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Thalidomide up-regulates prostate-specific antigen secretion from LNCaP cells

Abstract Thalidomide has been shown to have species- and metabolic-dependent antiangiogenic activity in vitro and in vivo, suggesting its potential in treating human angiogenesis-dependent pathologies such as solid tumors. Based on promising preclinical studies, thalidomide has entered phase II clinical trials for prostate, brain, breast cancer, and Kaposi's sarcoma. However, the antiangiogenic mechanism of action is largely unresolved, as are its effects on tumor-associated gene expression, cytokine secretion, etc. We have investigated the effects of thalidomide on: 1) the secretion of prostate-specific antigen (PSA) in a human androgen-dependent prostate cell line; 2) growth and viability of human prostate cells; and 3) differential gene expression profiles of thalidomide-treated vs untreated human prostate cells. A human androgen-dependent prostate carcinoma cell line (LNCaP) and a human androgen-independent prostate carcinoma cell line (PC-3) were incubated with thalidomide 0.6, 6, or 60 μg/mL for 5–6 days. Secreted PSA from LNCaP cells was measured using a commercial enzyme-linked immunosorbant assay. Cell viability studies were conducted in both LNCaP and PC-3 cells using the same thalidomide concentrations. Furthermore, the differential gene expression of thalidomide-treated LNCaP cells was compared to that of untreated control cells using a commercially available human cancer cDNA expression array system. Thalidomide-treated LNCaP cells demonstrated increased PSA/cell levels at all concentrations tested compared to untreated control cells. Thalidomide demonstrated a cytostatic effect in LNCaP cells but had no appreciable effect on PC-3 cell viability compared to untreated control cells. Comparison of cDNA expression arrays hybridized with thalidomide-treated LNCaP cDNA probes suggests that thalidomide may up- or downregulate expression of angiogenesis-related genes, i.e., vitronectin, but these differential effects require further verification. Thalidomide over a range of doses has demonstrated nontoxic, cytostatic activity in LNCaP cells and significant upregulation of LNCaP cell PSA secretion in vitro. Furthermore, preliminary data from cDNA nucleic acid arrays of thalidomide-treated LNCaP cells suggest that thalidomide upregulates a potential angiogenic modulatory protein, the vitronectin precursor, which may eventually link thalidomide's antiangiogenic activity with modulation of angiogenic vascular integrin pathways.

Key words Thalidomide · PSA · LNCaP cells · PC-3 cells · cDNA array

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Introduction

Thalidomide (N-phthalidoglutarimide) was developed in the 1950s by Chemic Gruilenthal, Stolberg, Germany as an effective sedative and antiemetic. While no toxicity was reported based on studies in rodent models during its development, it was tragically linked to dysmelia (stunted limb growth) of newborns following maternal usage [21, 22]. The discovery of this causal association between thalidomide usage in pregnant women and the development of dysmelia led to the eventual withdrawal of thalidomide from general circulation. However, despite its notoriety as a potent human teratogen, thalidomide has proven therapeutic value for a number of human pathologies, and has remained in continual clinical use since the 1960s.

The therapeutic history of thalidomide began in 1964, when Sheskin serendipitously discovered it to be an effective treatment for erythema nodosum leprosum associated with lepromatous leprosy [32]. This discovery consequently led to other clinical evaluations, based on the belief that thalidomide had general antiinflammatory or immunosuppressive properties. Trials are currently being conducted in chronic discoid lupus erythematosus, Bechet's syndrome, prurigo nodularis, ulcerative colitis [2], rheumatoid arthritis [16], AIDS [14, 24], Kaposi's sarcoma [5], graft-versus-host disease [35], inflammatory dermatoses [13], and other conditions [18–20]. Neubert and colleagues have made progress in elucidating the mechanism of the general antiinflammatory activity of thalidomide by describing the downregulation of alpha and beta subunits of cell adhesion receptors, particularly the beta integrin subunits, on circulating leukocytes of marmosets and humans [26, 27, 29]. Based on these studies, Neubert et al. hypothesized that the antiinflammatory effects of thalidomide may be explained by impaired leukocyte diapedesis into the extracellular matrix at inflammatory sites and that its teratogenicity may also be partially explained by suppressed expression of adhesion factors in the developing fetus [23, 26–29].

New evidence of a potential mechanism of the teratogenicity of thalidomide came in 1994 when D'Amato et al. demonstrated that thalidomide and a teratogenic analogue, EM-12, inhibited basic fibroblast growth factor (bFGF)-induced angiogenesis in the in vivo rabbit cornea micropocket assay [8]. However, neither the parent compound nor EM-12 had angioinhibitory activity in endothelial cell cultures or in the chick chorioallantoic membrane (CAM) assay [8]. They and others hypothesized that hepatic metabolism of thalidomide is required to form an antiangiogenic metabolite [2, 12]. In addition, they suggested that the teratogenic properties of thalidomide may be directly correlated to its antiangiogenic effect in the developing limb bud, and that thalidomide has potentially therapeutic applications in treating angiogenesis-dependent diseases such as proliferative retinopathy and solid tumors [8, 9, 12]. Recently, two angiogenic pathways have been described that are mediated by distinct alpha-v integrins in the CAM assay: a bFGF-induced angiogenic pathway dependent on alpha-v beta-3; and a distinct vascular endothelial growth factor-induced pathway dependent on alpha-v beta-5 [4, 10]. The discovery of these two pathways has provided new evidence to suggest, in agreement with the data produced by Neubert et al., that the antiangiogenic activity of thalidomide may be related to pharmacological modulation of angiogenic vascular integrins.

Despite the therapeutic promise of thalidomide as an antiangiogenic agent, studies of its proposed antitumor activity in rodent models have produced contradictory results. One study suggested that thalidomide promotes metastasis of prostate adenocarcinoma cells (PA-III) implanted in rats, while another suggested that it does not have antiangiogenic properties in mice implanted with either B16-F10 melanoma or CT-26 colon carcinoma cells [17, 30]. Our laboratory has previously shown that the antiangiogenic activity of thalidomide requires metabolic activation which is species dependent, showing activity in humans and rabbits [3]. Rodent microsomes failed to induce an antiangiogenic response in the rat aortic ring assay, suggesting an explanation for the lack of antitumor activity in the in vivo rodent models [3, 17, 30].

Elucidating the mechanisms of the antiangiogenic or antitumor activity of thalidomide will provide potentially new therapeutic targets. Studies have shown that thalidomide accelerates the degradation of tumor necrosis factor-alpha (TNF-α) mRNA two-fold in human monocytes, with a corresponding decrease in both in vitro and in vivo TNF-α levels [25, 31]. This cytokine modulating activity may indirectly benefit anticancer treatment by restraining TNF-α production, as suggested by D'Amato et al [8]. However, this evidence of cytokine modulating activity led to an investigation of whether thalidomide modulates expression of tumor-associated proteins and/or tumor-associated gene expression in vitro.

Prostate-specific antigen (PSA), a 33-kDa glycoprotein and a member of the kallikrein family of serine proteases, is secreted by prostate epithelial cells and by most metastatic tumors originating from the prostate gland [1, 6]. Elevated serum PSA levels in patients with prostate cancer, benign prostatic hyperplasia, and prostatitis have been consistently observed [11]. Since PSA has been evaluated as a diagnostic and prognostic surrogate maker for patients with prostate cancer, it is an important protein to assay in vitro. In this article we report the effect of thalidomide on PSA production by human prostate cells in vitro, and evaluate differential gene expression with thalidomide treatment using cDNA expression arrays.

Materials and methods

Cell culture

The human androgen-dependent prostate carcinoma cell line LNCaP and the human androgen-independent prostate carcinoma cell line PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown as directed. Cells, passage 25 for LNCaP and passage 22 for PC-3, were plated at 30,000 cells/2-cm well. Thalidomide was a gift from EntreMed, Inc. (Rockville, MD, USA) and prepared as a stock solution in dimethyl sulfoxide (DMSO). Serial dilutions were prepared in culture medium to produce final concentrations of 60, 6.0, and 0.6 μg/mL. The final DMSO concentration was 0.5%. Cells were treated daily with medium containing thalidomide.

PSA quantification

Cell culture supernatants were collected every 24 h. Adherent cells were trypsinized and counted using a Coulter Z1 counter (Coulter Corp., Hialeah, FL, USA). PSA secreted into the supernatant was measured using the Tandem-E PSA assay (Hybritech, La Jolla,

CA, USA) according to the company's instructions. The amount of PSA secreted per cell was calculated for every 24 h period.

Human cDNA expression arrays

An Atlas™ Human Cancer cDNA Expression Array (Clontech Laboratories, Palo Alto, CA, USA) was used to compare differential gene expression between thalidomide-treated vs untreated LNCaP cell cultures. LNCaP cells were treated with thalidomide 8 μg/mL and human microsomes (0.2 mg/mL) in culture medium, DMSO 0.5%, and an NADPH-generating system consisting of 0.1 M Tris buffer, 0.015 M $MgCl_2$, 4 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase 20 U/mL, and NADP+ 0.8 mg/mL to allow microsomal drug metabolism for 3 h. RNA isolation and preparation, cDNA hybridization, and interpretation of results were conducted according to the company's instructions. Poly A^+ RNA enrichment was performed using an Oligotex© mRNA Kit (Qiagen, Chatsworth, CA, USA). Phosphorimage analysis was performed on a STORM™ (Molecular Dynamics, Inc., Sunnyvale, CA, USA) and ImageQuaNT™ (Molecular Dynamics, Inc.) software was used for quantification analyses.

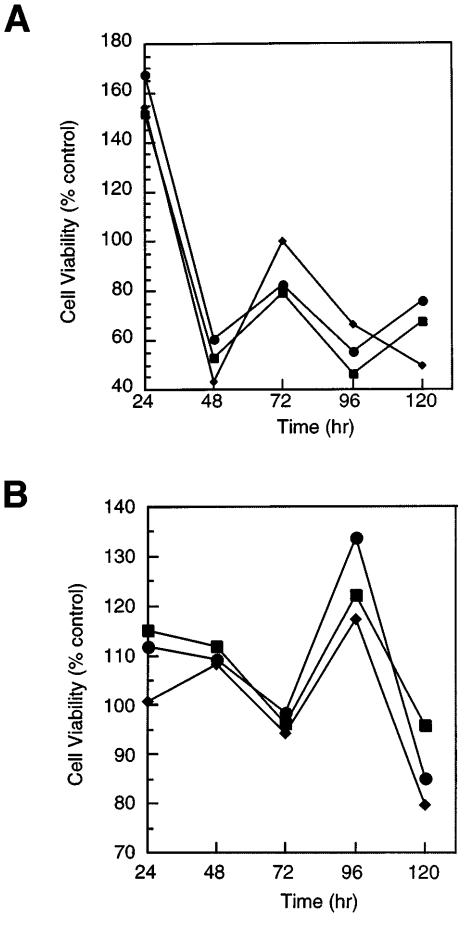


Fig. 1 Cell viability of LNCaP or PC-3 cells determined every 24 h following initial exposure to various concentrations of thalidomide. Each result is the mean of cell counts obtained from two wells and normalized to controls. **A**) Percentage viability curves for LNCaP cells treated with thalidomide 0.6 μg/mL (●), 6 μg/mL (■), and 60 μg/mL (▲). **B**) Percentage viability curves for PC-3 cells treated with thalidomide 0.6 μg/mL (●), 6 μg/mL (■), and 60 μg/mL (▲)

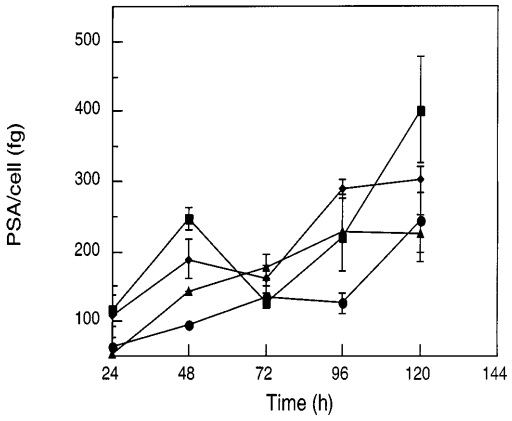


Fig. 2 Amount of PSA secreted normalized to cell number every 24 h following initial exposure to thalidomide. Each value represents the mean of the PSA values measured for each well divided by the corresponding cell number (±SD) normalized to control. ● untreated; ■ thalidomide 0.6 μg/mL; ◆ thalidomide 6 μg/mL; and ▲ thalidomide 60 μg/mL

Statistics

The Student *t* test was used to determine statistical significance at the 95% confidence interval ($P < 0.05$).

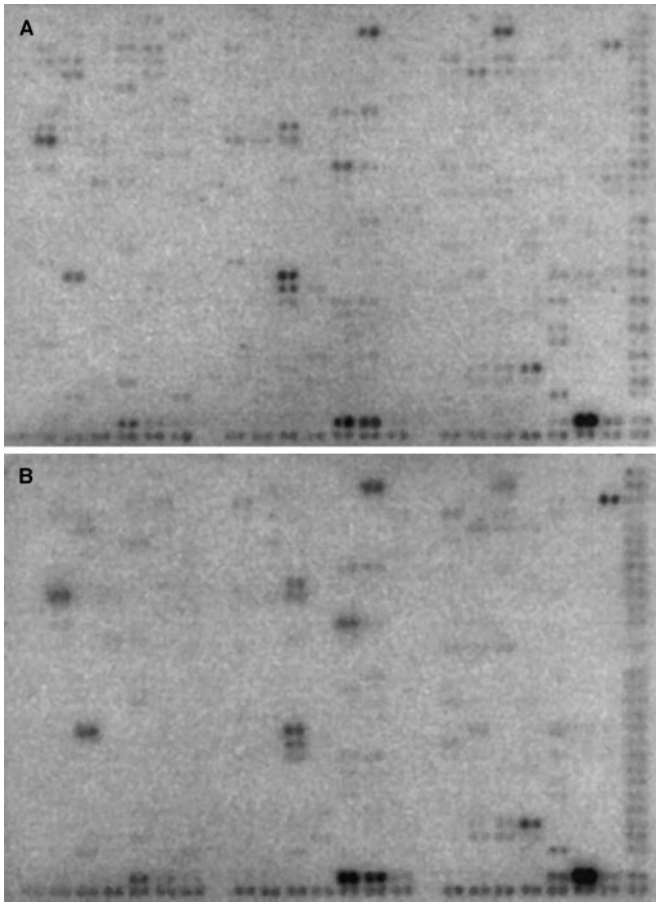


Fig. 3 Raw images of cDNA arrays of **A**) untreated LNCaP cells and **B**) LNCaP cells treated with thalidomide 8.0 μg/mL and human microsomes

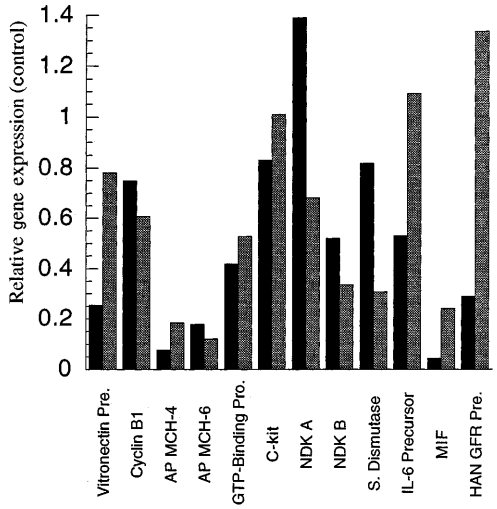


Fig. 4 Relative gene expression in thalidomide-treated (▒) and untreated (■) LNCaP cells. The genes listed are: Vitronection Pre., vitronectin precursor; AP-MCH-4 and -6, apoptotic protease MCH-4 and -6; GTP-Binding Pro., GTP-binding protein; NDK A and B, nucleoside diphosphate kinase A and B; S. Dismutase, superoxide dismutase; HAN GFR Pre., high-affinity nerve growth factor receptor precursor

Results

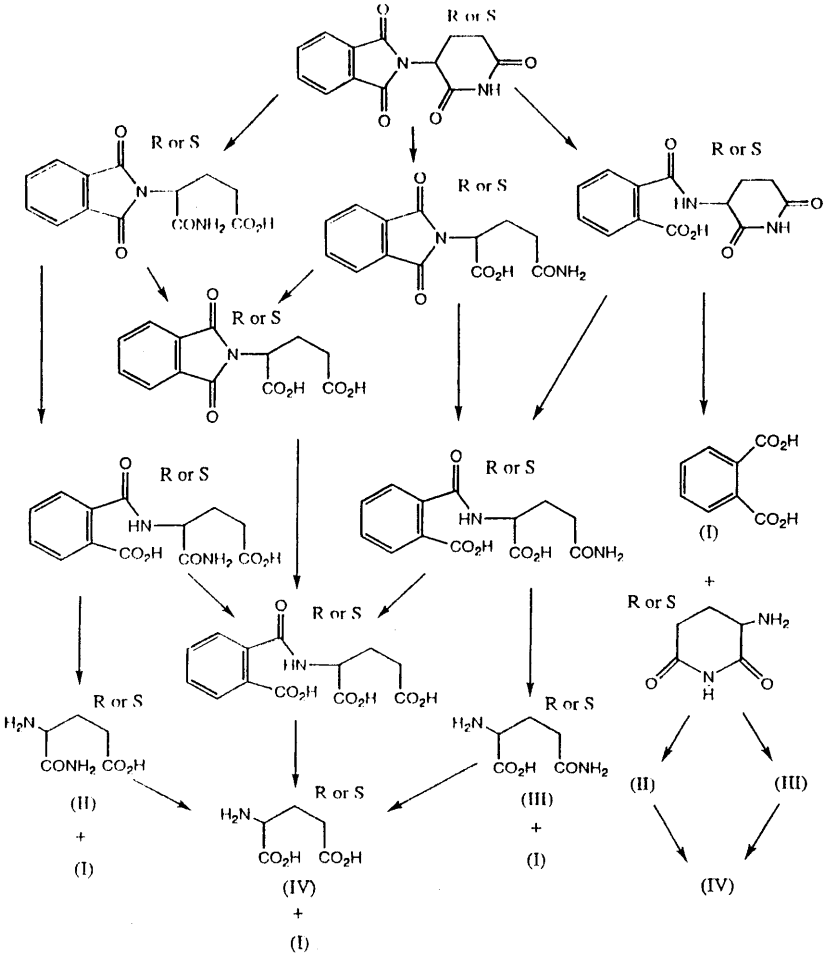
Figure 1 shows the relative growth curves for LNCaP cells treated with thalidomide at various concentrations (0.6, 6, and 60 μg/mL) versus untreated controls. It was noted that after 96 h of thalidomide treatment, LNCaP cell growth at all drug concentrations tested was significantly stunted compared to untreated controls. However, there were no statistically significant differences in LNCaP cell growth among the three thalidomide treatment groups (Fig. 1).

The cytostatic effect observed in thalidomide-treated LNCaP cells was not observed in PC-3 cells. The growth curves for PC-3 cells treated with various concentrations of thalidomide (0.6, 6, 60 μg/mL) are shown in Fig. 1B. There was no statistical difference between untreated controls and any drug concentration tested.

Figure 2 depicts the secreted PSA levels per LNCaP cell with daily administration of thalidomide 0.6, 6, or 60 μg/mL or control medium. It was noted that starting at 96 h all drug concentrations tested produced an increase in PSA levels that showed a trend toward statistical significance.

Preliminary data using cDNA nucleic acid arrays for thalidomide-treated and untreated LNCaP cells are

Fig. 5 Hydrolytic pathway for degradation of thalidomide



shown in Fig. 3. After background correction and normalization to a housekeeping gene, the identity of either novel gene transcripts or those that varied by at least 50% between the two arrays were considered for closer analysis. The normalized gene transcripts that fit this criteria are shown in Fig. 4. It was noted that the transcripts encoding the vitronectin precursor and the high-affinity nerve growth factor receptor precursor were up-regulated more than two-fold in thalidomide-treated LNCaP cells compared to untreated controls.

Discussion

The biologic activities of thalidomide are complex and the exact mechanisms of action of its therapeutic effect in a number of human pathologies remain to be described in detail. While thalidomide has shown immunosuppressive and antiinflammatory properties [19, 20], it has also shown species-dependent and metabolic-dependent antiangiogenic activity in vivo and in vitro [3, 8]. The antiangiogenic property of thalidomide has direct implications in the treatment of solid tumors [8, 9, 23]. Furthermore, since thalidomide is a known cytokine modulator (TNF-α in human monocytes) [25, 31], we decided to investigate whether thalidomide had any modulatory activity in an in vitro system using human prostate cell lines.

In the present study, the PSA modulatory activity of thalidomide was evaluated in the human androgen-dependent prostate cell line LNCaP, leading us to the conclusion that thalidomide treatment, regardless of the concentration tested, eventually has a statistically significant effect on secreted PSA per cell compared to controls (Fig. 2). Furthermore, thalidomide did not show cytotoxicity for either LNCaP cells or PC-3 cells at the concentrations tested (Figs. 1A and B). The drug also demonstrated a cytostatic effect in LNCaP cells that was not detected in PC-3 cells (Fig. 1A and B). Since the parent compound is degraded rapidly in an in vitro system by spontaneous hydrolysis (pH greater than 6 [4, 33]), our findings suggest that active hydrolytic derivatives are responsible for the PSA modulatory activity and the cytostatic activity of thalidomide in LNCaP cells. Thalidomide undergoes hydrolysis to form three primary products (4-phthalimidoglutaramic acid, 2 phthalimidoglutaramic acid, and alpha-(o-carboxybenzamido)glutarimide) and eight minor products [7, 15, 34]. The structure of thalidomide and the hydrolysis pathway for degradation are shown schematically in

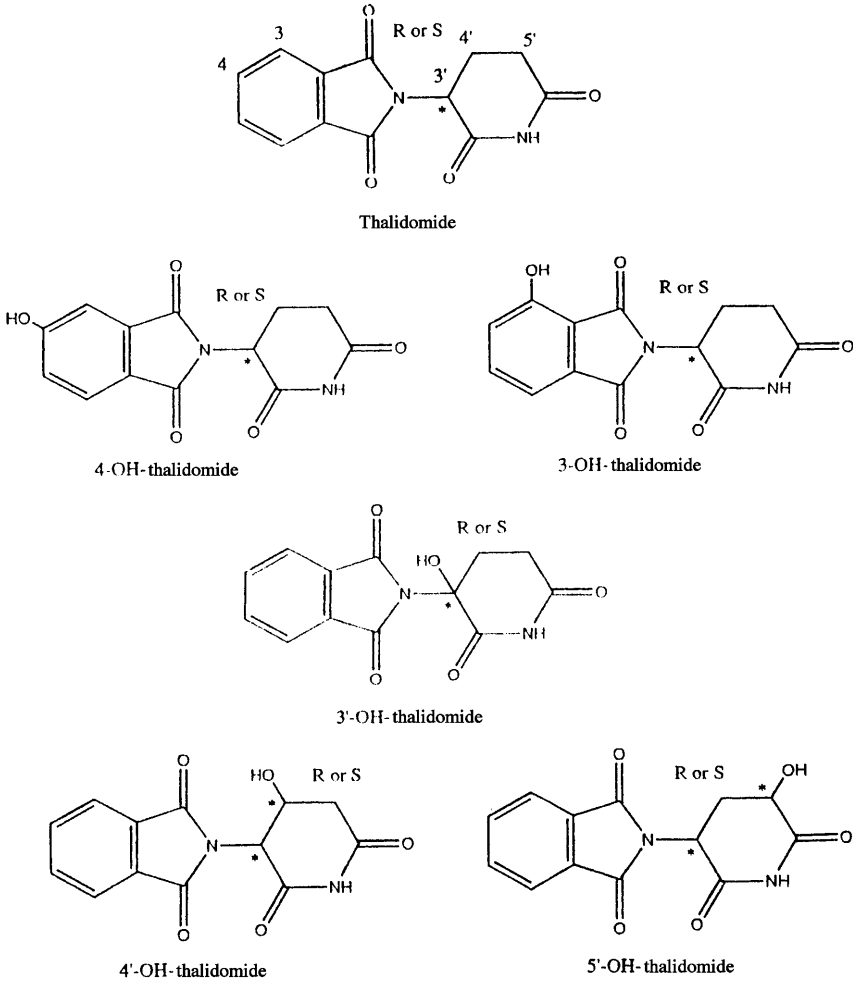


Fig. 6 Known metabolites of thalidomide

Fig. 5. However, it is probable that similar PSA modulatory effects will be detected using in vitro conditions that induce metabolic activation of thalidomide. The structures of five known metabolites of thalidomide are shown in Fig. 6. Further in vitro studies using LNCaP and other cell lines will be conducted to confirm this hypothesis.

The preliminary human cDNA array data suggest that the hydrolytic products of thalidomide also modulate gene expression in the LNCaP prostate tumor cell line (Figs. 4, 5). While these data will require further studies to be confirmed, we note the upregulation of the vitronectin precursor gene transcript in thalidomide-treated cells versus untreated controls. Vitronectin is the ligand for a documented angiogenesis-mediator cell adhesion receptor, integrin alpha-v beta-5 (or vitronectin receptor) [10]. Determining how this observation is relevant to the distinct angiogenic alpha-v integrin pathways described by Friedlander et al. [10] requires further investigation.

In conclusion, thalidomide treatment demonstrates evidence of PSA modulation and cytostatic activity in one human androgen-dependent prostate carcinoma cell line (LNCaP). These data require further verification, but may have direct implications for anticipating PSA modulation in the clinic during thalidomide treatment of prostate cancer. Preliminary cDNA gene expression array data suggest that thalidomide also modulates tumor-associated genes in vitro. We observed a significant upregulation of the high-affinity nerve growth factor receptor precursor transcript. The relationship of this finding to the known central nervous system toxicities of thalidomide treatment, specifically peripheral neuropathy, is not known and requires evaluation. Furthermore, a possible link between thalidomide and modulation of a specific alpha-v integrin ligand, vitronectin, was noted. Further investigation is needed.

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