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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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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 However, in 1961, it was withdrawn from the market due to its unsuspected teratogenic activity [1].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.).

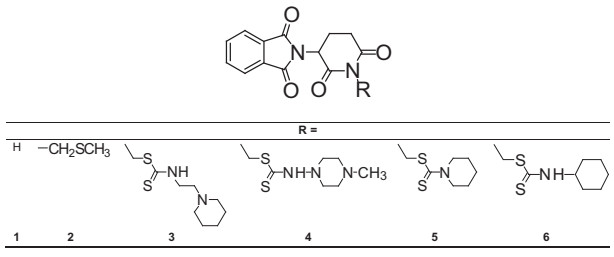

Figure 1 Thalidomide **1** and its sulfur analogs **2**–**6**.

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].