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ARTICLE

Thalidomide dithiocarbamate and dithioate derivatives induce apoptosis through inhibition of histone deacetylases and induction of caspases

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Dithioate analogs

Abstract Anti-cancer effect and mechanism of cell death were investigated in a battery of five thalidomide analogs containing one sulfur atom **2** or two sulfur atoms **3–6** and were compared with thalidomide **1** activity. The cytotoxic effect of thalidomide analogs **2–6** against Hep-G2, 1301, and HCT-116 cells was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Apoptosis and necrosis cell percentage was stained by ethidium bromide and acridine orange, DNA fragmentation, inhibition of histone deacetylase (HDAC), and total caspases were assayed by universal procedures and kits. We report here for the anti-cancer activity of thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** against Hep-G2 and HCT-116 cells, which was more cytotoxic than thalidomide itself, and that the cytotoxicity was associated with DNA fragmentation and was due to apoptosis and not necrosis. Moreover, we suggest that the cell death pathway is evoked by thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** in human hepatocellular carcinoma cells through multiple consequences that trigger apoptotic cell death; involving the enhancement of DNA fragmentation, the activation of caspases, and the induction of histone acetylation. In conclusion, thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** are promising anti-cancer agents more than thalidomide.

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1. Introduction

Thalidomide **1** has been introduced to the pharmaceutical market, as a quite successful sedative, in 1956. However, in 1961, it was withdrawn from the market due to its unsuspected teratogenic activity [1]. In the recent decade, thalidomide was re-evaluated and has attracted significant attention due to its selective inhibitory activity to tumor necrosis factor-α (TNF-α) [2], which is a clinically important activity against

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serious diseases such as rheumatoid arthritis, AIDS, leprosy, Crohn's disease, and various cancers [3]. Thus, in 1998, Thalidomide has received FDA approval for the treatment of erythema nodosum leprosum (ENL) [4]. A distinct and clear potent antitumor activity of a series of novel isosteric thalidomide analogs, which were designed and synthesized was revealed in our previous study. *N*-Methylthiomethyl thalidomide **2**, thalidomide dithiocarbamate analogs **3**, **4** and **6** and thalidomide dithioate analog **5** were the most potent antitumor analogs compared to thalidomide itself [5,6].

The four selected thalidomide dithiocarbamate and dithioate analogs **3**, **4**, **5**, and **6** possessed a high significant reduction in tumor volume (T.V.), antimitotic, apoptotic and necrotic activities against solid tumor [5,6]. The antioxidative activity of these compounds as the level of hepatic lipid peroxidation was decreased and levels of antioxidant enzymes like superoxide dismutase (SOD) and catalase were elevated. The selected analogs also demonstrated proapoptotic activity by progressive increase of Fas-L immunostaining expression compared with tumors treated with thalidomide as well as the untreated one. Moreover, the selected analogs showed a reduction in Ki67 protein and vascular endothelial growth factor (VEGF) staining in tumor cells from treated-animals [5,6].

Despite their encouraging and promising results, their mode of actions is still indistinct. Drawing inspiration from these results, we extend our previous work for better understanding of the mechanisms of action of these novel compounds shown in Fig. 1 and more investigation on the effect of increasing the sulfur content in the thalidomide analogs as antitumor compounds.

2. Materials and methods

2.1. Cell culture

Three human cell lines were used in testing anti-cancer activity including: lymphoblastic leukemia (1301 cells, T-lymphocytes), a generous gift from The Training Center of DakoCytomation, Elly, UK), hepatocellular carcinoma (Hep-G2) and colon carcinoma (HCT-116) (ATCC, VA, USA). Cells were routinely cultured at 37 °C in humidified air containing 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium), which was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate, 2 mM L-glutamine and 250 ng/ml amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment. All culture material was obtained from Cambrex BioScience (Copenhagen, Denmark), and all chemicals were from Sigma (USA). The tested compounds were dissolved in dimethyl sulfoxide (DMSO, 99.9%, HPLC grade) and then diluted into 1000-fold for experiments. In all cellular experiments, results were compared with DMSO-treated cells. Compound dilutions were endotoxin free as examined by endotoxin using Pyrogent® Ultra gel clot assay. All experiments were repeated four times, unless mentioned, and the data were represented as (mean ± S.D.).

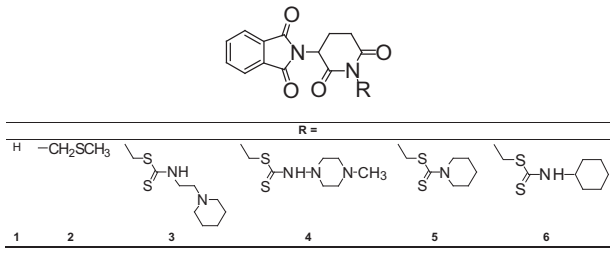

Figure 1 Thalidomide **1** and its sulfur analogs **2**–**6**.

2.2. Cytotoxicity assay

The cytotoxic effect of thalidomide analogs **2**–**6** against Hep-G2, 1301, and HCT-116 cells was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [7]. In metabolically active cells, MTT (a yellow tetrazolium salt) is reduced by mitochondrial dehydrogenases into insoluble purple formazan crystals that were solubilized by the addition of a detergent [8]. Cells (5×10^4 cells/well) were incubated with various concentrations of the compound at 37 °C in a FBS-free medium, before submitting to MTT assay. The absorbance was measured with an ELISA reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth inhibitory concentration IC_{50} values were estimated from the line equation of the dose–dependent curve of each compound.

2.3. Apoptosis and necrosis staining

The type of the cell death in Hep-G2 cells was investigated in the treated and untreated cells using acridine orange/ethidium bromide staining. In brief, the cells were plated at glass slides and treated with IC_{50} of each compound and incubated for 12 h. A mixture of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide was prepared in PBS. The cell uptake of the stain was monitored under a fluorescence microscope, and the apoptotic, necrotic, and viable cells were counted [9,10].

2.4. DNA fragmentation

Hep-G2 cells were treated with 30% of IC_{50} of each compound for 24 h. DNA fragmentation was essentially assayed as reported previously [11]. Briefly, the pellets of the treated and untreated Hep-G2 cells were re-suspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48 °C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000*g*. Pellets were re-suspended in 500 μl TE-buffer and samples were precipitated by adding 500 μl of 10% trichloroacetic acid at 48 °C. Samples were pelleted at 4000 rpm for 10 min and the supernatant was removed. After addition of 300 μl of 5% trichloroacetic acid, samples were boiled for 15 min. DNA contents were quantified using the diphenylamine reagent [9]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet [12].

2.5. Histone deacetylase activity

The activity of HDAC was measured in Hep-G2 cells, using a colorimetric assay kit (BioVision, Mountain View, kit No. K331-100). Cells were treated with 30% of IC_{50} of each compound for 24 h. The procedure involves the use of the HDAC colorimetric substrate (Boc-Lys(Ac)-pNA), which comprises an acetylated lysine side chain and is incubated with a sample containing nuclear extract. Deactivation sensitizes the substrate, and treatment with the lysine developer produces a chromophore, which can be analyzed using a colorimetric plate reader. HeLa cell nuclear extract was used as a positive control. A standard curve was prepared using the known amount of the deacetylated standard (Boc-Lys-pNA) included in the kit [13]. A similar volume of control sample was added to 100 ng/ml trichostatin A (TSA), as a known inhibitor of HDAC activity.

2.6. Evaluation of caspases activity

Hep-G2 cells were treated with 30% of IC_{50} of each compound for different time points. The lysates of the treated and untreated Hep-G2 cells were submitted to total caspases kit according to the manufacturer's instructions. Red Multi-Caspase Staining Kit (#PK-CA577-K190), PromoKine, Heidelberg, Germany was used for an analysis of total caspases in a black microtiter plate with fluorescence plate reader at Ex. = 540 nm and Em. = 570 nm. The assay utilizes the caspase family inhibitor VAD-FMK conjugated to sulfo-rhodamine (Red-VAD-FMK) as the fluorescent in situ marker. A comparison between the fluorescence readings of the treated sample and the untreated cells allows determination of the fold increase in the total caspases.

2.7. Statistical analysis

All values were expressed at the mean ± S.D. of four measurements. Data were statistically analyzed using Statistical Package for Social Scientists (SPSS) 10.00 for windows (SPSS Inc., Chicago, USA). Student's unpaired *t*-test as well as a one-way analysis of variance (ANOVA) test was used to detect statistical significance. *P* value more than 0.05 was considered insignificant.

3. Results and discussion

Exploring the cytotoxic effect of different doses of thalidomide analogs **2**–**6** on the solid tumor cell lines Hep-G2 and HCT-116 and the hematopoietic tumor cell line 1301 using MTT assay, a metabolic cytotoxicity assay, indicated that all tested compounds possessed variable degrees of cytotoxicity against Hep-G2 and HCT-116 cells, however thalidomide and its analogs **3** and **5** exhibited a high cytotoxic effect against both cell lines (IC_{50} < 20 μg/ml) and thalidomide dithiocarbamate analog **5** was the most cytotoxic compound as concluded from its low IC_{50} values as shown in Fig. 2A. On the other hand, submission of hematopoietic tumor cells; 1301 cells, to MTT assay revealed that only thalidomide and its analogs **3** and **5** had cytotoxic effect in the following order **3** > **1** > **5**, as concluded from their IC_{50} values (Fig. 2A, while thalidomide analogs **2**, **4**, and **6** induced the proliferation of 1301 cells (T-lymphocytes)

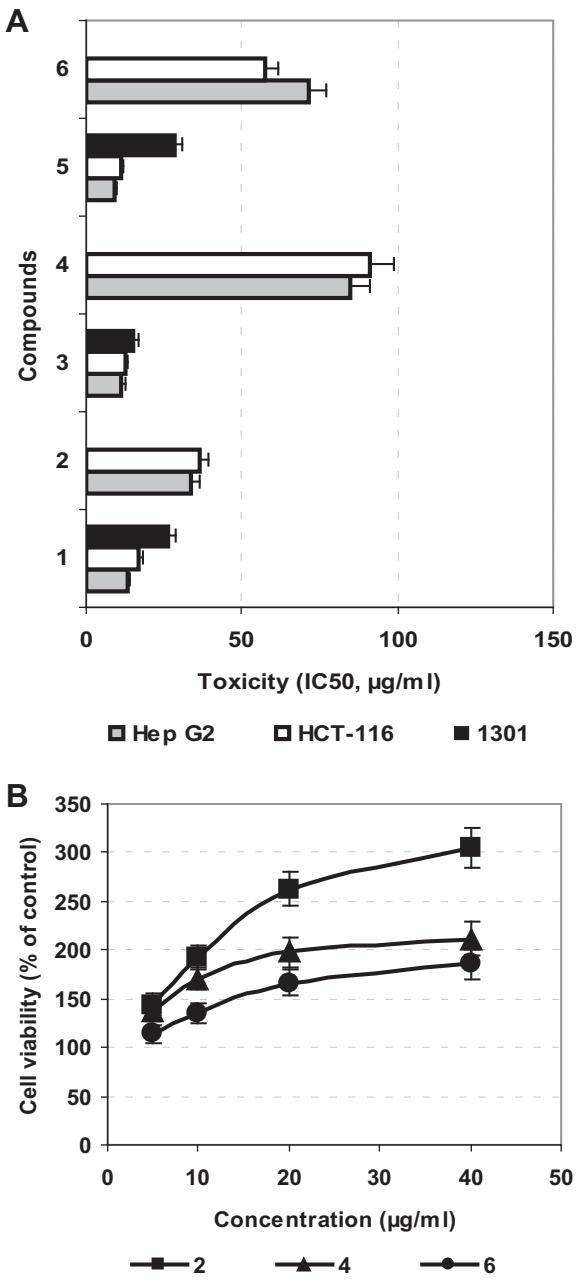

Figure 2 (A) The cytotoxic effect of thalidomide and its analogs **2**–**6** on different human cancer cell lines including Hep-G2 cells (gray bars), HCT-116 cells (white bars), and 1301 cells (black bars), as measured by MTT assay. The results are represented as IC_{50} values (μg/ml, mean ± S.D., $n = 4$). (B) The proliferative effect of thalidomide analogs **2**, **4**, and **6** on 1301 cells (T-lymphocytes), as measured by MTT assay. The results are represented as the number of folds of control (mean ± S.D., $n = 4$).

in a dose dependant manner up to 3.05-folds of control at a dose of 40 μg/ml for methylthiomethylthalidomide **2** (Fig. 2B). This finding suggested that thalidomide analogs **2**, **4**, and **6** are immunostimulant agents that can drastically induce T-lymphocyte proliferation; a suggestion that needs to be explored further. The original chemical structure of thalidomide is known as a selective agent that kills cancer cells, but induces T cell proliferation [14]. Sun et al. [15], indicated

that thalidomide can inhibit the proliferation of the human hepatoma SMMC-7721 cells in vitro. They suggested from their findings that the induction of apoptosis and inhibition of angiogenesis may possibly be two mechanisms for its anti-cancer action [15].

A previous in vitro study also revealed the thalidomide embryotoxicity in the embryonic stem cell test based on human induced pluripotent stem cells [16], while due to the known cell specificity of thalidomide, another study reported that the treatment with 10 μg/ml thalidomide to human myeloid KBM-5 cells for 24 h resulted in only 7% of cell viability inhibition [17]. Wang et al. [18], prepared 5 nitric oxide-donating thalidomide analogs that exhibited a cytotoxic effect against three human tumor cell lines (HepG2, lung cancer A549 cells, and prostate cancer PC-3 cells) stronger than that of 5-fluorouracil. They noticed that this cytotoxic activity is closely related to the type of NO donors, and the length of the spacers connecting to NO donors.

Cell death is generally classified into two large categories: apoptosis, representing "active" programed cell death, and necrosis, representing "passive" cell death without (known) underlying regulatory mechanisms, both are distinguished by well defined morphological and biochemical features. Necrosis is characterized by cell swelling, disruption and rapid disintegration of the cell membrane [19]. In contrast, during apoptosis the cells undergo nuclear and cytoplasmic shrinkage, the chromatin is condensed and fragmented, and the cells are finally broken into multiple membrane-surrounded bodies (apoptotic bodies) [20]. In the consequences of the high cytotoxic effect of the compounds against Hep-G2 cells, we find that the degree of DNA fragmentation, which was assayed by diphenylamine was induced dramatically by most of the tested compounds (Fig. 3A), thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** were the most DNA damaging agents (Fig. 3A).

To investigate the type of cell death (apoptosis or necrosis) induced in Hep-G2 cells after treating with the cytotoxic thalidomide and its analogs **2**, **3**, and **5**, we used acridine orange/ethidium bromide staining to distinguish between apoptotic, necrotic, and viable cells. The counting indicated that in untreated cells the range of apoptotic and necrotic cell number was 4–8%, and 1–4%, respectively. On the other hand, the test compounds led to a significant dose dependant induction of apoptosis ($P < 0.01$–0.001), where the cells had mainly condensed chromatin (Fig. 3B) and there was a low population percentage of the necrotic cells (Fig. 3B). Sun et al. [15], reported that thalidomide treatment resulted in a ladder pattern of DNA fragments in the human hepatoma SMMC-7721 cells and the cell nuclei were condensed and fragmented.

Gene transcription from DNA, which is tightly bound to the nucleosome core (histone proteins), is suppressed due to decreased accessibility of transcription factors (e.g. NF-κB) to their DNA binding motifs [21]. Acetylation of lysine residues in the N-terminal tails of the core histones results in uncoiling of the DNA, allowing increased accessibility for transcription factor binding [22]. The dynamic process of acetylation is reversible and is regulated by two families of proteins: the histone acetyltransferase and the HDAC [21]. HDACs remove the acetyl moieties from the ε-acetamido groups of lysine residues of histones, causing rewinding of DNA and hence silencing gene transcription. It has been reported that HDACs play an essential role in the regulation of cell proliferation and apoptosis [23].

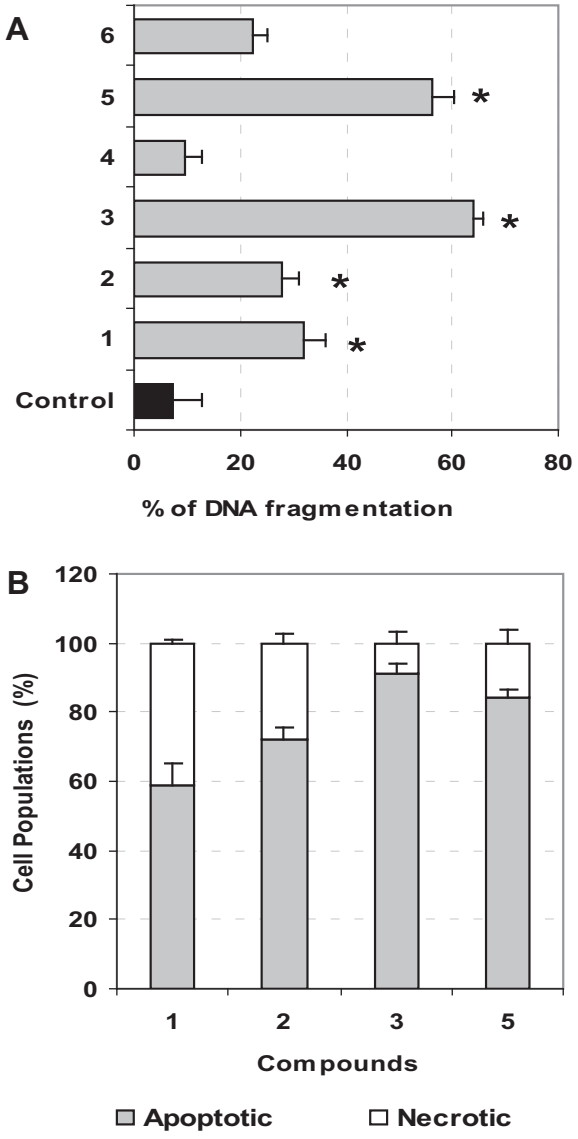

Figure 3 (A) The effect of thalidomide and its analogs **2**–**6** on the % of DNA fragmentation in Hep-G2 cells, compared with vehicle-treated cells. The amount of fragmented DNA was determined with the diphenylamine reaction (mean ± S.D., $n = 4$). (B) Analysis of cell death type (apoptosis and necrosis) in Hep-G2 cells treated with the cytotoxic thalidomide and its analogs **2**, **3**, and **5** as monitored by ethidium bromide/acridine orange staining (mean ± S.D., $n = 4$). The percentage was calculated among the total dead cells population only (the living cells were excluded).

To determine if decreased deacetylation is involved in net increased DNA fragmentation and apoptosis in thalidomide analog-treated Hep-G2 cells, we measured HDAC activity using the substrate Boc-Lys(Ac)-pNA and a colorimetric detection. TSA was used as a known inhibitor of HDAC activity. TSA addition to the control inhibited 53.9% of HDAC activity (Fig. 4A), while the treatment with different compounds led to a drastic decline in HDAC activity to the following order **5** > **3** > **1** > **2** > **6** > **4** ($P < 0.05$–0.001) of the control activity (Fig. 4A), which means that there is an induction of the histone acetylation. It is known that acetylation of

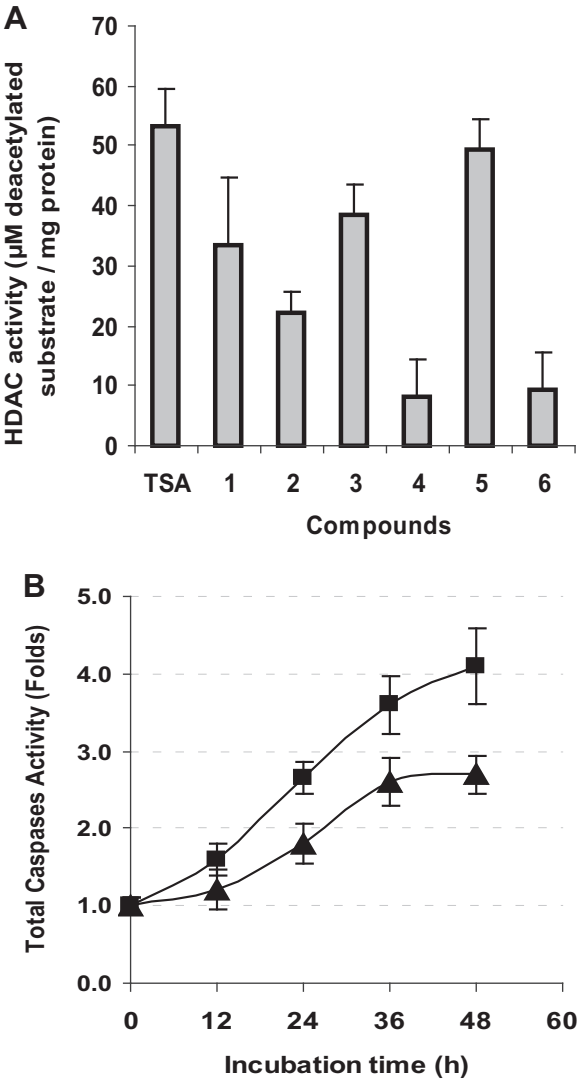

Figure 4 (A) The inhibitory effect of thalidomide and its analogs **2**–**6** against HDAC activity of Hep-G2 cells. The data were compared with the HDAC activity of the control cells pellet that was treated with TSA (100 ng/ml), as a known inhibitor. The results are represented as the percentage of inhibition of HDAC (mean ± S.D., $n = 4$). (B) Effect of thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** on the activity of total caspases activity of Hep-G2 cells after different incubation times. Data are represented as folds of control (mean ± S.D., $n = 4$).

the histones that form the nucleosome core around which DNA is coiled may cause unwinding of condensed chromatin [24]. Decondensation of the chromatin allows access of transcription factors to consensus sites on DNA, leading to the transcription of inflammatory mediators thereby enhancing the inflammatory response [25,26].

The HDAC inhibitors are classified as second-generation proteasome inhibitors such as heat shock protein 90 inhibitors, 2-methoxyestradiol, histone deacetylase, fibroblast growth factor receptor 3 inhibitors, insulin-like growth factor 1 receptor inhibitors, that all can treat multiple myeloma [27]. A previous study reported that a series of hydroxamic acid derivatives bearing a cyclic amide/imide group as a linker and/or cap structure, prepared during structural development studies based on thalidomide showed class-selective potent HDAC-inhibitory activity [28]. They also reported that the structure–activity relationship studies revealed that the steric character of the substituent introduced at the cyclic amide/imide nitrogen atom, the presence of the amide/imide carbonyl group, the hydroxamic acid structure, the shape of the linking group, and the distance between the zinc-binding hydroxamic acid group and the cap structure are all important for HDAC-inhibitory activity and class selectivity [28]. Podhorecka et al. [29], reported that thalidomide led to a modest increase in the phosphorylation of histone H2AX on Ser139 (gammaH2AX), reporter of DNA damage, but they found that thalidomide did not affect the expression of activated caspase-3 in ex vivo short-term cell cultures of peripheral blood cells from newly diagnosed untreated patients.

Caspases are cysteine proteases produced as inactive zymogens that cleave their substrates at aspartic acid residues contained within a tetrapeptide recognition motif. Activation of initiator caspases (procaspase-8, -9 and -10) leads to the proteolytic activation of downstream effector caspases (caspase-3, -6 and -7) that cleave specific substrates [30]. DNA fragmentation is due to cleavage and inactivation of ICAD, the initiator of CAD (caspase-activated DNase). In addition, the activation of several kinases by caspase cleavage contributes to the membrane remodeling and active blebbing observed in apoptotic cells [31]. Due to promising activities of thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** as cytotoxic, DNA fragmentation and apoptosis inducers and HDAC inhibitors we investigated the possible involvement of caspase activation of these activities, total caspase was assayed through time and revealed that there was a significant time-dependent increase in the caspase level in Hep-G2 cells ($P < 0.01$), when treated with either of thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** (Fig. 4B). Accordingly, the induced-apoptosis is associated with an induced activation of the net caspases.

The thalidomide analogs **3**, **4**, **5** and **6**, containing two sulfur atoms, are more potent than the *N*-methylthiomethyl thalidomide **2** with only one sulfur atom, which means that the increase in the sulfur content, enhances the anti-tumor activity of these analogs. On the other hand, thalidomide analogs **3** and **5** are more potent than **4** and **6**. On structural bases both thalidomide analogs **3** and **5** are almost identical except the CH_2–CH_2 linker in dithiocarbamate **3**, which links the dithiocarbamate group and the pipridinyl moiety, but in dithioate analog **5** the dithio ester group is directly connected to the pipridinyl moiety. This suggests that the activity might refer to the efficacious chelation of dithiocarbamate **3** (Thalidomide–CH_2–S–CS–NH–CH_2–CH_2–N pipridinyl moiety) compared with the chelation of dithioate **5** (Thalidomide–CH_2–S–CS–N pipridinyl moiety). Thus, it is clear that the chelation efficiency increases through the binding capability between the DNA and the dithiocarbamate group in analog **3** rather than the dithioate in analog **5** due to the presence of hydrogen atom of the imine nitrogen that belongs to the dithiocarbamate group.

As reported recently, treatment with thalidomide resulted in the apoptosis of mouse breast cancer cells in a time- and dose-dependent manner as demonstrated by caspase-3 enzyme activity [32]. Sun et al. [15], reported that thalidomide treatment led to an elevation of the apoptosis rate and to a dose-dependent induction in caspase-3 in the human hepatoma SMMC-7721 cells. On the other hand, Keller et al. [33], reported that thalid-

omide inhibited the activity and the activation of caspase-1, which is required for unconventional protein secretion of pro-inflammatory cytokines such as IL-1 and the proangiogenic fibroblast growth factor 2. The authors suggested that the latter finding implicated that the pharmacological activity is exerted by a metabolite of thalidomide [33]. Further investigation, designing and synthesis of a novel thalidomide as well as phthalimide dithiocarbamate analogs are now under consideration in order to reveal the mechanism of action and understanding the role of each part (phthalimide part and glutarimide part) constituting the thalidomide molecule.

Taken together, we report here the anti-cancer activity of thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** against Hep-G2 and HCT-116 cells, which was more cytotoxic than thalidomide itself and that cytotoxicity was associated with DNA fragmentation and was due to apoptosis and not necrosis. Moreover, we suggest that the cell death pathway is evoked by thalidomide dithiocarbamate analog **3** and thalidomide dithioate analog **5** in human hepatocellular carcinoma cells through multiple consequences that trigger apoptotic cell death; involving the enhancement of DNA fragmentation, the activation of caspases, and the induction of histone acetylation.

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