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Review Article

Thalidomide and Its Analogs as Anticancer Agents

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Abstract

Thalidomide has reemerged as a promising anticancer and anti-inflammatory drug despite its devastating congenital birth defects. Many thalidomide derivatives with enhanced antiangiogenic and immunomodulatory effects or greater cytokine inhibition accompanied by less adverse toxicities than the parent drug have been developed. The mechanisms of action of thalidomide and its analogs are complex and not yet fully understood, but studies indicate that their antiangiogenic and immunomodulatory effects play important roles. Thalidomide and lenalidomide have been approved for the treatment of multiple myeloma and myelodysplastic syndrome. The powerful antiangiogenic, anti-inflammatory, and apoptotic effects mean that thalidomide and its immunomodulatory derivatives will continue to be explored in the treatment of a variety of cancers and inflammatory diseases. (*Tzu Chi Med J* 2008;20(3):188–195)

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1. Introduction

Thalidomide was initially marketed in Germany in 1956 as a sedative and antiemetic for the treatment of morning sickness. It was widely introduced into clinical practice in Europe, Australia, Canada, Japan, and other countries around the world due to its presumed low toxicity. The association between thalidomide and congenital birth defects, e.g., limb malformations, was first reported in 1961, and the drug was immediately withdrawn from the market (see reference 1 for a review of the history of thalidomide). Interestingly, thalidomide was never approved by the US Food and Drug Administration (FDA) because of concerns over its neuropathic effects.

The drug resurfaced in 1965 when it was discovered serendipitously that the drug was remarkably effective in lessening the skin condition of patients with erythema nodosum leprosum (2). But it was not until 1998 that the US FDA approved thalidomide for indications under strict patient guidelines. Its effects have been found to result from its immunomodulating and anti-inflammatory actions (3,4). It has been suggested that the limb malformations might be secondary to the inhibition of vasculogenesis during limb bud development (5). Consequently, the antiangiogenic

effect of thalidomide might be used to prevent the growth of blood vessels recruited by solid tumors. Indeed, thalidomide has been shown to inhibit basic fibroblast growth factor-mediated angiogenesis in a rabbit cornea micropocket model [5].

The broad spectrum of activities of thalidomide indicates that it may be used in a variety of clinical conditions, and further suggests that it probably exerts these effects through multiple mechanisms. Many clinical trials using thalidomide and its derivatives as anticancer agents have been conducted [6–9]. Here, we review the basic science, and focus primarily on the mechanisms of action of thalidomide and its derivatives as anticancer agents. The enormous body of work on thalidomide and its derivatives precludes an exhaustive coverage of the literature.

2. Chemical properties and metabolism

Thalidomide consists of a racemic mixture of S(–) and R(+) enantiomers, with the former appearing to exert potent actions in immunomodulation (the reduction of tumor necrosis factor [TNF]-α release), sedation and teratogenicity, while the latter has also demonstrated equally potent inhibition of TNF-α release from activated human mononuclear blood cells [10]. The rapid interconversion of the two enantiomers under physiological conditions renders its separation impractical, and further implies its potential teratogenic effect whenever thalidomide is prescribed. In an aqueous solution with a pH greater than 6, thalidomide undergoes rapid spontaneous hydrolysis into a plethora of degradation products which appear to be devoid of antiangiogenic activity [11].

Thalidomide is metabolized into five metabolites, each of which undergoes spontaneous hydrolysis into inactive products [11]. The metabolites appear to be generated by CYP2C19 with minor contributions from CYP2C9 and CYP1A1 [7]. None of the metabolites exhibited antiangiogenic activity in human models and only high concentrations of 5′-hydroxythalidomide inhibited angiogenesis in a rat aortic ring assay [12]. Bauer et al [11] reported that the antiangiogenic and antiproliferative effects of thalidomide in a rat aorta model and human aortic endothelial cells, respectively, were observed only with the addition of human or rabbit microsomes but not rat microsomes, indicating that the metabolites rather than thalidomide *per se* were responsible for the pharmacological activities, including probably the teratogenic effects. However, the antiangiogenic activity of thalidomide in the absence of microsomes has also been suggested [7]. The relationship between biotransformation/hydrolysis products and effects (or relevant mechanisms of action) has been reviewed [13].

Although thalidomide exhibits serious teratogenic effects, it possesses significant immunomodulatory activities, especially the inhibition of TNF-α, and considerable effort has been expended in the development of analogs with reduced toxicities. Two classes of compounds have been developed using thalidomide as the template. Both classes of compounds exhibited potent inhibition of TNF-α but with differential cytokine modulation and T cell activation [14]. Immunomodulatory derivatives of thalidomide are termed IMiDs. Lenalidomide and actimid are two IMiDs. Lenalidomide has been approved by the FDA for the treatment of myelodysplastic syndrome with chromosome 5q31 deletion and multiple myeloma in 2005 and 2006, respectively. Actimid is being investigated for the treatment of myelodysplastic syndrome and myeloma. Still another group of derivatives, selected cytokine inhibitory drugs (SelCiDs), are being investigated for diverse clinical applications. An early [15] and several more recent reviews [6,9,16,17] on immunomodulation by these drugs have been published.

3. Mechanisms of action of thalidomide

3.1. Antiangiogenic activity

In 1971, Folkman formulated the hypothesis that tumor growth is dependent on angiogenesis [18]. Although the precise mechanisms of teratogenic action of thalidomide remain unclear, the fact that limb bud formation depends heavily on angiogenesis led to the idea that teratogenic and antiangiogenic actions were related. Angiogenesis is the formation of new blood vessels from pre-existing microvessels through complex mechanisms that involve various growth factors, e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) [19]. Initial studies demonstrated that thalidomide inhibited TNF-α-induced angiogenesis in a rabbit cornea micropocket model [5] and inhibited microvessel formation in an isolated rat aorta model [11]. A more recent study using an *in utero* chicken embryonic chorioallantoic membrane (CAM) assay found that VEGF plus bFGF-induced vessel formation was inhibited by thalidomide, and the antiangiogenic effect of thalidomide was increased by preincubation with human but not rat microsomes [20]. Furthermore, two putative hydroxylated thalidomides were found to exhibit antiangiogenic action in the CAM assay. The role of VEGF in tumor angiogenesis has been reviewed [21].

What are the molecular mechanisms of the antiangiogenic action of thalidomide? Thalidomide has been found to inhibit TNF-α production from lipopolysaccharide (LPS)-induced human monocytes and mouse

macrophages by increasing its mRNA degradation [22,23]. TNF-α has been observed to be a potent inhibitor of endothelial cell growth *in vitro*. The angiogenic action of TNF-α in a rabbit cornea model was suggested to result from secondary inflammation mediated by leukocyte infiltration because, in this model system, a high dose of TNF-α did not suppress angiogenesis induced by bFGF, but instead stimulated neovascularization and exerted an inflammatory response [24]. Further evidence suggested that prostaglandins [25], platelet-activating factor [26], and B61 and its Eck receptor [27] may mediate TNF-α-induced angiogenesis. Still, TNF-α may promote angiogenesis by upregulating the expression of endothelial integrin, which is crucial for angiogenesis [28]. It should be pointed out that TNF-α, by interacting with its receptor, can activate a wide range of transcription factors and gene products, and consequently can directly influence a diverse spectrum of cellular activities [29]. TNF-α is considered a potential target in therapy for solid tumors. For example, TNF-α antagonists have been successfully developed for the treatment of inflammatory diseases and tumors [30].

Hypoxia-inducible factor 1α (HIF-1α) is a transcription factor that senses reduced oxygen tension and responds with increased transcription of hypoxia-inducible genes involved in angiogenesis. Accumulation of HIF-1α protein but not its mRNA was induced by TNF-α in kidney cells via a nuclear factor (NF)-κB dependent pathway [31]. Consequently, thalidomide may decrease the expression of HIF-1α, and hence the inhibition of angiogenesis, through reduction of TNF-α production [7]. NF-κB is an evolutionarily conserved, inducible transcription factor that plays important roles in a very large variety of biological processes. It has been demonstrated that angiogenesis requires NF-κB activation [32]. The activity of NF-κB is controlled by an inhibitory protein, Ik-Bα. Phosphorylation of Ik-Bα by IkB kinase leads to its degradation and allows active NF-κB to enter the nucleus to activate gene transcription. Thalidomide has been shown to block NF-κB activity and cytokine-mediated expression of NF-κB-regulated genes such as interleukin (IL)-8 through suppression of IkB kinase activity in human Jurkat T cell lymphocytes and human endothelial cell cultures [33,34]. Thus, thalidomide may regulate TNF-induced angiogenesis through suppression of the expression of VEGF, bFGF and IL-8. Furthermore, thalidomide has been demonstrated to inhibit endothelial cell proliferation *in vitro*, which appears to correlate with suppression of NF-κB activation [35]. It is important to mention that endothelial cells and VEGF are actively involved in vascular development [19]. In addition, thalidomide and its analogs have been observed to reduce NF-κB-dependent HIV-1 replication *in vitro* in human monocyte-derived macrophages [36].

3.2. Immunomodulatory and anti-inflammatory activity

Inhibition of immune/inflammatory responses and angiogenesis by thalidomide is probably interrelated because cytokines such as TNF-α, and NF-κB have been found to be involved in both processes [33,34]. Thalidomide increased the levels of both intra- and extracellular IL-2 in cultures of human mononuclear cells activated with mitogens or antigen without affecting proliferation or IL-2 receptors [37]. IL-2 may possess antitumor activity or may modulate the immune system to induce anticancer activity. The serum level of TNF-α was reduced while that of interferon (IFN)-γ was increased in erythema nodosum leprosum patients following treatment with thalidomide [38]. In contrast to its suppressive effects in the inflammatory response, thalidomide has been reported to act as a costimulator in enhancing the response of T cells to T-cell receptor complex, resulting in increased secretion of IL-2 and IFN-γ and increased numbers of NK cells leading to enhanced cytotoxicity in multiple myeloma [39]. Under the condition of T cell costimulation, thalidomide enhanced TNF-α generation by $CD4^+$ and $CD8^+$ T lymphocytes activated by anti-CD3, and elevated serum TNF-α levels in advanced cancer patients treated with an immunomodulatory derivative of thalidomide [40].

IL-12 plays an important role in the development of cellular immune responses. IL-12 stimulates IFN-γ secretion and proliferation of activated T cells and NK cells, and enhances T and NK cell-mediated cytotoxicity [41]. Thalidomide inhibited IL-12 production by monocytes activated by LPS [42]. However, similar to TNF-α, IL-12 was increased by thalidomide and its analogs *in vitro* and *in vivo* under the condition of T cell activation [14,43]. IL-6 has been demonstrated to be a major growth factor for multiple myeloma [44]. The expression of IL-6 and its mRNA in mitogen-activated blood mononuclear cells was selectively inhibited by thalidomide but the expression of IL-2, IL-4 and IL-10, which was markedly inhibited by dexamethasone, was unaffected [45]. However, levels of TNF-α, IL-1β and IL-6 were inhibited while IL-10 was largely increased by thalidomide in LPS-stimulated blood mononuclear cells [14]. IL-8 is a potent neutrophil chemoattractant that can be released from endothelial cells. Thalidomide enhanced the TNF-α-induced but inhibited the LPS-induced IL-8 release from human umbilical vein endothelial cells [46].

Cyclooxygenase-2 (COX-2) is an important pharmacological target in the treatment of pathological angiogenesis, including cancer and chronic inflammatory diseases [47]. In contrast to COX-1, which is constitutively expressed and plays a homeostatic role, COX-2 is an immediate early gene induced by many stimuli including LPS, cytokines and growth factors.

COX-2 has been reported to be required for endothelial migration and rat corneal angiogenesis [48,49]. Thalidomide inhibited LPS-mediated induction of COX-2 protein and mRNA in murine macrophages by decreasing its mRNA stability without affecting its transcription [50]. This effect might partly explain the antiangiogenic activity of thalidomide.

The migration and invasion potentials of cancer cells play critical roles in tumor metastasis. Adhesion molecules facilitate the adherence of tumor cells to the endothelium and are required for the development of metastasis. Blunting of TNF-α-induced upregulation of adhesion molecules (ICAM-1, VCAM-1, E-selectin) on human umbilical vein endothelial cells by thalidomide has been demonstrated [51]. Thalidomide also directly induced tumor cell apoptosis and G1 growth arrest in multiple myeloma cell lines and patient multiple myeloma cells that were resistant to anticancer drugs and dexamethasone [52]. Interestingly, the effect of dexamethasone was enhanced by thalidomide and inhibited by IL-6 [52].

The multiple effects of thalidomide demonstrated both *in vitro* and *in vivo*, albeit more difficult to show in the former, include antiangiogenic, anti-inflammatory and immunomodulatory actions, downregulation of adhesion molecules, direct apoptotic effects and growth arrest. These effects suggest that the drug and its derivatives may be useful for the treatment of various diseases. Indeed, thalidomide and its derivatives have been approved or are under clinical trials for a variety of diseases, including erythema nodosum leprosum, multiple myeloma, myelodysplastic syndrome, melanoma, prostate cancer, renal cell carcinoma, Crohn's disease, rheumatoid arthritis, Behcet's disease, and many other diseases.

4. Immunomodulatory (IMiDs) and selective cytokine inhibitory (SelCiDs) thalidomide derivatives

To minimize the teratogenic effect and enhance the anti-TNF-α activity of thalidomide, two classes of derivatives have been developed [14]. Both classes are much more potent TNF-α inhibitors than the parent drug thalidomide yet exhibit different pharmacological spectra. One class has been found to be potent phosphodiesterase (PDE) 4 inhibitors that suppressed TNF-α production, exhibited a modest inhibitory effect on LPS-induced IL-1β and IL-12, modestly increased anti-inflammatory IL-10 generation, and did not affect IL-6 and T cell activation. These compounds appear to exhibit selective anti-inflammatory actions and are termed selected cytokine inhibitory drugs (SelCiDs). The other class did not inhibit PDE 4 but instead demonstrated broad inhibitory effects on the LPS-mediated release of TNF-α, IL-1β, IL-6 and IL-12 while potently increasing IL-10 production. In addition to their strong anti-inflammatory effects, these compounds also potently stimulated T cell proliferation as well as IL-2 and IFN-γ production. These compounds are termed IMiDs (immunomodulatory imide drugs). This review will focus on thalidomide and IMiDs, in particular lenalidomide (Revlimid, CC-5013), because the latter has been approved for the treatment of multiple myeloma and myelodysplastic syndrome and tested in many clinical trials for other diseases. The immunomodulatory and nonimmunomodulatory effects of these drugs will be presented and compared, if data are available.

4.1. Immunomodulatory effects

Thalidomide and IMiDs exert potent immunomodulatory properties including inhibition of TNF-α, IL-1β, IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF), and stimulation of IL-10 in LPS-stimulated monocytes and macrophages. Lenalidomide and CC-4047 (Actimid) have been found to be up to 50,000 times more potent TNF-α antagonists in LPS-stimulated models than the parent compound [53,54]. Levels of IL-1β, IL-6 and GM-CSF were also inhibited whereas IL-10 was increased by lenalidomide and CC-4047, although with varying degrees of potency compared to thalidomide [14]. However, IMiDs may increase TNF-α production to potentiate immune responses under the condition of T cell activation. For example, CC-4047 increased TNF-α generation by $CD4^+$ and $CD8^+$ T lymphocytes stimulated by anti-CD3 [40]. In a clinical trial, thalidomide increased plasma TNF-α level in patients with toxic epidermal necrolysis, and this was associated with increased mortality [55]. Similarly, lenalidomide has been found to increase TNF-α level in patients with metastatic melanoma without excess morbidity or mortality [56].

IL-12 plays an important role in the development of host immune response. IL-12 enhances the expansion and activity of T and NK cells to boost innate and adaptive immunity [41]. IL-12 is produced primarily by antigen-presenting cells (APC; monocytes, macrophages and dendritic cells) in T cell-dependent and -independent pathways. LPS directly stimulated T cell-independent IL-12 generation by APC, which was suppressed by thalidomide and IMiDs. In the T cell-dependent pathway, the production of IL-12 by the APCs was induced by the interaction of CD40 on the surface of the APC, with CD40L (CD40 ligand) on the surface of the activated T cells [57,58]. Analogous to TNF-α, thalidomide and IMiDs exerted bidirectional effects on IL-12 production. IL-12 production was suppressed by IMiDs in LPS-stimulated monocytes but stimulated by thalidomide and its analogs *in vitro* and *in vivo* under the conditions of T cell stimulation [14,43]. Thalidomide and IMiDs also upregulated

the expression of CD40L on the surface of T cells [14,43]. The time course of activation indicated that IL-12 production resulted from drug-induced T cell activation [43].

Expression of cell surface adhesion molecules plays important roles in response to inflammatory stimuli and cancer metastasis. Thalidomide was found to reduce the density of TNF-α-induced cell surface adhesion molecules ICAM-1, VCAM-1 and E-selectin on human umbilical vein endothelial cells, and L-selectin on human leukocytes *in vitro* [51]. Blocking the expression of these molecules may partly explain the antivasculitic effect of thalidomide. Lenalidomide has been demonstrated to inhibit the binding of multiple myeloma cells to bone marrow stromal cells, resulting in reduced production of IL-6, VEGF and TNF-α, blockade of angiogenesis, and stimulation of host anti-multiple myeloma NK cell immunity [39,52,59].

One of the major distinctive differences between IMiDs and SelCiDs is that the latter class of drugs exhibits little or no T cell activation. SelCiDs are PDE 4 inhibitors, and increasing cAMP levels in T cells during the early phase of mitogen or antigen activation results in a decreased proliferative potential [60]. In contrast, IMiDs are potent costimulators of T cells and increased cell proliferation in a dose-dependent manner [14]. Similar to thalidomide, these compounds exerted a greater effect on $CD8^+$ cells than on $CD4^+$ cells. When T cells were stimulated by anti-CD3, thalidomide and IMiDs caused a marked increase in IL-2, IFN-γ and IL-12, and induced the upregulation of CD40L on the surface of T cells [14,43]. IMiDs are 100 to 1000 times more potent than thalidomide in costimulating T cells that have been partially activated by the T cell receptor complex [15]. IMiDs were demonstrated to enhance T cell cytokine production through potentiation of the transcription factor AP-1 [61].

The multiple effects of thalidomide and IMiDs on the immune system reflect the intricacies of the interplay among the myriad components participating in innate and adaptive immunity. Drugs that exhibit broad immunomodulatory activities are potential candidates for the treatment of various diseases. Major questions concerning the adverse profiles of these drugs will be presented below.

4.2 Nonimmunomodulatory effects

Thalidomide and IMiDs have been found to exert antiangiogenic properties independent of their immunomodulatory effects [5,62]. Using a novel *in vitro* multicellular human umbilical vein endothelial cell culture and rat aorta assay, IMiDs and SelCiDs were shown to be significantly more potent than thalidomide in inhibiting angiogenesis. Furthermore, the antiangiogenic effect did not correlate with their TNF-α and PDE 4 inhibitory efficacies [62]. In this study, an IMiD was observed to exhibit antitumor activity using a murine rectum polypoid carcinoma cell line xenograft. The tumor growth rate was reduced and accompanied by extensive necrosis. IMiDs were more potent than thalidomide in reducing the microvessel density in murine lymphoma xenograft models [63]. It has been pointed out that in multiple myeloma, the close proximity interaction between bone marrow stromal cells and patient multiple myeloma cells significantly increases the levels of proangiogenic factor VEGF and IL-6 [6,39,52,59]. IL-6 is a major growth factor for multiple myeloma [44]. Thalidomide and CC-4047 (Actimid, an IMiD) significantly decreased the expression of VEGF and IL-6, leading to the inhibition of angiogenesis in multiple myeloma cells [59].

In addition to their direct antiangiogenic action, thalidomide and IMiDs inhibited, albeit modestly, DNA synthesis in human multiple myeloma cell lines and cells from patients [52]. IMiDs also inhibited the proliferation of doxorubicin- and melphalan-resistant multiple myeloma cells by 20–50% [6,64]. Several studies have shown that thalidomide and IMiDs directly induce apoptosis or growth arrest in multiple myeloma cells [52], inhibit the generation of VEGF and IL-6 [59], and stimulate NK cell anti-multiple myeloma immunity [39]. Another study showed that IMiDs activated caspase-8, enhanced multiple myeloma cell sensitivity to Fas-induced apoptosis, and downregulated NF-κB activity as well as the expression of apoptosis inhibitory protein [65].

4.3. Adverse effects

Thalidomide was withdrawn from the market shortly after its introduction into clinical practice because of its teratogenic effects in babies born to women who took the drug during the first trimester of pregnancy. Neurologic adverse effects of thalidomide are common and often represent the limiting toxicity after repeated administration. Somnolence and constipation are dose-related, and peripheral neuropathy may occur following prolonged administration [66]. Thalidomide may increase the risk of thromboembolic complications in cancer patients. The prevalence of thrombosis was about 5% with thalidomide monotherapy but increased about threefold when thalidomide was combined with corticosteroids or cytotoxic drugs [67]. Enoxaparin (40 mg, subcutaneous, qd) but not low dose warfarin (1 mg/day) reduced the incidence of deep vein thrombosis (DVT) in a randomized trial of patients with multiple myeloma treated with thalidomide and chemotherapy [68].

Several phase I studies in patients with solid tumors or multiple myeloma showed that the toxicities associated with lenalidomide, in contrast to thalidomide,

were primarily Grades 1 and 2 according to National Cancer Institute Common Toxicity Criteria. Sedation, somnolence, constipation and neuropathy were not reported (17). Thrombocytopenia and neutropenia were found, but their occurrence was dose-limiting and developed during the second month of treatment. Interestingly, in a phase I study in patients with refractory or relapsed multiple myeloma, CC-4047 (Actimid) appeared to retain some of the adverse effects of thalidomide, such as constipation, neuropathy and DVT (69). More importantly, lenalidomide has not been found to be teratogenic in rabbits, a sensitive species for thalidomide-induced birth defects (16). Thus, it appears that IMiDs, especially lenalidomide, lack the adverse effects of thalidomide or exhibit reduced side effects. However, larger populations need to be evaluated to understand the frequency of adverse effects, and women of childbearing age taking these medications should take necessary measures to prevent conception.

5. IMiDs in cancer therapy

Given that IMiDs are potent inhibitors of monocyte inflammatory cytokine generation and angiogenesis, and are also strong costimulators of T cell activation, it is no wonder that these drugs have been widely tested in hundreds of clinical trials worldwide. Lenalidomide has been approved by the US FDA for the treatment of myelodysplastic syndrome and multiple myeloma. In addition, lenalidomide and Actimid alone or in combination with other therapeutic agents have been evaluated in melanoma, mantle cell carcinoma, relapsed/refractory multiple myeloma, prostate cancer, renal cell carcinoma, and many other inflammatory diseases. For readers interested in clinical trials currently underway in the US, please refer to www.cancer.gov/clinicaltrials.

6. Conclusions

The serendipitous discovery of the anti-inflammatory effects of thalidomide has generated interest not only in understanding the mechanisms of thalidomide and its IMiDs but also in the application of these drugs for the treatment of cancers and inflammatory diseases. Studies have clearly demonstrated that IMiDs exhibit greater immunomodulatory and antiangiogenic efficacy than the parent drug, and an apparent apoptotic effect accompanied by a lack of thalidomide's adverse effects, in particular teratogenicity. The IMiDs therefore exhibit novel mechanisms of anticancer activity and offer important alternatives for the treatment of at least several cancers previously lacking appropriate drugs.

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