

Immunomodulatory analogs of thalidomide inhibit growth of Hs Sultan cells and angiogenesis *in vivo*

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We have previously shown that thalidomide and its potent immunomodulatory derivatives (IMiDs) inhibit the *in vitro* growth of multiple myeloma (MM) cell lines and patient MM cells that are resistant to conventional therapy. In this study, we further characterize the effect of these drugs on growth of B cell malignancies and angiogenesis. We established a beige-nude-xid (BNX) mouse model to allow for simultaneous *in vivo* measurement of both anti-tumor and anti-angiogenic effects of thalidomide and its analogs. Daily treatment (50 mg/kg/d) with thalidomide or IMiDs was nontoxic. The IMiDs were significantly more potent than thalidomide *in vivo* in suppressing tumor growth, evidenced by decreased tumor volume and prolonged survival, as well as mediating anti-angiogenic effects, as determined by decreased microvessel density. Our results therefore show that the IMiDs have more potent direct anti-tumor and anti-angiogenic effects than thalidomide *in vivo*, providing the framework for clinical protocols evaluating these agents in MM and other B cell neoplasms.

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Introduction

It has recently been demonstrated that microvessel density (MVD) in multiple myeloma (MM) patients' bone marrow (BM) correlates with disease progression and poor prognosis.^{1–3} These data, together with the known anti-angiogenic properties of thalidomide,⁴ provided the rationale for the use of thalidomide in the treatment of patients with MM.⁵ Remarkably, thalidomide induced clinical responses in 32% of MM patients whose disease was refractory to conventional and even to high-dose chemotherapy,⁵ suggesting that thalidomide can overcome *in vivo* drug resistance. Besides alkylating agents, corticosteroids and anthracyclines, thalidomide therefore represents a novel class of agents for the treatment of MM.^{6–8}

We have previously shown that the potent immunomodulatory thalidomide analogs (IMiDs)⁹ induce a dose-dependent inhibition of proliferation, either apoptosis or growth arrest, even in MM cell lines and MM patient cells resistant to conventional chemotherapy.¹⁰ Moreover, thalidomide and the IMiDs enhance the anti-MM activity of dexamethasone (Dex); and conversely, are inhibited by interleukin (IL)-6. Furthermore, our *in vitro* studies also demonstrate that the induction of IL-6 and VEGF secretion triggered by MM cell binding to bone marrow stromal cells (BMSC) is abrogated in the presence of thalidomide and the IMiDs.¹¹

Given the anti-angiogenic properties of thalidomide^{4,12} and the demonstration of its clinical utility for the treatment of MM,⁵ in the present study we investigated whether thalidomide and the IMiDs inhibit *in vivo* cell growth and angiogen-

esis in a beige-nude-xid mouse tumor model. For this purpose we established a tumor model allowing measurement of both tumor growth and angiogenesis *in vivo*. These studies further elucidate mechanisms of anti-tumor activity of thalidomide and the IMiDs, and provide the framework for clinical trials of these agents in hematologic malignancies.

Materials and methods

Cells and culture conditions

Hs Sultan human Burkitt cells¹³ were obtained from the American Tissue Type Culture Collection (Rockville, MD, USA). It is an Epstein–Barr virus-immortalized B cell line established from the plasmacytoma of a myeloma patient. The cell line was cultured in RPMI-1640 media (Sigma Chemical, St Louis, MO, USA) containing 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine (GIBCO, Grand Island, NY, USA), 25 U/ml penicillin, and 25 µg/ml streptomycin (GIBCO).

Thalidomide and analogs

For *in vivo* animal experiments, thalidomide and its analogues (50 mg/kg) were suspended in 0.5% carboxymethylcellulose, vortexed with sterile glass beads for 5 min, and administered intraperitoneally (i.p.) in a volume of 0.1 ml daily. Control mice received 0.1 ml of the vehicle i.p. All drugs were prepared immediately before use.

Mice

Five- to 8-week-old female beige-nude-xid (BNX) mice were obtained from Frederick Cancer Research and Development Center (Frederick, MD, USA). All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana-Farber Cancer Institute. Animals were anesthetized by isoflurane inhalation before blood withdrawal and observed daily for signs of toxicity. Animals were killed by CO₂ asphyxiation.

Tumor model

To determine the anti-tumor activity of thalidomide and the IMiDs, BNX mice were inoculated with 3×10^7 Hs Sultan Burkitt cells in 100 µl of RPMI-1640 media together with matrigel 100 µl (Becton Dickinson, Bedford, MA, USA). In our first model, drug treatment was started on day +1 after tumor cell injection; in our second model, drug therapy was initiated on day +1 after the development of measurable tumor. In each case, drug injection was performed daily i.p. Serial caliper

measurements of perpendicular diameters were used to calculate tumor volume using the following formula: (shortest diameter)² × (longest diameter) × 0.52. Animals were killed if the tumor was ≥ 2 cm or necrotic.

Microvessel staining and counting

Tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin according to standard histological procedures. After deparaffinization, tissue sections were pre-treated with proteinase K (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 37°C for 30 min before staining with rat anti-mouse CD-31 Ab (Pharmingen, San Diego, CA, USA). Positive staining was detected using secondary biotinylated rabbit anti-rat Ab, followed by incubation with streptavidin-horseradish peroxidase (DAKO, Carpinteria, CA, USA). 3-Amino-9-ethylene carbazol (DAKO) was used as the chromogenic substrate, and sections were counterstained with Gill's Hematoxylin (Fisher Scientific, Fair Lawn, NJ, USA). MVD was determined by light microscopy according to the procedure of Weidner *et al.*¹⁴ MVD was assessed without knowledge of the treatment of mice. Areas of most intense neovascularization were identified by scanning tumor sections at low power ($\times 40$), and then counted at high magnification ($\times 400$). At least five separate $\times 400$ fields were analyzed by two investigators in a blinded fashion using double-headed light microscopy.

Statistical analysis

Student's two-tailed *t*-test was used to calculate the statistical significance of observed differences between groups of drug treated and control animals, and results are presented as mean \pm s.e.m. or as average \pm s.d. To analyze the statistical differences in survival, the prism computer program was used.

Results

Effect of thalidomide and IMiDs on *in vivo* tumor growth

We have previously shown that the IMiDs inhibit proliferation of both Hs Sultan and MM.1S cells (IC_{50} 0.01–0.1 μ M), associated with apoptosis in MM.1S cells and growth arrest in Hs Sultan cells.¹⁰ We therefore chose these two cell lines for our *in vivo* pre-experiments. Due to low tumorigenicity (<50%) of MM.1S cell line in mice, we focused on Hs Sultan Burkitt cells to evaluate the *in vivo* anti-tumor activity of thalidomide and the IMiDs. Mice were treated with either vehicle methylcellulose only (control); or with thalidomide, IMiD1 or IMiD3 (50 mg/kg) daily.

In our first experimental model, we started daily treatment one day after tumor cell injection. Animals in the control group ($n = 5$) developed tumor at a median of 15 (range 7–18) days and were killed due to tumor progression (size ≥ 2 cm or necrotic) at a median of 26 (range 21–32) days post tumor cell injection. After day 18 of treatment with IMiD1 ($n = 5$) or IMiD3 ($n = 5$), there was a significant inhibition of tumor growth ($P < 0.05$), compared either to the group treated with thalidomide ($n = 5$) or the control group ($n = 5$) (Figure

1). Importantly, animals in the cohort treated with IMiD1 showed an almost complete suppression of tumor growth until they were killed on day 35.

The focus of our second tumor model was to study the effects of thalidomide and the IMiDs on already established tumors. Treatment was therefore started on the day when tumor became palpable (average 177 mm³), at a median of 6 (range 4–15) days after tumor inoculation. All drug-treated animals demonstrated significantly prolonged survival: IMiD1 ($n = 6$) > IMiD3 ($n = 8$) > thalidomide ($n = 11$) > control group ($n = 12$), (control vs thalidomide $P < 0.0001$, thalidomide vs IMiD3 $P = 0.0013$, thalidomide vs IMiD1 $P = 0.0015$) (Figure 2a). All IMiD1-treated animals were alive at day 45, whereas all animals in either the control or thalidomide-treated group required sacrifice due to tumor growth. Furthermore, tumor growth was already significantly delayed by days 5 to 10 in all treated groups. For example, tumor volumes on day 10 were significantly lower in treated groups than control group: control vs thalidomide, $P = 0.0084$; control vs IMiD3, $P = 0.0002$; and control vs IMiD1, $P = 0.0002$ (Figure 2b). IMiD1 inhibited tumor growth to a greater extent than did thalidomide and IMiD3 ($P < 0.05$). Importantly IMiDs led to complete tumor regression in five of 14 mice. These five animals with complete remission of tumor received daily drug injections for an additional 10 days: in two of these animals, the tumor recurred 16 and 27 days after discontinuation of treatment; the remaining three mice showed sustained complete tumor remission, with follow-up extending to 102 days. In all experiments, no signs of toxicity or weight loss were observed in either control or drug treated animals.

Microvessel density

Immunohistochemical staining with antibody to CD31 was used to investigate whether the neovascularization of tumors was also inhibited by treatment with thalidomide or the

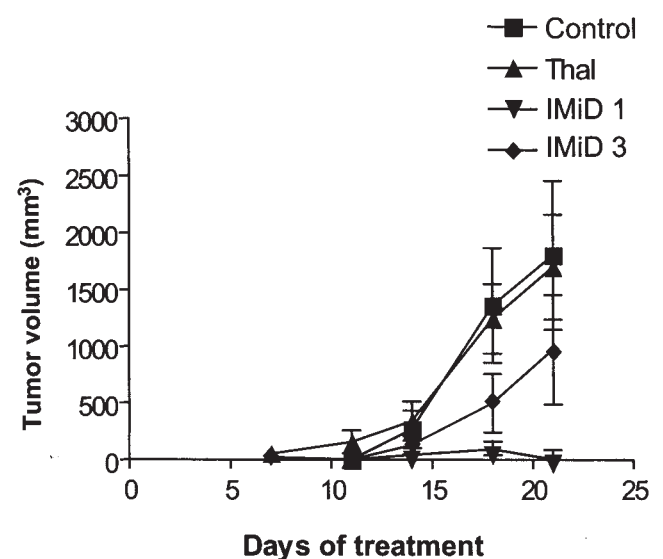


Figure 1 Effect of thalidomide and IMiDs on tumor cell growth *in vivo*. BNX mice were inoculated with Hs Sultan tumor cells 3×10^7 , and daily i.p. treatment with either thalidomide, IMiD1, IMiD3 (50 mg/kg) or methylcellulose control was started 1 day later. Tumor volume (mean \pm s.e.m. mm³) was compared in drug-treated vs control animals.

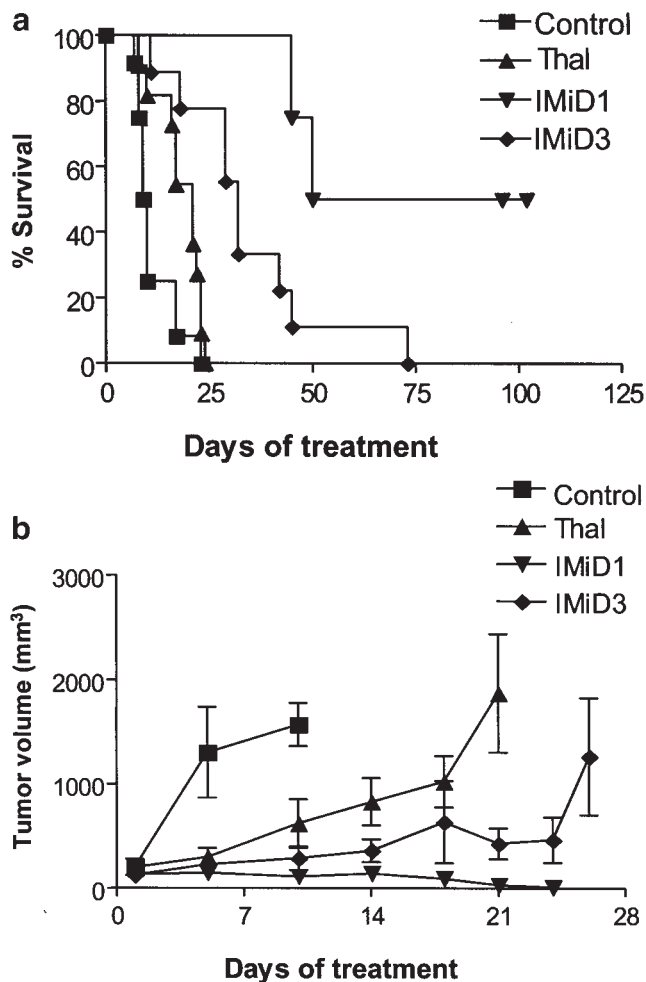


Figure 2 Effect of thalidomide and IMiDs on *in vivo* growth of established tumors. BNX mice were inoculated with 3×10^7 Hs Sultan tumor cells, and daily i.p. treatment with either thalidomide, IMiD1, IMiD3 (50 mg/kg) or methylcellulose control was started 1 day after the development of measurable tumor. (a) Survival of animals is calculated from the start of treatment; mice were killed if tumor was ≥ 2 cm or necrotic. (b) Tumor volume (mean \pm s.e.m. mm³) was calculated from the start of treatment.

IMiDs. Although tumors were heterogeneous in their MVD, areas of highest neovascularization were found by scanning the tumor sections at low power at the margins of the tumors. There was a trend towards lower MVD in thalidomide-treated animals compared to the control group, but the difference was not significant ($P < 0.06$) (Figure 3). Importantly, tumors from IMiD1 and IMiD3-treated animals showed significantly lower MVD ($P = 0.0022$ and $P = 0.0003$, respectively) than tumors from the control group (Figure 3).

Discussion

It has been demonstrated that thalidomide results in an overall response rate of 32% in patients with relapsed or refractory MM.⁵ Although the empirical use of thalidomide in MM was predicated upon the increased angiogenesis in MM bone marrow and the anti-angiogenic effects of thalidomide, the mechanisms whereby thalidomide mediates its anti-MM activity are unknown. We have previously demonstrated direct effects of thalidomide against MM cells *in vitro*, but only at high con-

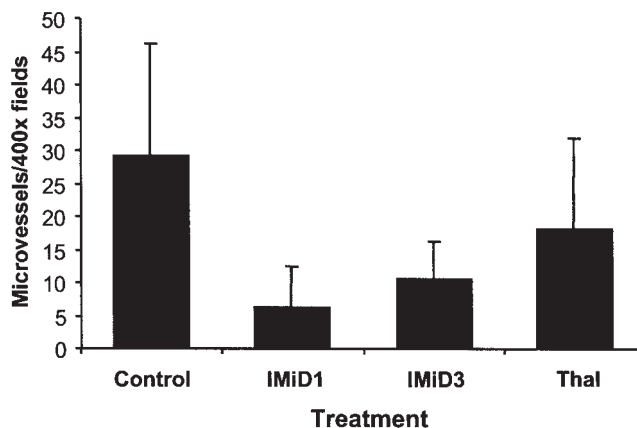


Figure 3 Effect of thalidomide and IMiDs on *in vivo* angiogenesis. MVD was assessed as described in Materials and methods. Resected tumors from mice treated with thalidomide, IMiD1, IMiD3 (50 mg/kg) or methylcellulose were stained for CD31 expression, and MVD was determined by enumerating vessels in five high power ($\times 400$) magnification fields per slide using light microscopy. Values represent the average \pm s.d.

centrations (100 μ M), which are not achievable in patient plasma.⁶ In contrast, the IMiDs are 50 000 times more potent than thalidomide in inhibiting TNF- α production from LPS-stimulated peripheral blood mononuclear cells *in vitro*.^{15,16} Although these analogs are also more potent inducers of T cell proliferation and secretion of IFN- γ and IL-2,⁹ we were unable to show CTL activity.⁶ However, these drugs augmented natural killer cell cytotoxicity against autologous MM cells,⁶ and inhibit IL-1 β and IL-6 secretion from PBMCs.⁹ We first demonstrated a direct effect of the IMiDs against human MM cell lines and patient cells *in vitro*, including drug-resistant cell lines: IMiD1 and IMiD3 achieved 50% inhibition of DNA synthesis at concentration (0.01–0.1 μ M), corresponding to serum levels that are readily achievable.¹⁰ These drugs induce either growth arrest or apoptosis, associated with activation of RAFTK, in both MM cell lines and patient cells. We further demonstrated that these drugs abrogate the increased IL-6 and VEGF secretion triggered by MM cell adhesion to BMSC.¹¹ In the current study, we investigated the effects of IMiDs on tumor growth and angiogenesis *in vivo*.

For these studies, we used a model system in beige-nude-xid mice, with B, T and NK cell defects, as well as an impaired motility and chemotaxis of macrophages; irradiation of the animals therefore was not necessary. Inoculation of 3×10^7 Hs Sultan Burkitt cells together with matrigel subcutaneously achieved almost 100% tumorigenicity. The advantage of this model was the early and simultaneous onset of tumor development in injected mice. This homogeneous pattern of tumor development in all animals provided a highly reproducible *in vivo* tumor model. Although this model could also be established with other MM cell lines, the low tumorigenicity (50% for MM.1S cells), as well as the variation in the time of tumor onset and lack of reproducibility, precluded their use. We therefore utilized Hs Sultan cell line for our characterization of effects of the IMiDs on tumor cells and angiogenesis *in vivo*. Although this mouse model does not include interaction of the tumor cells to their microenvironment, the model allows the study of direct cell killing and the effect on neo-angiogenesis.

In our first model, we initiated drug treatment on the day after tumor cell injection, and showed that tumor growth was

significantly inhibited and survival significantly prolonged in animals treated with IMiD1 and IMiD3. Importantly, IMiD1 completely inhibited tumor development. These data suggest that the IMiDs can effectively treat a lower burden of tumor cells. To more accurately reflect the clinical setting, we next tested the IMiDs in a second model in animals with already established tumors. Importantly, even when treatment was initiated after the tumor was established, we observed an almost complete and sustained remission of tumors until day 45 of IMiD1 treatment in all animals. Animals with complete remissions were treated for an additional 10 days, and five of 14 animals that achieved complete response remained tumor free for up to 102 days, suggesting the potential for sustained responses. However, relapse of tumors was noted in two of these mice, and re-treatment with IMiDs did not induce a second remission. Therefore it also appears that resistance to the IMiDs may develop *in vivo*. Our model therefore appears to be useful not only to evaluate *in vivo* mechanisms of action of IMiDs against B cell malignancies, but also to aid in delineating mechanisms of resistance to IMiDs.

To enhance the tumorigenicity of our cell lines, we mixed tumor cells with matrigel prior to injection. Apart from enhanced tumorigenicity, this system also permitted assessment of the effect of thalidomide and IMiDs on tumor-associated neo-angiogenesis. Tumor sections were immunohistochemically stained for mouse CD31 (PECAM) expressed on endothelial cells, and evaluated for tumor MVD. Animals treated with IMiD1 and IMiD3 showed a significant inhibition of neovascularization compared to thalidomide-treated animals and control animals, suggesting that IMiDs are more potent than thalidomide not only in inhibiting tumor cell growth, but also in blocking angiogenesis. Direct anti-angiogenic effect of thalidomide has been demonstrated previously, using the rabbit corneal assay.⁴ The decrease in the microvessel density (MVD) in IMiDs-treated tumors could be due to direct tumor cell killing with concomitant decrease in pro-angiogenic factors secreted by tumor cells. However, considering the fact that at the time of sacrifice, tumors were all the same size, the decrease in MVD suggests a direct anti-angiogenic effect. In conclusion, this study demonstrates further *in vivo* evidence for activity of the IMiDs against Burkitt cells and tumor-related angiogenesis. This study provided the framework for testing IMiDs in new clinical trials to target both the tumor cell and its microenvironment, to overcome classical drug resistance, and to achieve improved outcome in this presently incurable disease. A phase I clinical trial in MM has recently been completed, demonstrating stabilization of disease or response in 79% of relapsed and refractory relapsed MM patients,¹⁷ and a phase II trial in MM is currently ongoing.

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