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A Mini-Review on Thalidomide: Chemistry, Mechanisms of Action, Therapeutic Potential and Anti-Angiogenic Properties in Multiple Myeloma

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Abstract

Thalidomide is a drug with interesting therapeutic properties but also with severe side effects, which require a careful and monitored use. Potential immunomodulatory, anti-inflammatory, anti-angiogenic and sedative properties make thalidomide a good candidate for the treatment of several diseases such as multiple myeloma. Through an increase in the degradation of TNFα-mRNA, thalidomide reduces the production of TNFα by monocytes and macrophages stimulated by lipopolysaccharide or by T lymphocytes induced by mitogenic stimuli. The decreased level of TNFα alters the mechanisms of intracellular transduction by preventing the activation of NF-kB and by decreasing the synthesis of proteins, in particular IL-6, involved in cell proliferation, inflammation, angiogenesis and protection from apoptosis. Furthermore, thalidomide affects VEGF levels by down-regulating its expression. Nowadays, new safer and less toxic drugs, analogs of thalidomide, are emerging as beneficial for a more targeted treatment of multiple myeloma and several other diseases such as Crohn's disease, rheumatoid arthritis, sarcoidosis, erythema nodosum leprosum, graft-versus-host disease.

1. INTRODUCTION

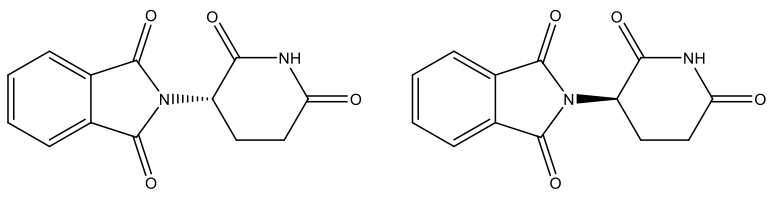
Thalidomide, a former antiemetic and sedative, recorded one of the most tragic chapters in the era of modern drug discovery. It is a glutamic acid derivative that was developed by the West German Pharmaceutical Company Chemie Grünenthal GmbH and imported to the market in 46 countries as a drug for the treatment of nausea during pregnancy [1]. In 1957, thalidomide was also prescribed as a sedative due to its higher safety compared to barbiturates. In the early 60s, however, it was clear that the use of thalidomide during pregnancy was associated with major congenital anomalies: severe birth defects including amelia (lack of limb) and phocomelia (seal limbs) [2,3], as well as deformities of internal organs, cleft lip and palate, and abnormal eyes and ears with up to 12.000 babies affected [4]. Fortunately, the approval of thalidomide was delayed, thanks to Dr. Frances Kelsey from the Food and Drug Administration (FDA), so that only a limited number of Americans suffered the consequences of its use [5]. In 1962, thalidomide was withdrawn from all markets in the world with the exception of Brazil. It is necessary to clarify that when thalidomide was launched on the market the toxicity studies, as well as teratogenicity, were not included in the standard drug safety protocol. After this tragic event, scientists started to testing thalidomide for other applications and, in 1965, Jacob Sheshkin, a dermatologist from Israel, found that its administration to an insomniac patient with erythema nodosum leprosum (ENL) not only relieved the insomnia, but also resulted in improvement of patient's skin lesions within several days of treatment [6]. As a result, in July 1998 the FDA approved, for the first time in the US, the marketing of thalidomide for the treatment of cutaneous manifestations of ENL. In addition to its sedative effect, thalidomide is used as anti-inflammatory drug and immunosuppressive [7]. To minimize the risk of teratogenicity related to thalidomide, the Celgene Corporation (Waren, New Jersey) developed a precise program in order to regulate provision, dose, administration and use of thalidomide [8]. This program, known as "System for Thalidomide and Prescribing Safety" uses a three-pronged approach: control access to the drug, inform doctors, pharmacists and patients, and monitor compliance. Nowadays, thalidomide is a drug widely used for the treatment of many diseases. Wu et al. [9] provided a

review on the more common off-label uses of thalidomide in dermatologic conditions including apthous stomatitis, Behcet's syndrome, lupus erythematosus, prurigo nodularis, as well as novel uses in the treatment of Kaposi's sarcoma, pyoderma gangrenosum, and lichen planus. Some cancers including myelodysplastic syndrome, melanoma, and prostate cancer as well as inflammatory conditions such as Crohn's disease have been treated with thalidomide [10, 11]. Indeed, studies regarding the effects of thalidomide on Multiple Myeloma (MM) attracted significant interest. On a worldwide scale, it is estimated that about 86 000 incident cases occur annually, accounting for about 0.8% of all new cancer cases. About 63 000 subjects are reported to die from the disease each year, accounting for 0.9% of all cancer deaths [12]. MM is a malignant disease affecting senior adults and characterized by the proliferation and accumulation of B lymphocytes and plasma cells in the bone marrow, and by an increased production of monoclonal protein detectable in serum and/or urine [13]. MM plasma cells produce several cytokines that induce stromal cells to produce other pro-MM cytokines or the direct interactions between stromal and MM plasma cells also resulting in the production of cytokines. Moreover, this type of disorder is characterized by osteolytic bone lesions, anemia and hypercalcemia [14]. There are some options to treat MM: chemotherapy and other drugs, the use of bisphosphonates, radiation, surgery, stem cell transplant, plasmapheresis. The most convenient treatment should be assessed by an expert team and it depends on individual cases. In the context of chemotherapy, the most used agent is the dexamethasone, alone or in combination with other agents such as cisplatin and thalidomide. Thalidomide has become the first of a series of new immunomodulatory agents (analogs of thalidomide) [15]. In addition, in the twenty-first century a new class of drugs has been introduced: proteasome inhibitors, including bortezomib. Each of these drugs is currently used in different disease stages and with different directions. These drugs are continually controlled and the main purpose of the researchers is to optimize them and to reduce their negative and side effects. In this context, the identification of new compounds endowed with a better pharmacological profile than thalidomide should be envisaged. One of these, lenalidomide, is already used for the treatment of

the refractory myeloma and it is in phase 2 and 3 clinical trials [16,17]. It is possible that the combination therapy of thalidomide with other anticancer agents or monoclonal antibody could lead to additive or synergistic interactions and also reduce the possibility of chemical resistance [18, 19]. The purpose of this paper is to review the potency of this drug in particular on the treatment of MM.

1.1 Chemistry and metabolism of thalidomide

Thalidomide, 2-(2,6-dioxopiperidin-3-yl)isoindole-1,3-dione or α-(*N*-phthalimido)glutarimide, has a chiral carbon atom, thus it can exists in the form of two enantiomers: (+)-(*R*)-thalidomide and (–)-(*S*)-thalidomide (Fig. 1). As it is commonly known, the *R*-thalidomide is a safe sedative, while the *S*-enantiomer, the distomer, is responsible for the well known teratogenic effects of the drug [20]. Recently, it has been shown that cereblon, the ubiquitously expressed E3 ligase protein identified as the primarily molecular target of thalidomide and its analogs, preferentially binds the *S*-enantiomer, further underlining the importance of chirality for these molecules [21]. The chemical formula of thalidomide is $C_{13}H_{10}N_2O_4$ and its molecular weight is 258.2 g/mol. Its color is white crystalline and its melting point is 217 °C. It is insoluble in ether and benzene and it has a low solubility in water, methanol, ethanol and glacial acetic acid. The metabolism of the drug occurs by spontaneous non-enzymatic hydrolysis to physiological pH with the formation of twenty metabolites. Thalidomide can still be metabolized by cytochrome P450 2C19 with the formation of molecules that may act as anti-angiogenic [22].



S-enantiomer *R*-enantiomer

Figure 1. Chemical structure of thalidomide enantiomers

1.2 Mechanism of action of thalidomide

Thalidomide is marketed as a racemate of the two enantiomers. Studies indicated that the antiangiogenic and teratogenic effects involve the *S*-enantiomer, while the *R*-enantiomer was responsible for sedation [23,24]. Thalidomide has been shown to exert multiple actions as (i) an anti-proliferative effect on blood cells and stromal cells of the bone marrow, (ii) a reduction of the expression of cytokines such as IL-6, IL-1β, TNF-α, bFGF, VEGF, interferon-gamma (INF-γ) and possibly NF-κB, (iii) a modulation of the expression of adhesion molecules and the immune system by stimulating the proliferation of cytotoxic T cells and by inducing the production of anti-inflammatory cytokines, (iv) an inhibition of cyclooxygenase (COX)2, and (v) an anti-angiogenic effect in endothelial cells by modulation of the expression of adhesion molecules and the production of angiogenic factors such as bFGF and VEGF [25–30].

1.3 Effects of thalidomide on potential targets in Multiple Myeloma

The pathophysiology of MM manifests by various steps (Fig. 2): a) initial asymptomatic malignant condition, defined as “Gammopathy of undetermined significance (MGUS)”, seen in approximately 1% of the population aged over 50 years; b) intermediate stage between MGUS and MM, defined as “smoldering MM”, which does not present the MM clinical symptoms, but often progresses to symptomatic MM; c) stadium of asymptomatic MM intramedullary, whose clinical symptoms are manifested; d) terminal stage of extramedullary MM, in which cancer cells are detectable in the blood. The tumor progression process is due to sequential molecular events affecting the plasma cell clone and changes of the bone marrow microenvironment. The microenvironment changes include the secretion of particular cytokines crucial for growth, progression and diffusion of malignant cells of MM [31]. Among the cytokines, our attention focus on IL-6 (interleukin-6), VEGF and TNF-α and the effect of thalidomide on each of them.

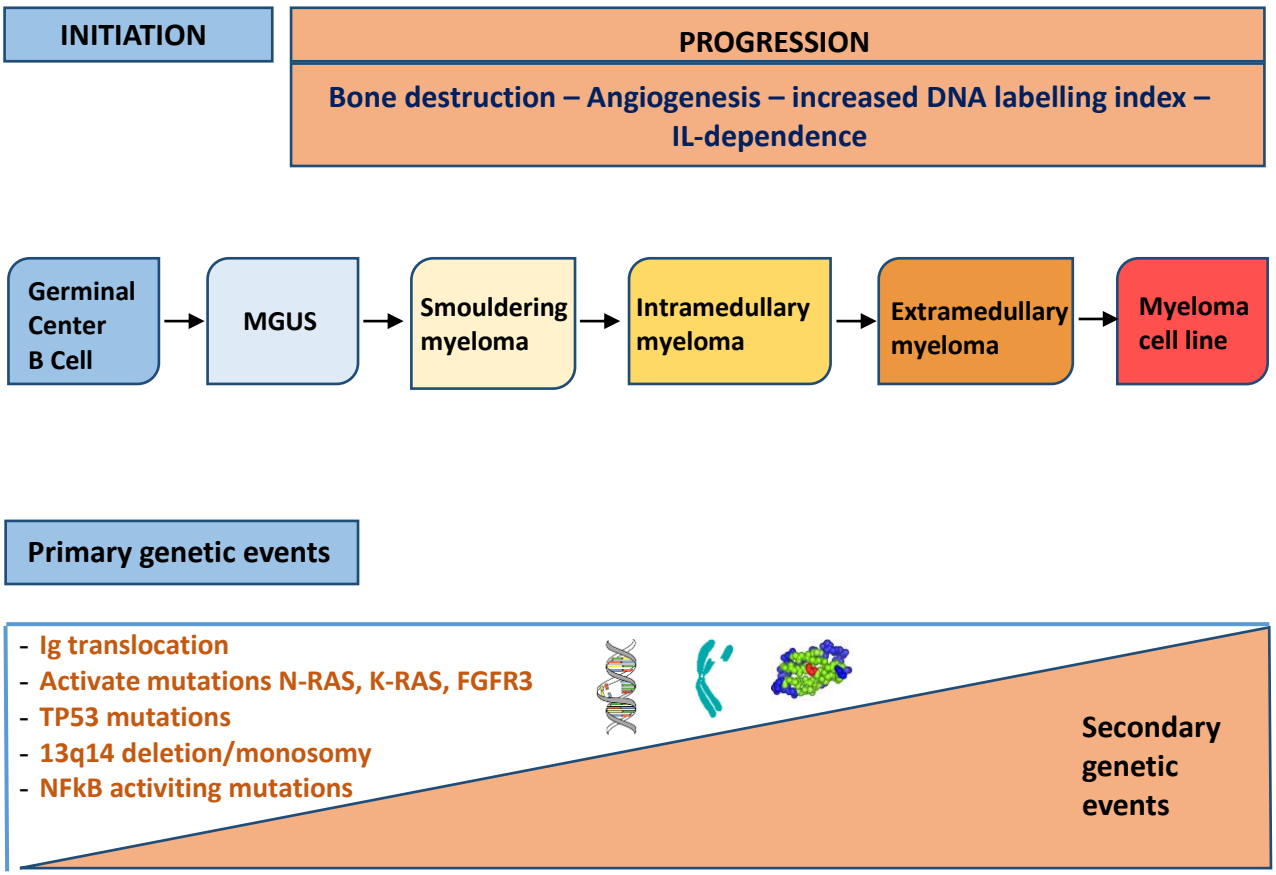


Figure 2. Evolution of Multiple Myeloma

1.3.1. The effect of thalidomide on TNF-α

Among the pro-inflammatory cytokines, TNF-α is the most studied in relation to thalidomide. In particular, in MM, the role of TNF-α is explicated both directly, through activation of different pathways pro-proliferative and anti-apoptotic as MAPK/ERK, NF-kB, PI3K/AKT pathway in MM cells, and indirectly through the stimulation of IL-6 secretion by the bone marrow stromal cells, where it activates NF-kB. Furthermore, TNF-α up-regulates the expression of adhesion molecules on both the MM cells (CD49d, VLA-4; CD11a, LFA-1) and the bone marrow stromal cells (CD54, ICAM-1, CD106, VCAM-1); the increased adhesion between these cells stimulates the secretion of the main proliferative factor IL-6 by the bone marrow stromal cells and stimulates cell adhesion mediated drug resistance (CAM-DR) [32]. In MM, TNF-α leads to an up-regulation of adhesion molecules and contributes to the survival of neoplastic cells by protecting them from apoptosis. TNF-α induces secretion of other cytokines, including IL-6, that favor the proliferation of neoplastic

cells [32]. Thalidomide has been demonstrated to reduce TNF-α production by human monocytes stimulated by LPS [27], by human alveolar macrophages from tuberculosis patients stimulated by LPS [33] and by human alveolar macrophages stimulated by LPS from patients with interstitial lung disease [34]. The reduced production of TNF-α, in these cells, appears to be mediated by an accelerated degradation of mRNA of TNF-α. In particular, mRNA degradation occurs leading to a decrease in half-life of the molecule from 30 minutes to 17 minutes [25,26]. Moreover, thalidomide seems to be able to block the activity of NF-kb transcription factor involved in the immune response, cell growth and regulation of the levels of TNF-α [35,35]. In addition, thalidomide has been shown to bind α1-acid glycoprotein (AGP), an acute-phase protein with potential TNF-α potentiating activity, suggesting that it may act by binding to and inhibiting AGP; however, the physiologic role of AGP in the *in vivo* regulation of TNF-α is not yet clear [37]. Then, starting from this effect of thalidomide, new thalidomide analogs have been developed to selectively inhibit TNF-α [38].

1.3.2. The effect of thalidomide on interleukine-6 (IL-6).

IL-6 is the interleukin that acts as a pro-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma [39]. Moreover, IL-6 is involved in the process of proliferation of myeloma cells in MM [40]. Stromal cells and myeloma plasma cells in the microenvironment, in fact, increase the release of cytokines such as IL-6. The transcription of IL-6 depends on the activation NF-kB which is activated by TNF-α. In normal condition, IL-6 is able to stimulate the differentiation of B cells to plasma cells but not their proliferation. In contrast, in myeloma cells, IL-6 triggers the cell proliferation (via RAS-MAPK), promotes the survival (JAK-STAT), increases resistance to apoptosis induced by dexamethasone (activation of the PI3K-AKT signaling pathway), stimulates VEGF secretion and inhibits monocyte differentiation to dendritic cells hindering the immune response in order to fight the tumor [41–44]. In addition, it has recently been found that IL-6 may play an important role in dephosphorylation of retinoblastoma

protein (pRB). In particular, IL-6 was demonstrated to down-regulate dephosphorylated pRB and to promote its phosphorylated form, suggesting that it can play an important role in promoting the growth of MM cells via phosphorylation of pRB protein [45]. *In vitro* studies showed that thalidomide decreases IL-6 levels from human lung fibroblasts [46]. Moreover, thalidomide decreased IL-6 levels from bone marrow stromal cells, MM cells and their co-cultures. In the context of these studies and given that IL-6 promotes the growth and survival of cells in MM, it is conceivable to consider it as a potential target for thalidomide [47,48].

1.3.3. The effect of thalidomide on VEGF

VEGF is a molecule involved in the angiogenetic process. Angiogenesis is a phenomenon that leads to the formation of new blood vessels. During tumor growth, angiogenesis becomes a very important process for tumor progression and dissemination. The mechanisms involved in the regulation of tumor angiogenesis, nowadays, are extremely complex and still not completely known. Angiogenesis in tumor happens through the same stages of physiological angiogenesis; however, it results in a more marked increase of proliferative activity of the endothelial cells leading to functional differences and structural vascular plexus [49]. The angiogenic switch, that is to say the passage from one phase of avascular growth toward a vascular growth, is determined by unbalanced pro- and anti-angiogenic factors in the tumor microenvironment, both directly secreted by tumor cells and indirectly secreted by supporting cells of the microenvironment promoted by the same tumor [50]. VEGF seems to play a central role in angiogenesis, on which many factors and conditions act, as inducers or promoters: the hypoxic stimulus, many oncogenes (ras, bcl-2, VHL), growth factors as TNF-α, HGF (hepatocyte growth factor), EGF (epidermal growth factor), G-CSF (granulocyte-colony stimulating factor), cytokines such as IL-1 (interleukin-1), IL-6 and IL-8, IFN-γ (gamma-interferon), proteases such as MMP-9 (matrix metalloproteinase 9 or 11 gelatinase B) and MMP-2 (matrix metalloproteinase 2 or gelatinase A) [51-53]. However, VEGF is not the only factor involved in the process of angiogenesis. Perineoplastic area, in fact, is the scene of a complex

inflammatory interaction between tumor cells, monocytes and granulocytes: macrophages induce endothelial cell proliferation and are often found in contact with the walls of neovascularization; metalloproteinases as MMP-9, synthesized by neutrophils attracted locally, play an important role in the vascular remodeling; FGF (Fibroblast Growth Factor), released by the tumor, exerts a selective recruitment on vascular cells; angiopoietin-1 (Ang1) induces the recruitment of pericytes to endothelial cells and the incorporation in the vessel wall of smooth muscle cells; angiopoietin-2 (Ang2) acts, however, rejecting the recruitment of pericytes and smooth muscle cells. Expression profiling studies have identified endothelial cells as the primary source of Ang2 [54]. A recently published study contributed significantly to elucidate the anti-angiogenic effect of thalidomide in patients with active MM compared to inactive MM or to patients with MGUS (Monoclonal gammopathy of undetermined significance). The effect of thalidomide on the expression of the major angiogenic factors secreted by endothelial cells (VEGF, bFGF, HGF, IGF-1, Ang1 and Ang2) was analyzed by comparing MM endothelial cells (MMEC) of patients with active and inactive disease, endothelial cells of patients with MGUS, Kaposi's sarcoma cell line and human umbilical vein endothelial cells (HUVEC). As a result, in active MM, thalidomide at concentrations of 10 μM and 20 μM down-regulates all genes except Ang1 in a dose dependent manner. Both doses inhibit the proliferation and capillarygenesis of these cells in vitro [30]. Another interesting study about anti-angiogenic activity of zoledronic acid that acts on autocrine loop VEGF-VEGFR-2 in endothelial cells of patients with MM, demonstrated that zoledronic acid inhibits certain angiogenic functions such as proliferation, chemotaxis and capillarygenesis on Matrigel. At the molecular level it down-regulates the expression of VEGF or its receptor [55]. Thus, VEGF represents a good target for both thalidomide and new types of drugs with antiangiogenic activity.

1.3.4 The effect of thalidomide on Cereblon

Interesting is also the effect of thalidomide and its analogs on cereblon (CRBN) [56]. Cereblon is a protein encoded by the CRBN gene in humans. It is a component of a protein complex called E3

ubiquitin ligase, important for the degradations of target proteins. When thalidomide and its analogs bind CRBN, the enzyme complex E3 ubiquitin ligase attaches ubiquitin molecules to the transcription factors IKZF1 (Ikaros) and IKZF3 (Aiolos) [57]. This link induces the breakdown of these molecules. Ikaros and Aiolos represent the survival factors for myeloma plasma cells in MM disease [58]. For this reason, the use of these drugs is effective during the MM treatment. Which is the role of Ikaros and Aiolos in blood disorder? Ikaros and Aiolos are two zinc-finger transcription factors required for viability of many MM cell lines. In particular, Ikaros is able to bind and activate IRF4 promoter, a transcription factor essential for MM proliferation through Myc activation. If Ikaros is degraded, the reduction of the IRF4 expression occurs. Aiolos is a repressor of interluekine-2 (IL-2) promoter [58]. The loss of Aiolos induces the T-cell to produce IL-2, the one of the key cytokines with pleiotropic effects on immune system [59]. Therefore, the final result of the interaction between thalidomide (and derivatives) and CRBN is the block of cancer and its progression.

1.4 Use of analogs of thalidomide in new therapies for the treatment of Multiple Myeloma

In recent years, new analogs of thalidomide, obtained by chemical modification of thalidomide, have been designed in order to optimize their immunological and anticancer properties and overcome the original devastating side effects [60]. Thus, a series of immunomodulatory drugs (IMiDs) have been developed (Table 1). Furthermore, another class of thalidomide analogs termed SelCIDs (Selective Cytokine Inhibitory Drugs) consists of type 4 phosphodiesterase (PDE4) inhibitors [61-63].

Analogs of thalidomide	Mechanism of action	Effect
IMiDs	- Inhibition of endothelial cell migration *in vitro* models. - Inhibition of VEGF, βFGF, TNF-α and IL-6 secreted by BMSC.	Anti-angiogenesis
	- Inhibition of endothelial cell integrins.	Downregulation of adhesion molecules
	- Downregulation of TNF-α from LPS-stimulated monocytes and IL-6. - Block of the activity of NFkb transcription factor.	Anti-inflammatory properties
SelCIDs	- inhibition of PDE4	Anti-inflammatory properties

Table 1. Thalidomide analogs

The structural development studies of thalidomide analogs are based on different activities: TNF-α production regulators, androgen antagonists, aminopeptidase inhibitors, α-glucosidase inhibitors, thymidine phosphorylase inhibitors, COX inhibitors and nitric oxide synthase (NOS) inhibitors [64]. In particular, IMiDs are potent stimulators of T cells and thus can be considered the promoters of the immune response to tumor cells. CC-5013 or lenalidomide, CC-4047 or pomalidomide (Fig. 3) and ENMD-0995 are representative compounds of this class.

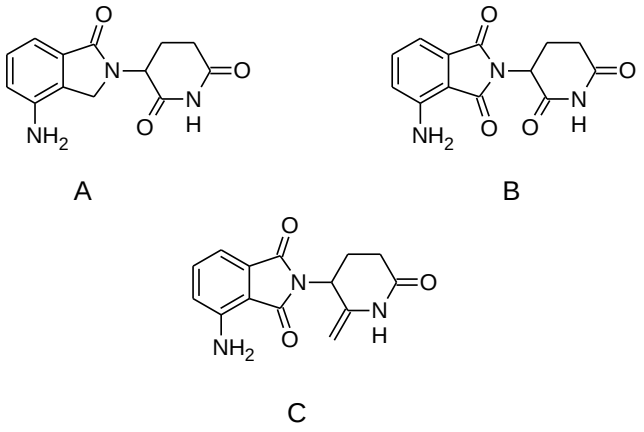


Figure 3. Structures of lenalidomide (A), pomalidomide (B) and ENMD-0995 (C).

Results for IMiDs are very interesting because of their anti-angiogenic activity which has been demonstrated *in vivo*. This activity is secondary to the inhibition of the secretion of angiogenic cytokines including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) from both tumor and stromal cells [65]. In addition, these compounds have been shown to have an inhibitory power against the endothelial cell migration and adhesion of these cells. Probably this is due to down-regulation of endothelial cells integrins [66]. For these reasons, the IMiDs are widely used for the treatment of MM; the treatment with IMiDs or thalidomide inhibited the up-regulation of VEGF and IL-6 from co-cultures of bone marrow stromal and MM cells [47]. SelCIDs were shown to potently inhibit PDE4, a phosphodiesterase isoenzyme found in human myeloid and lymphoid lineage cells [67] that functions to maintain cyclic adenosine monophosphate (cAMP) at low intracellular levels, resulting in modulation of LPS-induced cytokines [68]. For example, the TNF-α synthesis can be blocked increasing intracellular levels of cAMP. Normally, cAMP is converted into AMP through the action of PDE4. However, in the presence of PDE4 inhibitors, cAMP levels remain high, causing the activation of protein kinase A (PKA). Activation of PKA prevents transcription factors such as NF-kB from promoting transcription of the gene encoding TNF-α, thereby resulting in a decrease in TNF-α synthesis. Early reports showed that one of the SelCID analogs (SelCID-3) was consistently effective in reducing tumor cell viability in a variety of solid tumor lines [69]. In any case, this type of compounds seems to have an anti-inflammatory role.

Selective PDE4 inhibitors may be grouped into three broad classes: catechol esters (rolipram), bicyclic heteroaromatics (nitraquazone) and xanthine derivatives (denbufylline) [70]. The SelCID and IMiD analogs differ in terms of their effects on cytokine production after activation of either monocytes or T-cells [71]. Another study demonstrates that thalidomide and the IMiDs act directly on MM cells, in the absence of accessory bone marrow or T-cells. It is also possible that the anti-MM effect of these agents may be mediated by cytokines, given their known inhibitory effects on TNF-α, IL-1β and IL-6 [72,73]. In a previous paragraph, the attention was fixed on the binding of CRBN with thalidomide and its analogs. In the case of lenalidomide, when the interaction between this drug and CRBN occurs, the ubiquitination and degradation of the regulatory enzyme casein kinase 1α (Ck1-α) is induced. Ck1-α has a role in the biology of DEL(5q) MDS (Myelodysplastic syndrome) [74] and it is a therapeutic target in myeloid malignancies [75]. The activity of this drug on CRBN makes it more effective than other compounds in the treatment of MDS. All in, the powerful anticancer properties of these drugs mean that they are now emerging from thalidomide's shadow as useful anticancer agents in the treatment of MM.

CONCLUSION

This review is an attempt to summarize studies regarding the activity of thalidomide and its analogs in the treatment of MM. The return of this drug on the market was very significant because of its interesting anticancer properties. Thanks to this event, studies on the possible molecular targets for thalidomide are attracting growing interest of researchers. VEGF is one of the targets of greater interest. It is necessary to act on the bone marrow microenvironment and, in particular, on the process of angiogenesis that occurs in the bone marrow microenvironment during the progression of the tumor, rather than on the tumor cells themselves, in order to provide effective advantages and reach significant results. Thalidomide and its analogs could open new avenues and could help the discovery of new molecular targets. Understanding the precise mechanisms of action will help in

the rational design of new thalidomide analogs with beneficial activity in specific neoplasms and in optimizing their clinical applications.

LIST OF ABBREVIATIONS

bFGF = basic fibroblast growth factor

CAM-DR = cell adhesion mediated drug resistance

COX = cyclooxygenase

CRBN = cereblon

ENL = erythema nodosum leprosum

ERK = extracellular signal-regulated kinase

FDA = Food and Drug Administration

FGF = Fibroblast Growth Factor

IL-6 = interleukin-6

IMiDs = Immunomodulatory imide Drugs

INF-γ = interferon gamma

LPS = lipopolysaccharide

MAPK = Ras–mitogen-activated protein kinase

MM = Multiple myeloma

NF-κB = nuclear factor κB

PKA = protein kinase A

PI3K = Phosphoinositide 3-kinase

SelCIDs = Selective Cytokine Inhibitory Drugs

TNF-α = tumor necrosis factor α

VEGF = vascular endothelial growth factor

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