S-3-Amino-phthalimido-glutarimide Inhibits Angiogenesis and Growth of B-Cell Neoplasias in Mice


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ABSTRACT

Thalidomide has recently been shown to be useful in the treatment of multiple myeloma and may also be useful in the treatment of other hematological malignancies. We have identified a new derivative of thalidomide, S-3-[3-amino-phthalimido]-glutarimide (S-3APG) with dual activity against B-cell neoplasias. S-3APG was able to directly inhibit the proliferation of myeloma and Burkitt’s lymphoma cell lines in vitro without showing toxicity to normal bone marrow stromal cells or hematopoietic progenitor cells. In vivo, S-3APG treatment of drug resistant myeloma cell tumors in mice was able to produce complete and sustained regressions without any observed toxicity. Additionally, S-3APG induced complete regressions of Burkitt’s lymphoma cell tumors. Furthermore, S-3APG inhibited angiogenesis more potently than thalidomide in the murine corneal micropocket model. We conclude that S-3APG is a powerful anti-myeloma and anti-B-cell-lymphoma agent that has both anti-proliferative and antiangiogenic effects.

INTRODUCTION

Thalidomide was originally developed in Europe as a sedative but withdrawn from the market because of its association with teratogenicity. The recent discovery of its immunomodulatory (1) and antiangiogenic (2) activity has led to increased exploration of its therapeutic utility. Current and potential applications include erythema nodosum leprsum, renal cell carcinoma, Kaposi’s sarcoma, and other cancers (3, 4). Recent reports that increased bone marrow microvessel density correlated with disease progression, and poor prognosis in multiple myeloma patients (5–7) provided a rationale for the use of thalidomide in the management of this disease. Indeed, 32% of myeloma patients whose disease was refractory to conventional, and even high-dose, chemotherapy demonstrated a clinical response to thalidomide (8). In addition, 31% of AML patients demonstrated a clinical response to thalidomide in an early trial (9), which suggests that thalidomide’s efficacy in hematological malignancies may not be limited to myeloma. For this reason, new derivatives are being developed to enhance the antitumor activity of thalidomide (10, 11).

The mechanism of action of thalidomide in multiple myeloma is currently under investigation. Three effects have been proposed. First, the growth of new vessels is thought to be necessary to sustain myeloma tumor growth in the marrow and angiogenic growth factors may directly stimulate myeloma cell growth (12–17). Therefore, thalidomide may mediate its anti-myeloma effect by the inhibition of angiogenesis and angiogenic factors. Although thalidomide treatment of myeloma patients did not significantly lower bone marrow microvessel density, other more sensitive markers of angiogenesis have recently been reported to be affected (18). A 10-fold decrease of bone marrow-activated endothelial cells in myeloma patients that respond to thalidomide recently reported. Also, plasma levels of VEGF and bFGF decreased significantly in responders. Interestingly a similar finding was just reported for AML patients who responded to thalidomide (9). This subject was recently reviewed by Folkman (19).

Second, thalidomide may act directly on myeloma cells (20) and/or BMSC by inhibiting myeloma-cell-BMSC adhesion or the secretion of cytokines. Finally, thalidomide may stimulate T-cell and natural killer cell response (1, 21). Elucidation of the mechanism by which thalidomide inhibits multiple myeloma is complicated by the fact that thalidomide is metabolized in vivo to various derivatives, each of which may have independent effects. Indeed, thalidomide and its breakdown products require metabolism by liver microsomes for activity in angiogenesis assays in vitro (22). In the case of other hematological malignancies (such as AML), thalidomide’s efficacy has yet to be conclusively demonstrated, but in tumors in which it is active, it is likely to act via a pathway similar to that observed in myeloma.

To further explore the activity of thalidomide, we synthesized and screened monosubstituted derivatives looking for compounds with direct effects on myeloma cells or angiogenesis. From this screening, we identified a synthetic amino derivative of thalidomide, S-3APG, with promising activity against both myeloma and Burkitt’s lymphoma. This compound was previously reported to have cytokine-modulatory (compound S-5a in Ref. 11) and teratogenic (23) activity.

MATERIALS AND METHODS

Cell Culture. Dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) human myeloma cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL) and ANBL-6 cell line by Diane Jelinek (Mayo Clinic, Rochester, MN). Hs Sultan human Burkitt’s lymphoma cells (24) were obtained from the American Type Culture Collection (Manassas, VA). Patient myeloma cells (96% CD38+, CD45RA−) were purified from patient bone marrow samples, as described previously (25). BMSCs were generated from patient bone marrow samples as described previously (26). These cells were cultured in RPMI 1640 (Sigma Chemical, St. Louis, MO) containing 10% FBS, 2 mM l-glutamine (Life Technologies, Inc., Grand Island, NY), 25 units/ml penicillin, and 25 μg/ml streptomycin (Life Technologies, Inc.). MM.1R cells were cultured with dexamethasone to confirm their lack of drug sensitivity. Human umbilical vein endothelial cells P168 (Biowhittaker, Walkersville, MD) were maintained in EGM2MV growth media (Biowhittaker). LLC cells were cultured in DMEM, containing 10% FBS, 2 mM l-glutamine, 25 units/ml penicillin, and 25 μg/ml streptomycin. MG-63 cells (American Type Culture Collection) were cultured in DMEM...
containing 7% FBS, 2 mM l-glutamine, 25 units/ml penicillin, and 25 μg/ml streptomycin. Bovine capillary endothelial cells were cultured in DMEM containing 10% FBS, 2 mM l-glutamine, and 1 ng/ml bFGF (Scios Nova, Sunnyvale, CA). For coculture assays, 3 x 10^5 stromal cells/well were seeded into a 6-well plate (3 ml/well). After 24 h, supernatant with unattached cells was removed and replaced with conditioned medium containing 3 x 10^3/3 ml MM.1S cells and DMSO, thalidomide, or S-3APG. Cell proliferation was measured using thymidine incorporation and colorimetric assays as described (10).

**Thalidomide and Analogues.** For in vitro assays, thalidomide and 3APG (EntreMed, Rockville, MD) in DMSO (Sigma) were diluted in culture medium immediately before use. For animal experiments, thalidomide and analogues (50 mg/kg) were suspended immediately before use in 0.5% carboxymethylcellulose, vortexed with sterile glass beads for >5 min, and administered i.p. in a volume of 0.1 ml daily. Control mice received 0.1 ml of the vehicle i.p.

**Colony-forming Unit Assays.** Immunomagnetic enrichment of human CD34 cells from leukapheresis products was performed as described previously (27). Hematopoietic colony formation was analyzed using a standard methyl cellulose-based assay (Methocult GF H4434, Stem Cell Technologies, Vancouver, Canada). CD34 cells were cultured in IMDM with 20% FBS in the absence or presence of IL-3, and 2μg/ml IL-6 for 72 h. Stimulated or unstimulated CD34+ selected cells were seeded at 1.5 x 10^3 cells/ml, together with S-3APG or thalidomide. Colonies were scored after 14 days.

**Tumor Models.** To determine the in vivo antitumor activity of thalidomide and S-3APG, immunodeficient mice were inoculated s.c. with 3 x 10^5 human Hs Sultan (5–8-week-old BNX mice, Frederick Cancer Research and Development Center, Frederick, MD), RPMI-8226 (BNX mice) or MM.1R (beige-SCID mice) cells together with Matrigel (Becton Dickinson, Bedford, MA) in a volume of 200 μL. The murine Lewis Lung Carcinoma tumor line was maintained in vivo passages as described previously (28). We passaged 3 x 10^5 LLC cells from tumor-bearing mice s.c. into C57BL/6J recipients (6–8-weeks-old, male, The Jackson Laboratory, Bar Harbor, ME), and treatment was started after the development of measurable tumor. Daily i.p. drug injections were performed until death, for 10 days after complete resection (Hs Sultan cells) or for 90 days (MM.1R cells). Serial caliper measurements of perpendicularly diameters were used to calculate tumor volume using the following formula: (shortest diameter)^2 x (longest diameter) x 0.52. Animals were killed if the tumor was ≥2 cm^3 or necrotic. All of the animal studies were conducted according to protocols approved by the Animal Ethics Committees of the Dana-Farber Cancer Institute and Children’s Hospital. Animals were anesthetized by isoflurane inhalation before all procedures and observed daily for signs of toxicity. Animals were killed by CO2 asphyxiation.

**Mouse Corneal Micropocket Assay.** The corneal micropocket assay was performed in C57BL/6J mice as described previously (29) using pellets containing 80 ng of bFGF or human recombinant VEGF (R&D Systems, Minneapolis, MN). The treated groups received daily administration for 5 (bFGF) or 6 (VEGF) consecutive days of thalidomide or 3APG (50 mg/kg) suspended in 0.5% carboxymethylcellulose i.p. Treatment was started on the day of pellet implantation; control mice received only carboxymethylcellulose i.p. The area of vascular response was assessed on the 5th (bFGF) or 6th (VEGF) postoperative day using a slit lamp.

**Microvessel Staining and Counting.** Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and stained for CD-31 as described (30). Microvessel density was determined by light microscopy, according to the procedures of Weidner et al. (31). Examination was performed blinded simultaneously by two investigators using a double-headed light microscope. At least five separate high power magnification (×400) fields were analyzed.

**Statistical Analysis.** Student’s two-tailed t test was used to calculate the statistical significance of differences between groups for experiments (with the exception of experiments with multiple comparisons in which ANOVA was used). The results are presented as means ± SE. Statistical difference between the survival curves was performed using log rank test and Graphpad Prism software.

**RESULTS**

The initial characterization of S-3APG for activity included proliferation assays using a variety of cell types. In these assays, we observed that S-3APG directly inhibits cultured myeloma and lymphoma cell lines but does not affect NIH 3T3 fibroblasts, MG-63 osteogenic sarcoma cells, bovine capillary endothelial cells, or FBMD1 preadipocyte cells. In fact, more than one-half of the myeloma cell lines we tested were sensitive to S-3APG as assessed by the inhibition of DNA synthesis and cell proliferation (7 of 11 cell lines; Fig. 1). For example, 50% inhibition of proliferation (IC_{50}) of MM.1S cells with S-3APG was noted at ~10 nm, whereas RPMI-8226 cells were not significantly inhibited. Other cells of B-cell lineage are also likely to be affected by S-3APG as evidenced by the inhibition of the Hs Sultan cell line with similar IC_{50}. Furthermore, we studied MM.1R cells resistant to dexamethasone to determine whether there was cross-resistance between S-3APG and conventional anti-myeloma agents. These cells showed the same sensitivity to S-3APG as MM.1S (IC_{50} ~10 nm, data not shown). In contrast, thalidomide failed to inhibit any of the cell lines that we tested, except minimally at very high concentrations (100 μM; Fig. 2A–C).

To ascertain whether the BM microenvironment might exert a protective effect on myeloma or Burkitt’s lymphoma cells, S-3APG and thalidomide were added to cocultures of cell lines with BMSCs (Fig. 2D). Coculture of MM.1S or Hs Sultan with BMSCs did not alter the antiproliferative effect of S-3APG. Hs Sultan and MM.1S cell growth was still inhibited by S-3APG in a dose-dependent fashion, with IC_{50} ~10 nm, whereas thalidomide did not have a significant inhibitory effect up to 100 μM.

Because IL-6 has been found to be an important paracrine factor for myeloma growth, we used the IL-6-dependent cell line ANBL-6 to determine the effect of S-3APG and thalidomide on proliferation in the presence of IL-6 (2 ng/ml). S-3APG inhibited ANBL-6 cell growth in a dose-dependent fashion with an IC_{50} of 10 or 1 nm in the presence or absence of IL-6, respectively (Fig. 2E). Thus, IL-6 deprivation further sensitizes these cells to the effects of S-3APG.

Because extended passing in culture can affect cells’ sensitivity to drugs, we examined the activity of thalidomide or S-3APG on cells isolated from two different myeloma patients. The observed effects on patient cells was similar to those of other myeloma cell lines: ~10 nm IC_{50} for S-3APG and >100 μM for thalidomide (Fig. 2F). These results validate those obtained with myeloma cell lines.

We further tested whether there is any effect of thalidomide and thalidomide analogues on cell viability. To determine this, we performed the MTT assay with various concentrations of thalidomide and analogues for 24 h. The results showed that thalidomide and analogues had no significant effect on cell viability. In addition, we examined the effect of thalidomide and analogues on cell proliferation. We performed the BrdU incorporation assay with various concentrations of thalidomide and analogues for 24 h. The results showed that thalidomide and analogues had no significant effect on cell proliferation.

**Fig. 1.** Effect of 1 μM of S-3APG and thalidomide on myeloid cell lines. Proliferation of indicated cell lines in the presence of 1 μM of S-3APG or thalidomide. Values represent the percentage mean ± (±SD) [3H]thyminde (cpm) uptake of triplicate cultures compared with the mean of mock-treated triplicate cultures.
S-3APG (0.01–100 μM) on the other cells that contribute to the bone marrow microenvironment of myeloma. To investigate whether stimulated human hematopoietic progenitor cells show sensitivity to thalidomide or S-3APG, we examined their effect on CD34-positive cells in colony-forming assays in the presence of SCF, IL-3, and IL-6. Even at very high concentrations (100 μM), we did not observe inhibition of CD34 cell colony formation by S-3APG. Furthermore, proliferation of neither human umbilical vein endothelial cells nor BMSCs was affected by thalidomide or S-3APG at concentrations up to 100 μM (data not shown).

To examine the influence of S-3APG on Burkitt’s lymphoma tumor growth in vivo, we injected Hs Sultan cells into immunodeficient mice and started treatment with 50 mg/kg drug (the maximum tolerated dose of thalidomide) once the tumor was palpable (average size, 180 mm³). Treated animals showed significant inhibition of tumor growth and prolongation of survival with S-3APG compared with thalidomide (Fig. 3, A and B). Indeed, 6 of 15 animals treated with S-3APG were alive with complete tumor regression after 60 days of treatment. In contrast, all of the control and thalidomide treated animals had to be sacrificed before day 25 (Fig. 3B). Animals treated with 10 mg/kg drug showed no significant response (data not shown).

Next, we asked whether S-3APG was able to inhibit myeloma tumor growth in vivo. We used dexamethasone-resistant MM.1R myeloma cells in beige-SCID mice as our model. After 15 days, tumors in S-3APG-treated mice were all markedly inhibited compared with thalidomide-treated and control groups (Fig. 3C) with complete regressions in 3–6 weeks. S-3APG-treated mice also showed significant prolongation of survival compared with the thalidomide and control groups (Fig. 3D). All of the mice treated with S-3APG remained tumor free until day 100, when treatment was stopped. After 2 weeks without treatment, one mouse started to develop a tumor and was sacrificed at 6 weeks. Despite extended treatment, no signs of toxicity or weight loss relative to control were observed in the S-3APG-treated group, whereas some weight loss was observed among the thalidomide-treated mice.

Because thalidomide has been shown to be a potent antiangiogenic drug, we were interested in studying the antiangiogenic activity of S-3APG. We compared S-3APG with the R and the racemic (RS)
enantiomers as well as thalidomide in the mouse corneal micropocket assay. Treatment of C57BL/6 mice with S-3APG inhibited bFGF- or VEGF-induced corneal neovascular area by 54 or 35% in comparison with control animals, respectively. Furthermore, S-3APG showed a significantly stronger inhibition of bFGF- or VEGF-induced corneal neovascularization than did thalidomide. The R (+) and R/S (±) isomer of 3APG inhibited corneal neovascularization to the same extent as thalidomide (Fig. 4).

Further demonstration of the antiangiogenic activity of S-3APG was obtained by the demonstration that Hs Sultan tumors, treated with S-3APG, showed significantly lower microvessel density than did tumors of the control group [17 ± 6 microvessels/×400 field versus 29 ± 4, mean ± SE (P = 0.03)]. There was also a trend in thalidomide-treated tumors toward a lower microvessel density (21 ± 7) than the control group, but the difference was not statistically significant (Fig. 4). Examination of these animals for VEGF expression in the serum (by ELISA) and tumor (by immuno-histochemistry) indicated that there was no detectable VEGF in the serum, whereas VEGF staining in the tumors was unchanged between the treated and untreated groups. This was expected because S-3APG was also unable to affect in vitro VEGF secretion (as measured by ELISA) in any cell line tested including: IM-9, RPMI-8226, Hs Sultan, MM.1R, MM.1S, U266, AS, SV, and ARH-77 (data not shown).

Having shown that S-3APG acted as a strong antiangiogenic agent, we were interested in determining whether S-3APG would still have an antitumor effect in vivo on tumor lines resistant to the antiproliferative activity of S-3APG in vitro. Thus, we compared the in vivo effects of S-3APG and thalidomide on in vitro resistant myeloma cells in immunodeficient mice. Treatment of RPMI-8226 tumors with S-3APG suppressed the growth of this in vitro resistant myeloma line as compared with control and thalidomide-treated mice. Tumors in S-3APG-treated animals were significantly smaller than in thalidomide-treated animals beginning day 11, whereas the inhibition

Fig. 4. Effect of 3APG and thalidomide on angiogenesis in vivo. Inhibition of corneal neovascularization induced by (A) bFGF and (B) VEGF. Differences in bFGF-induced neovascularization between S-3APG and control were significant (n = 9 each; P < 0.0001), as were differences between S-3APG and thalidomide (n = 9 each; P < 0.01). Differences in VEGF-induced neovascularization between S-3APG and control were significant (n = 9 each; P < 0.001), as were differences between S-3APG and thalidomide (n = 9 each; P < 0.01). C, difference in corneal neovascularization between untreated (left) and S-3APG-treated (right) mice implanted with bFGF pellets. D, differences in microvessel density in untreated (left) and S-3APG-treated (right) tumors.
of tumor growth by thalidomide was not statistically significant (Fig. 3E). These tumors were also examined for VEGF expression and, although expression was observed, there was no apparent difference between the control and treated groups.

Given the strong immunomodulatory effects of S-3APG (11), we also wanted to examine the antitumor activity of S-3APG on a resistant tumor line in immunocompetent mice. For this, we used the mouse LLC model because LLC cells are insensitive to S-3APG in vitro (data not shown) and form tumors in immunocompetent mice. LLC tumors in mice treated with S-3APG, R/S-3APG or thalidomide were inhibited relative to control treated animals. We also observed significantly smaller tumors in S-3APG-treated mice compared with mice treated with R/S-3APG or thalidomide (Fig. 3F). Thus, the stronger antiangiogenic effects of the S-3APG translated into a stronger antitumor effect.

**DISCUSSION**

The ability of S-3APG to inhibit MM.1R or Hs Sultan tumors is the result of a direct antitumor effect and an indirect antiangiogenic activity. The direct antitumor activity is seen very potently in vitro. The antiangiogenic activity, with a mechanism likely similar to that of thalidomide, is demonstrated in vivo by three findings: (a) the direct inhibition of ocular neovascularization by S-3APG; (b) the inhibition of RPMI-8226 and LLC tumors, which are resistant to S-3APG in vitro; and (c) the reduction of MVD in treated tumors. Immune mediated effects can be ruled out because S-3APG is effective in both immune-competent and -incompetent animals. A combination of antiangiogenic and antitumor effects explains the mechanism behind the dramatic regressions obtained with MM.1R or Hs Sultan tumors, which are susceptible to both.

It is likely that metabolites of S-3APG are responsible for its antiangiogenic effects given the lack of direct effects on endothelial cells in vitro. However, the ability of S-3APG to directly inhibit cultured myeloma and Burkitt’s lymphoma cells without exogenous enzymatic activation suggest that its antitumor metabolite(s) is distinct from the antiangiogenic metabolite(s). Thus, different, although perhaps related, mechanisms are to be expected for the two activities. Identification of the mechanism responsible for S-3APG’s anti-myeloma effects may thus provide a target for further drug design.

In conclusion, we have shown that S-3APG is a powerful anti-myeloma and anti-Burkitt’s lymphoma agent that has antiproliferative and antiangiogenic effects without toxicity to cells of the bone marrow microenvironment and hematopoietic progenitor cells. It demonstrates superior in vivo antitumor activity compared with thalidomide and induces sustained regressions in murine models of Burkitt’s lymphoma and myeloma, including myeloma tumors resistant to conventional therapy. Additionally, S-3APG exhibited less toxicity than thalidomide in long-term treatment groups. Although the effects of thalidomide and its analogs in humans have proven difficult to predict from preclinical studies (likely due at least in part to a requirement for in vivo metabolism), we believe that S-3APG is a promising new therapy for multiple myeloma and related diseases. The drug is currently in late-stage preclinical development in preparation for clinical trials in the near future.

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