

Design and biological characterization of hybrid compounds of curcumin and thalidomide for multiple myeloma†

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In our efforts to develop effective treatment agents for human multiple myeloma (MM), a series of hybrid molecules based on the structures of thalidomide (**1**) and curcumin (**2**) were designed, synthesized, and biologically characterized in human multiple myeloma MM1S, RPMI8226, U266 cells, and human lung cancer A549 cells. The biological results showed that two hybrid compounds, **5** and **7**, exhibited significantly improved lethal effects towards all three human MM cell models compared to **1** or **2** alone, as well as the combination of **1** and **2**. Furthermore, mechanistic studies in U266 cells demonstrated that **5** and **7** can induce the production of reactive oxygen species (ROS) and cause G1/S arrest, thus leading to apoptosis and cell death. Additionally, they exhibited inhibitory effects on NFκB activation in A549 cells. Collectively, the results obtained from these hybrid compounds strongly encourage their further optimization as new leads to develop effective treatment agents for human MM.

Multiple myeloma (MM), an incurable plasma cell malignancy, is characterized by disordered growth of terminally differentiated plasma cells and is the second most common hematopoietic cancer.^{1,2} In the United States, 50 000 patients are currently affected by MM. Approximately 10 000 of these patients die each year.³ With the advances in understanding the molecular mechanisms of MM, novel agents with more defined molecular targets have been successfully developed and added to the armamentarium for treatment of MM, such as immunomodulatory drugs (IMiDs) including thalidomide (**1**, Fig. 1) and proteasome inhibitors.^{4,5} **1** was first introduced

to control morning sickness during pregnancy in the 1950s, but was withdrawn due to severe teratogenicity.^{6,7} **1** has been recently reintroduced as an effective MM treatment either as a stand-alone therapy or in combination with other chemotherapeutic agents, due to its activities in modulating the immune system and disturbing the microenvironment of tumor cell growth in bone marrow.^{8–11} Although the response rate and patient survival of MM patients have significantly improved upon treatment with **1**, certain toxicities are associated with it, such as venous thromboembolism,¹² that need to be managed. Curcumin (**2**, Fig. 1), a yellow spice and pigment isolated from the rhizome of *Curcuma longa*, has been traditionally used as a food coloring additive.¹³ Recently, **2** has attracted extensive interest as both a preventive and treatment agent in a variety of disease models, such as arthritis, cancer, and Alzheimer's disease (AD).^{14–18} Notably, **2** has been shown to exhibit cytotoxic effects in MM models and to potentiate the effects of other therapeutic agents, including **1**, in MM disease models.¹⁰

Recently, the “hybrid molecule” strategy has drawn increased attention in drug design and development.^{19–22} Conceptually, this strategy incorporates structural features that are essential to the biological activities of different drug structures into a single molecule. Compared to the combination of multiple drugs, hybrid molecules may provide certain advantages: (1) enhanced potency by self-synergy within one molecule that may not be achievable by a traditional combination of separately dosed agents that may miss the ideal timing window; (2) reduced risk of developing drug resistance; and (3) improved pharmacokinetic properties and reduced toxic side effects compared to the administration of multiple agents. Additionally, such hybrid molecules may provide the advantages of reduced cost and improved patient compliance, which are sometimes as significant as drug resistance and toxicity. Herein, we report the design and biological characterization of a series of hybrid compounds of **1** and **2** as potential treatment agents for MM.

Since the phenolic oxygen of **2** can be modified without a significant influence on its biological activity, compound **3**

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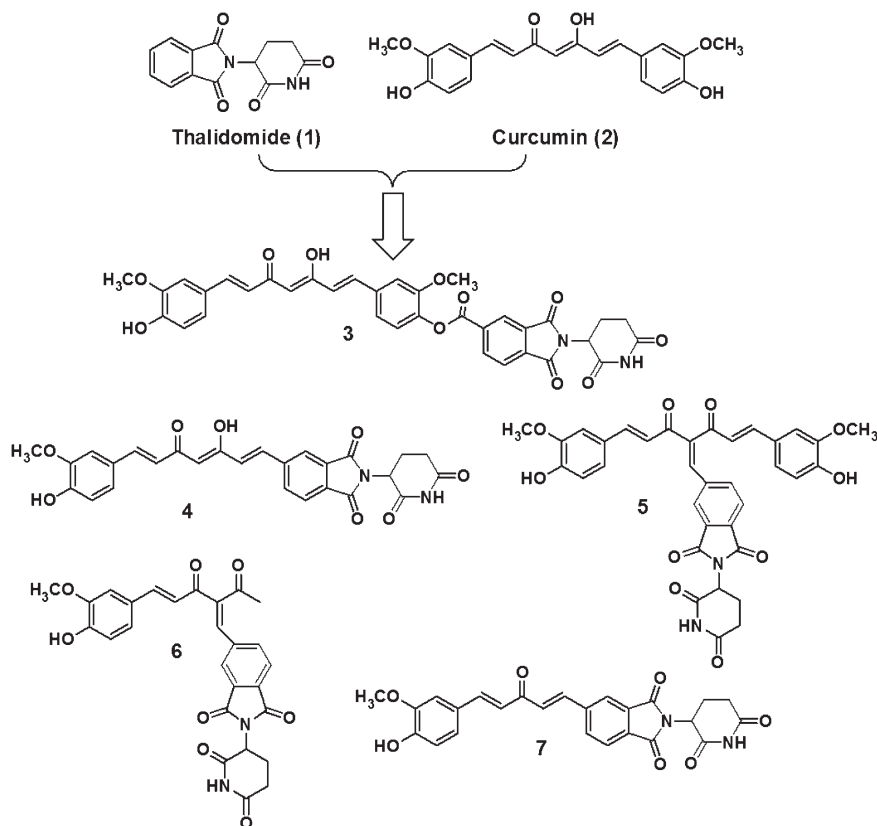


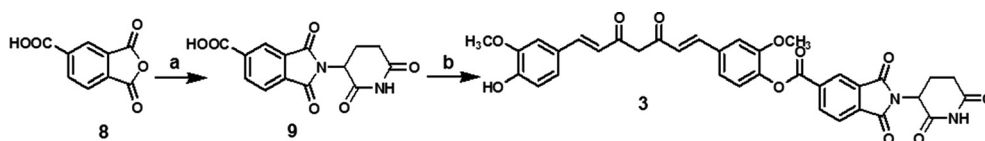
Fig. 1 Molecular design of curcumin–thalidomide hybrids.

was therefore designed to simply link **1** and **2** into one molecule through an ester linkage (Fig. 1). Hybrid **4** was designed using the phthalimide moiety of **1** to replace one of the 4-hydroxy-3-methoxy-phenyl rings of **2**. Hybrid **5** was constructed to incorporate the structural features of **1** and **2** through a benzylidene connection at the methylene position between the two carbonyls of **2**. The results from **4** and **5** will shed light on optimizing the positions for hybridization, as well as on whether both of the 4-hydroxy-3-methoxy-phenyl rings of **2** are needed for activity. Compound **6** will further confirm the importance of the 4-hydroxy-3-methoxy-phenyl rings of **2** on biological activity. Recently, a mono-ketone analog of **2** has been designed and shown to be more potent than **2**.²³ Therefore, **7** was designed to evaluate whether a mono-ketone hybrid will produce improved biological activities.

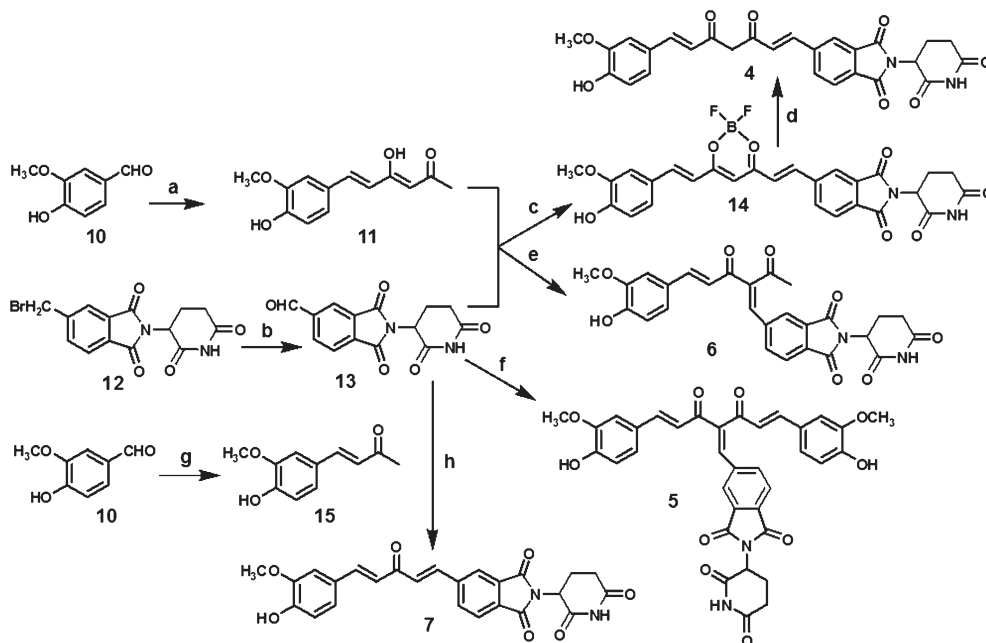
The syntheses of the designed compounds were achieved through the conditions listed in Schemes 1 and 2. Briefly, thalidomide carboxylic acid **9** was prepared from 1,2,4-tribenzene-carboxylic anhydride **8** by following reported procedures.²⁵

The coupling reaction of **9** with **2** in the presence of EDC in dichloromethane afforded compound **3** (Scheme 1). The bromide intermediate **12**, prepared by following a reported method,²⁴ was oxidized by IBX to afford the desired aldehyde **13**. To prepare compound **4**, intermediate **11** prepared from vanillin **10** was first reacted with $\text{BF}_3 \cdot \text{Et}_2\text{O}$ to form a complex, which was condensed with aldehyde **13** under Pabon reaction conditions to yield **14**. Refluxing of **14** in the mixture of MeOH and DMSO afforded **4**. The condensation reaction of aldehyde **13** with **11**, catalyzed by acetic acid and morpholine under microwave irradiation, yielded compound **6** as the major product. In the presence of piperidine and tributylborate, condensation of aldehyde **13** with curcumin **2** afforded **5**. Compound **7** was prepared by condensation of aldehyde **13** with **15**, which was prepared from vanillin **10** and acetone, in the presence of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in dioxane.²⁶

After synthesis, we initially tested these compounds in human MM1S, U266, and RPMI8226 cells at a single concentration of 10 μM to confirm their cytotoxic effects. **1** and **2**



Scheme 1 Reagents and conditions: (a) ref. 25; (b) curcumin, EDC, DMAP, DCM, 46%.



Scheme 2 Reagents and conditions: (a) i. B_2O_3 , acetylacetone, EtOAc, ii. vanillin, $(\text{BuO})_3\text{B}$, $n\text{BuNH}_2$, iii. 1 M HCl, 60%; (b) IBX, DMSO, 90%; (c) i. $\text{BF}_3\cdot\text{Et}_2\text{O}$, EtOAc, ii. aldehyde **13**, $(\text{BuO})_3\text{B}$, $n\text{BuNH}_2$, EtOAc, 39%; (d) MeOH, DMSO, 22%; (e) aldehyde **13**, B_2O_3 , AcOH, morpholine, microwave 200 W, 5 min, 25%; (f) curcumin, $(\text{BuO})_3\text{B}$, piperidine, EtOAc, 10%; (g) acetone, NaOH, 87%; (h) compound **15**, $\text{BF}_3\cdot\text{Et}_2\text{O}$, dioxane, 25%.

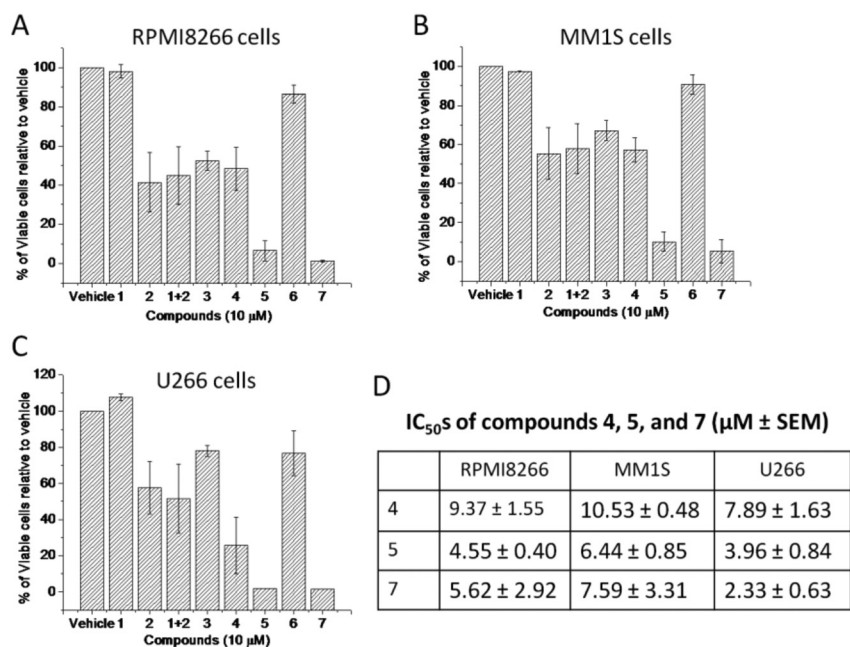


Fig. 2 Antiproliferative activity of hybrid molecules. (A) RPMI8226 cells, (B) MM1S cells, (C) U266 cells were treated with indicated compounds at $10 \mu\text{M}$ for 48 h and cell viability was analyzed by MTT (data are expressed as mean percentage viability with vehicle control set at 100% viability. Error bars represent SEM from three independent experiments and at least each data point was tested in triplicate in each experiment); (D) IC_{50}s (calculated based on the four parameter dose–response analysis function in Prism to fit five doses of 0.3, 1, 3, 10, $30 \mu\text{M}$ and corresponding viability) of indicated compounds in RPMI8226, MM1S, and U266 cells.

alone and the combination of **1** and **2** were compared as controls. As shown in Fig. 2A–2C, **2** ($10 \mu\text{M}$) decreased the cell growth of MM1S, U266, and RPMI8226 cells by 45.0%, 42.5%, and 58.6% respectively. However, **1** did not exhibit antiproliferative activity at this concentration by itself. This is

consistent with the literature report that a minimum of $50 \mu\text{M}$ concentration is needed for **1** to exhibit significant cytotoxic effects in MM cells.⁴ The combination of **1** and **2** exhibited comparable anti-proliferative activity as **2** alone. The ester analog **3** ($10 \mu\text{M}$) decreased the cell viability of MM1S, U266,

and RPMI8226 cells by 32.8%, 21.8%, and 47.5%, respectively, *i.e.*, is comparable to **2**. Compound **4** also showed comparable antiproliferative activities against MM1S, RPMI8226, and U266 cells to **2**. This may suggest that replacement of one of the 4-hydroxy-3-methoxy-phenyl rings of **2** with the phthalimide moiety can retain antiproliferative activity. Notably, compound **5** significantly inhibited the cell growth of all three cell lines by $\geq 90\%$ at $10\ \mu\text{M}$. On the other hand, **6** exhibited no significant activity against all three cell lines at this concentration, thus indicating that the 4-hydroxy-3-methoxy-benzylidene moiety is essential to the antiproliferative activity of **5**. The mono-ketone hybrid **7** also significantly inhibited the growth of all three cell lines compared to **5**. With these results in hand, we then tested **5** and **7** against MM1S, RPMI8226, and U266 cells in dose-response studies to obtain potency information. As shown in Fig. 2D, **5** exhibited IC_{50}s of 6.44 ± 0.85 , 4.55 ± 0.40 , and $3.96 \pm 0.84\ \mu\text{M}$ for MM1S, RPMI8226, and U266 cells, respectively, and **7** exhibited IC_{50}s of 7.59 ± 3.31 , 5.62 ± 2.92 , and $2.33 \pm 0.63\ \mu\text{M}$ for MM1S, RPMI8226, and U266 cells, respectively. In general, U266 cells are more sensitive to these hybrid compounds; therefore, U266 cells were employed for the following mechanistic studies.

Reactive oxygen species (ROS) can be double-edged swords, depending upon their concentrations and cellular localizations.^{27–30} Many anticancer agents, including **2**, have been demonstrated to exert their cytotoxic effects through the generation of ROS.³¹ Therefore, we decided to investigate whether **5** and **7** induce the production of ROS that could potentially lead to the cytotoxic effects observed in U266 cells. As shown in Fig. 3, **2** dose-dependently increased ROS production at 1 h treatment, and the level of ROS is reduced after longer exposure. Notably, **5** significantly increased the production of ROS at 1 h treatment in U266 cells at both 3 and $10\ \mu\text{M}$ concentrations (1.6 and 2.2 fold increase, respectively). Compound **7** also significantly increased the production of ROS at 1 h treatment in U266 cells at both 3 and $10\ \mu\text{M}$ concentrations. Interestingly, ROS induction by **7** at $3\ \mu\text{M}$ is stronger than that at $10\ \mu\text{M}$ at this time point (1.9 and 1.7 fold

increase, respectively). This dose-independent mode of action in ROS induction is possibly due to the unique mono-ketone structure of **7** and the antioxidant effects of the phenol group in this structure at higher concentrations, but further studies are warranted to better understand such a dose-independent nature. The ROS level steadily decreased with the increase of exposure time for both compounds, even to a level that is lower than the control after 6 h treatment. ROS have also been demonstrated to induce apoptosis or necrosis in many cancer cell models.^{27,30} Since our compounds induced significant oxidative stress in U266 cells, we next investigated whether **5** and **7** can induce apoptosis in U266 cells to investigate the molecular mechanisms of their cytotoxicity. As demonstrated in Fig. 3, because the ROS level decreased to normal or even below control level after 6 h treatment, we decided to analyze the apoptotic effects of **5** and **7** at 4, 6 and 24 h time points and to see whether there is any association between ROS production and induction of apoptosis if apoptotic effects are observed. As shown in Fig. 4, **5** and **7** induced mainly early apoptosis at $10\ \mu\text{M}$ concentrations after 6 h treatment (26.7% and 10.4% increase, respectively), but apoptosis was only slightly induced at $3\ \mu\text{M}$ concentrations for this time course. No apoptotic effects were observed at 4 h treatment (data not shown). Prolonged treatment (24 h) induced significant apoptosis by both compounds at both $3\ \mu\text{M}$ and $10\ \mu\text{M}$ concentrations (14.8% and 79.2% increase for **5**; 9.1% and 54.1% increase for **7**). Comparing the time course of ROS production and apoptosis induction, it may suggest that ROS production triggers the apoptosis of U266 cells upon treatment with compounds **5** and **7**. To further confirm the association between ROS production and apoptosis, an *N*-acetyl-cysteine (NAC, an ROS scavenger agent) rescue experiment was performed. As shown in Fig. 5, NAC (8 mM) did not induce apoptosis by itself; however, it completely blocked the apoptotic effects induced by **5** and **7** at this concentration, thus demonstrating that ROS production is a direct cause of **5**- or **7**-mediated apoptotic cell death of U266 cells.

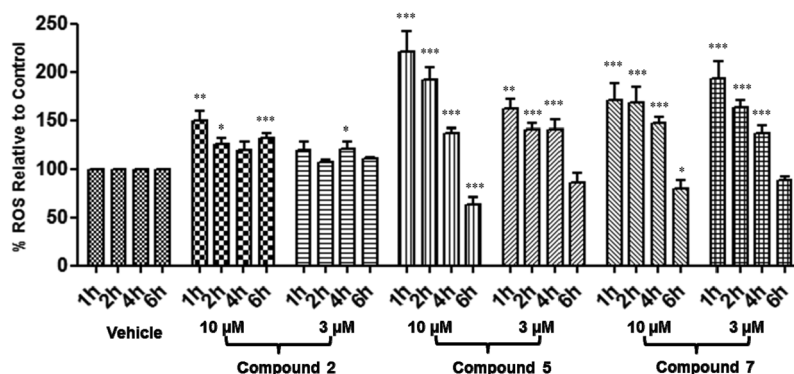


Fig. 3 ROS production in U266 cells. U266 cells were treated with indicated compounds at indicated concentrations for indicated intervals, then DCFH-DA ($10\ \mu\text{M}$) was loaded and fluorescence intensity was analyzed by flow cytometry. Data are expressed as mean percentage of ROS production with vehicle control set at 100% from three independent experiments (at least each data point was tested in triplicate in each experiment). Error bars represent SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. vehicle.

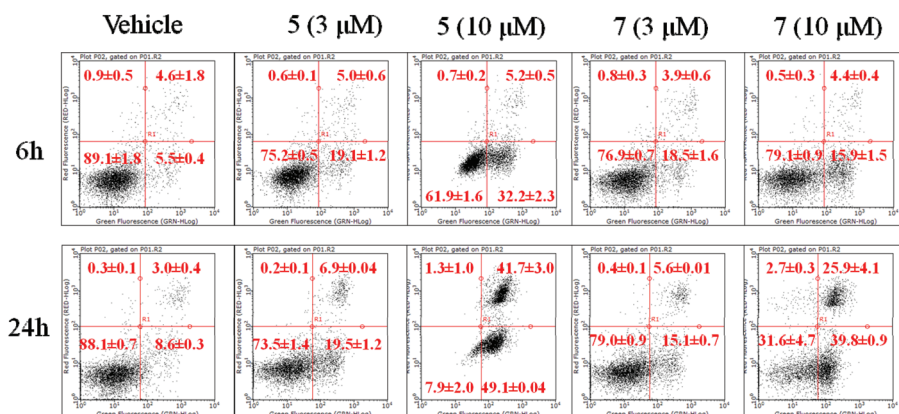


Fig. 4 Apoptotic effects of **5** and **7** on U266 cells. U266 cells were treated with indicated compounds at indicated concentrations for 6 h and 24 h, and then the cells were stained with Annexin V/PI and analyzed by flow cytometry. Data are expressed as mean value \pm SEM from two independent experiments.

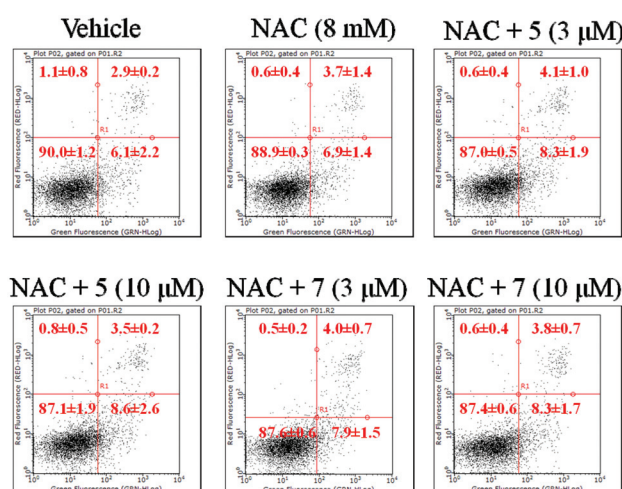


Fig. 5 Protection of U266 cells from **5**- and **7**-induced apoptosis by NAC. U266 cells were treated with NAC (8 mM) and indicated compounds for 24 h, and then cells were stained with Annexin V/PI and analyzed by flow cytometry. Data are expressed as mean value \pm SEM from two independent experiments.

ROS have also been implicated as having an important role in the progression from G₁ to S phase.³² Furthermore, **2** has been reported to induce a G₁/S arrest of cancer cells,^{33,34} and **1** to interfere with the cell cycle of MM cells.³⁵ Therefore, we determined the effects of **5** and **7** on the cell cycle of U266 cells. As shown in Fig. 6A, treatment with **5** or **7** (3 μM) significantly arrested U266 cells at the S phase (21.67% and 23.98% increase, respectively), an event accompanied by a decrease of the G₂/M population (16.36% and 22.74% decrease, respectively). This may suggest that ROS-induced cell cycle arrest at the S phase led to the apoptotic cell death of U266 cells upon treatment with **5** or **7**.

NFκB activation has been suggested to play a critical role in the survival of MM cells and in the progression of MM.^{36,37} Furthermore, both **1** and **2** have been shown to inhibit NFκB activation in cancer models.^{38,39} Our previous research has demonstrated that the upregulated NFκB signaling pathway can be blocked by curcumin analogues in lung cancer A549

cells.⁴⁰ Due to their well-defined cell morphology, we have employed A549 cells as a model system and developed a highly quantitative method to monitor the nuclear translocation as a NFκB activation readout in an automated imaging-based high content analysis (HCA) assay. Therefore we initially used this well-defined quantitative assay in the A549 model system to evaluate the effect of compounds **5** and **7** on NFκB activation. Notably, as shown in Fig. 6B, both **5** and **7** inhibited the activation of NFκB in A549 cells upon stimulation with TNFα (10 ng mL⁻¹) with an IC₅₀ of 5.2 and 5.6 μM, respectively, a potency comparable to the potency of growth inhibition on MM cells. Microscopy examination (Fig. 6C) also clearly demonstrated that **5** dose-dependently inhibited the activation and translocation of NFκB into the nucleus of A549 cells upon stimulation with TNFα.

In summary, hybrid compounds of thalidomide (**1**) and curcumin (**2**) were successfully designed and synthesized. The biological tests in human MM cells established that compounds **5** and **7**, designed hybrids of **1** and **2**, exhibited superior cytotoxic effects than **2** alone or the combination of **1** and **2**. Further mechanistic studies in U266 cells demonstrated that both **5** and **7** can induce significant apoptotic cell death of U266 cells that could be due to the induction of ROS production and cell arrest at the S phase. Furthermore, **5** and **7** both exhibited potent inhibition on NFκB activation in A549 cells. Taken together, the results demonstrated that the hybrid compounds exhibit all the properties reported for **1** and **2** but are significantly more potent than both of them, thus suggesting that the hybrid strategy in drug design could lead to novel compounds with improved biological activities. Furthermore, the results also strongly encourage further optimization of **5** and **7** as new lead compounds to develop more potent agents as treatment options for human MM.

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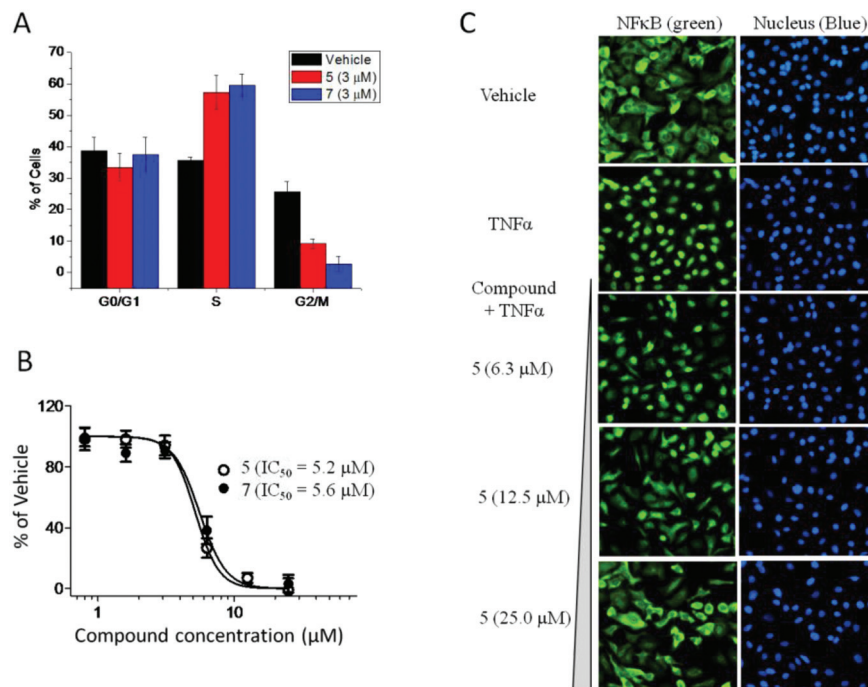


Fig. 6 Cell cycle arrest and inhibition of NFκB activation by **5** and **7**. (A) U266 cells were treated with indicated compounds for 24 h, DNA content was then analyzed by flow cytometry (data are expressed as mean value from three independent experiments and error bars represent SEM); (B) A549 cells were treated with **5** or **7** at indicated concentrations for 30 min, followed by stimulation with TNFα (10 ng mL⁻¹) for 30 min. Cells were fixed, stained, and analyzed by confocal microscopy; (C) images of samples treated with **5** from B.

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