of single endothelial cells. Thus, the antivascular effect of these compounds appears to involve both the loss of immature vessels and a decrease in new vessel formation, respectively. This pattern of vascular change is similar to that observed in tumors undergoing vascular endothelial growth factor withdrawal and suggests that tumor treatment with antiangiogenic agents may generally lead to preferential loss of smaller vessels. A possible implication is that the effects of angiogenesis inhibitor therapy may vary, depending on the complement of mature vessels in tumors.

Table 1: Effect of SelCID treatment on tumor vasculature

<table>
<thead>
<tr>
<th>Tumor Treatment</th>
<th>Dosage (mg/kg/day)</th>
<th>MVD per hpf (±SD)</th>
<th>SMA+ % (±SD)</th>
<th>SEC % (±SD)</th>
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<tbody>
<tr>
<td>K1735 Vehicle</td>
<td>None</td>
<td>11.70 ± 2.36</td>
<td>35.33 ± 5.03</td>
<td>18.33 ± 4.51</td>
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<tr>
<td>CC-7034 500</td>
<td>4.66 ± 1.60a</td>
<td>47.80 ± 18.94</td>
<td>10.60 ± 3.65a</td>
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<tr>
<td>CC-9088 100</td>
<td>6.68 ± 1.54a</td>
<td>55.00 ± 12.89a</td>
<td>12.40 ± 3.85a</td>
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<tr>
<td>RENCA Vehicle</td>
<td>None</td>
<td>9.27 ± 1.88</td>
<td>29.67 ± 12.66</td>
<td>14.00 ± 7.72</td>
</tr>
<tr>
<td>CC-7034 100</td>
<td>4.00 ± 1.38ab</td>
<td>54.20 ± 8.81ab</td>
<td>9.60 ± 5.77ab</td>
<td></td>
</tr>
<tr>
<td>CC-9088 250</td>
<td>2.75 ± 0.79b</td>
<td>51.00 ± 21.04</td>
<td>9.20 ± 3.05b</td>
<td></td>
</tr>
</tbody>
</table>

a,b Statistical significance compared to vehicle-treated tumors: " P < 0.01; " P < 0.05.
CC-7034- and CC-9088-treated tumors exhibited broad areas of staining for the hypoxia marker EF5 that were not seen in control tumors, implying that the reduction in tumor vessel density observed after treatment was physiologically significant. Treatment also caused a dramatic increase in tumor cell death. The colocalization of areas of EF5 staining and tumor cell death, together with the lack of tumor cell cytotoxicity in vitro, suggests that vascular inhibition mediates the antitumor effect of SelCIDs.

An unresolved issue is why different SelCID dosages are required to obtain efficacy in different tumor models. CC-7034 is effective against RENCA tumors at 100 mg/kg/day but is active against K1735 tumors only at higher doses, whereas CC-9088 demonstrates an opposite pattern of antitumor activity. One possibility is that the analogs antagonize different angiogenic factors. For example, RENCA tumors are known to express more vascular endothelial growth factor and less platelet-derived growth factor-B than K1735 tumors (data not shown). Another possibility is that there is a genetic component to SelCID activity such that each analog is most efficacious in a different mouse strain. There are many other possible explanations, and identifying the determinants of therapeutic efficacy may require elucidation of the molecular mechanism of SelCID antivascular activity. Regardless of the mechanism(s), our results illustrate that antivascular responses are unlikely to be determined solely by the therapeutic agent used, and there may be variability in the response of different tumors.

Finally, CC-7034 and CC-9088 are two structural analogs of thalidomide that exhibit potent antiangiogenic and antitumor activities. A number of recent studies have demonstrated the ability of thalidomide analogs to control tumor growth, postulating a variety of mechanisms including inhibition of tumor cell proliferation (31), augmentation of natural killer cell cytotoxicity (32), and alterations in expression of Bcl-2 family proteins (20). The ability of one analogue to suppress growth of a murine B-cell tumor model was associated with a decrease in tumor microvessel density (33). CC-7034 and CC-9088 are the first analogs in which control of tumor growth can be conclusively linked to a therapeutic reduction in tumor vascularity. Additionally, we have identified properties of tumor vessels conferring relative susceptibility and resistance to SelCID-induced regression. Given the promising initial findings regarding the use of thalidomide in cancer clinical trials, the results obtained with these SelCIDs merit further investigation of these novel PDE4 inhibitors as potential cancer therapeutic agents.

Acknowledgments

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References

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