ChemPubSoc Europe

DOI: 10.1002/cmdc.201300273

Structural Elaboration of a Natural Product: Identification of 3,3′-Diindolylmethane Aminophosphonate and Urea Derivatives as Potent Anticancer Agents

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An approach involving rational structural elaboration of the biologically active natural product diindolylmethane (DIM) with the incorporation of aminophosphonate and urea moieties toward the discovery of potent anticancer agents was considered. A four-step approach for the synthesis of DIM aminophosphonate and urea derivatives was established. These novel compounds showed potent anticancer activities in two representative kidney and colon cancer cell lines, low toxicity to normal cells, higher potency than the parent natural product DIM and etoposide, and potent inhibition of cancer cell migration. Biophysical and immunological studies, including DAPI nuclear staining, western blot analysis with apoptotic protein markers, flow cytometry, immunocytochemistry, and comet assays of the two most potent compounds revealed good efficacies in apoptosis and DNA damage. It was found that down-regulation of nuclear factor κB (NF-κB p65) could be an important mode of action in apoptosis, and the two most potent derivatives were found to be more potent than parent compound DIM in the down-regulation of NF-κB. Our results show the importance of structural elaboration of DIM by rational incorporation of aminophosphonate and urea moieties to produce potent anticancer agents; they also suggest that this approach using other structurally simple bioactive natural products as scaffolds holds promise for future drug discovery and development.

Introduction

Natural products play a key role in healthcare. They continued to be a major source of inspiration in drug discovery. About 40% of all marketed drugs are either natural products or their semisynthetic derivatives, while about 80% of all antibacterial drugs and 60% of all anticancer drugs are these types of compounds.[1] The use of herbal medicine in health care since ancient times has rooted the screening of natural extracts in the discovery of natural bioactive agents. As an extension, the biological screening of analogues, structurally simplified compounds inspired by natural products, has been found even more effective than screening of natural products alone. A popular example of this is morphine; its derivatization led to the development of potent analgesics such as codeine and etorphine, and structural simplification led to exploration of therapeutic agents such as morphinanes, benzomorphanes, and methadone.[2] Another well-known example is nakijiquinone;[3] the compounds of this class are inhibitors of Her2/Neu receptor tyrosine kinases. During the study of these natural products as inhibitors of a new set of receptor tyrosine kinases, it was found that none of the natural nakijiquinones exhibited significant inhibitory activity, though some of their analogues/derivatives showed activity in the low micromolar range. Investigation of the podophyllotoxin class of natural products eventually led to the development of semisynthetic derivatives etoposide and teniposide as efficient anticancer drugs.[4] In light of these examples, derivatization of bioactive complex natural products isolated from natural sources or synthesized via multiple steps is often considered. However, the type of chemistry that the natural products can undergo for derivatization or the comprehensive multistep synthetic approach frequently limits the preparation of analogues with relevant moieties incorporated or the generation of suitable libraries required for structure–activity relationship studies. Recently, an approach toward structural simplification of bioactive natural products, which can be completed through simple multicomponent syntheses, has shown much promise.[4a,5] Nevertheless, this approach may completely change the pharmacodynamic as well as pharmacokinetic profiles, which are also important components of the resulting class of compounds. In this regard, the pharmacologi-

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cally relevant elaboration of structurally simple bioactive natural products can be an important strategy.[6]

3,3′-Diindolylmethane (DIM) is a biologically active and abundant dietary compound found in *Brassica* genus vegetables such as broccoli, cabbage, and brussels sprouts.[7] It exhibits remarkable antitumor activities against several tumors[8] and is presently in clinical trials for the treatment of prostate cancer.[9] The anticancer activity of DIM has been found to be associated with down-regulation of the nuclear factor κB (NF-κB) transcription factor.[10,11] The inhibitory activities of DIM for angiogenesis and cancer invasion are also known,[12] and DIM has been found to possess leishmanicidal activity.[13] Diindolylalkane and their derivatives are found in bioactive metabolites of terrestrial and marine origin.[14] Several members of the indole class of anticancer natural products (Figure 1): vibrindole A, bisindolylmaleimide (e.g., enzasturin), and indolo-[2,3-*a*]carbazole (e.g., rebeccamycin, arcyriaflavins A–D, and staurosporine) structurally resemble DIM.[15]

For possible enhancement of therapeutic activities, the incorporation of various pharmaceutically relevant moieties into important scaffolds is common. In this context, the incorporation of aminophosphonate, especially its use as a bioisosteric replacement for amino acids, and the inclusion of urea moieties are valuable in drug discovery research.[16] The derivatization of vinblastine with a urea moiety at the C-20 position was found to enhance anticancer activity.[17] The anticancer and cytoprotective activities of flavagline are known to be improved by incorporation of an additional urea moiety.[18] Sorafenib, used in the treatment of renal and hepatocellular carcinoma, possesses a urea moiety which plays an important role in the inhibition of Raf kinase.[19] The α-aminophosphonate derivatives of carbazole also exhibit good anticancer activities.[20] Aminophosphonate derivatives of pyridothiazole[21] and thiourea-containing pseudopeptide[22] possess potent antitumor activities as well. Furthermore, the incorporation of urea and α-aminophosphonates as additional moieties on scaffolds are also known to improve the pharmacokinetic profiles of therapeutic agents. Based on all these aspects, DIM aminophosphonate and urea derivatives were evaluated as potential anticancer agents that could possibly possess enhanced activity over the parent compound, DIM.

In the present study, novel DIM aminophosphonate and urea derivatives that possess potent anticancer activities in kidney and colon cancer cells, low toxicity to normal cells, and potent inhibition in the migration of cancer cells were explored. Two of these synthesized compounds were found to be the most potent anticancer agents and exhibited higher activities than parent compound DIM and the clinically used anticancer drug etoposide. Biochemical and immunological techniques, including DAPI (4′-6-diamidino-2-phenylindole) nuclear staining, expression of representative apoptotic protein markers (caspase-3, PARP cleavage, and p53), caspase-3 staining (immunocytochemistry), flow cytometry, and comet assays revealed the activities of the investigated compounds in apoptosis and DNA damage. A luciferase-based promoter assay indicated the possible role of NF-κB in the apoptotic, anticancer, and antimetastatic activities of these DIM aminophosphonate and urea derivatives.

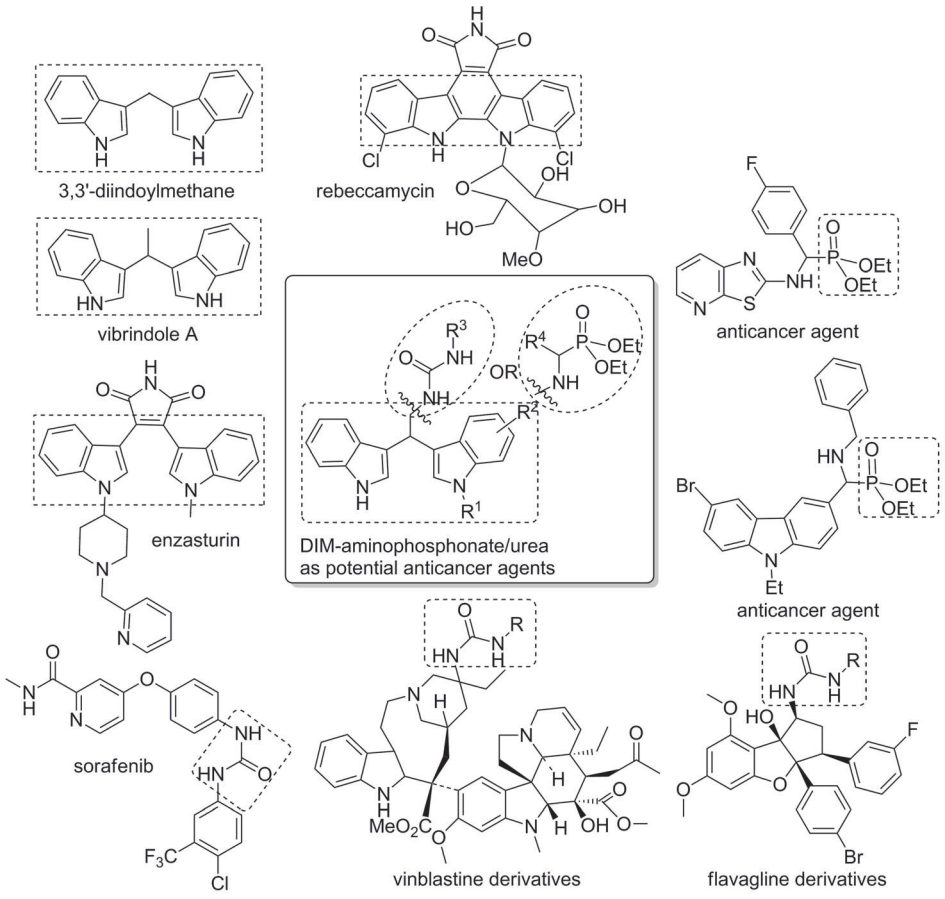
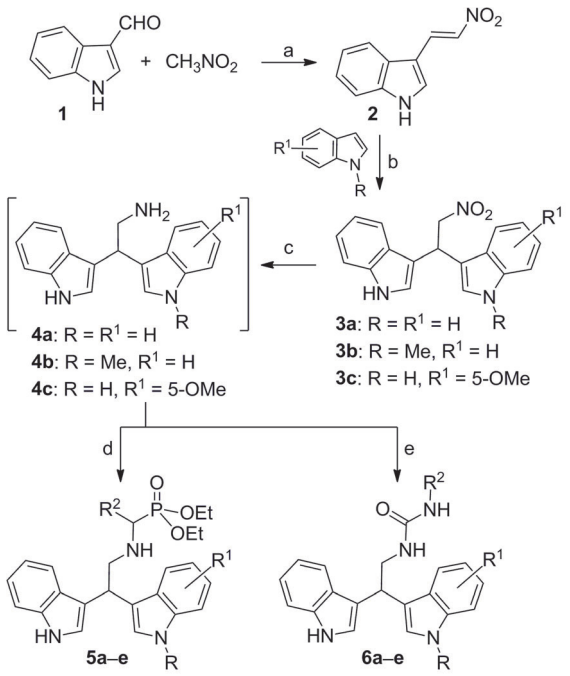


Figure 1. Examples of bisindole-containing natural/synthetic anticancer agents and therapeutic agents/drugs that possess aminophosphonate or urea as a pharmacologically important moiety. Based on these existing examples, DIM aminophosphonate and urea derivatives were postulated as potential anticancer agents.

Results and Discussion

Chemistry

For the synthesis of alkylaminophosphonate and alkylurea derivatives of DIM, a four-step method was established (Scheme 1). This methodology involved convenient preparation of 2,2-bis(indol-3-yl)nitroethanes and their required derivatization toward the preparation of de-



Scheme 1. Synthesis of DIM aminophosphonate and urea derivatives **5a**–**e** and **6a**–**e**. *Reagents and conditions*: a) NH_4OAc, AcOH, 110 °C, 4 h, 91%; b) NBS (10 mol%), CH_2Cl_2, 40 °C, 16 h, 67%; c) Zn dust, 6 N HCl, EtOH, RT, 1 h; d) R^2CHO, $H(O)P(OEt)_2$, $Mg(ClO_4)_2$ (10 mol%), EtOH, 50 °C, 46–56%; e) R^2NCO, CH_2Cl_2 (anhyd), RT, 1 h, 50–59%.

signed compounds for investigation. 3-(2′-Nitrovinyl)indole (**2**), a precursor for the synthesis of 2,2-bis(indol-3-yl)nitroethane (**3**), was prepared by Henry aldol condensation of indole-3-carboxaldehyde (**1**) with nitromethane.[23] Michael addition of 3-(2′-nitrovinyl)indole with NH-free/substituted indole by *N*-bromosuccinamide (NBS) catalysis produced 2,2-bis(indol-3-yl)nitroethane (**3**) in good yields.[24] The reduction of 2,2-bis(indol-3-yl)nitroethane (**3**) to 2,2-di(indol-3-yl)ethanamine (**4**) was found to be difficult. Various approaches were attempted for this reduction, such as catalytic hydrogenation using hydrogen, transfer hydrogenation,[25] use of hydride reducing agents,[26] and Clemmensen reduction[27] using zinc and HCl. Therefore, the process of reduction of 2,2-bis(indol-3-yl)nitroethane (**3**) to 2,2-di(indol-3-yl)ethanamine (**4**) and its in situ derivatization was considered. After several experiments, it was found that Clemmensen reduction using zinc and HCl of compound **3** to amine **4**, followed by quick extraction using ethyl acetate without chromatographic purification and Kabachnik–Fields reaction[28] provided DIM aminophosphonates (**5**) in good yields. Likewise, Clemmensen reduction of compound **3**, extraction, and subsequent reaction of **4** with isocyanate led to the formation of DIM ureas (**6**) in good yields. Following the established synthetic approach, various relevant substituted title compounds (**5a**–**e** and **6a**–**e**) were prepared (Figure 2). DIM, which was used as a control in the bioevaluation of investigated compounds, was prepared by a known reaction of indole with paraformaldehyde.

Biological studies

Antiproliferative activities of DIM aminophosphonates (5a–e) and ureas (6a–e)

To evaluate the effects of DIM aminophosphonate and urea derivatives (**5a**–**e** and **6a**–**e**) on anchorage-dependent cell viability, an MTT assay was performed using representative kidney cancer cell (HEK 293T) and normal kidney epithelial (Vero) cell lines.[6a] Cell viabilities were measured at different time periods (24, 48, and 72 h) after treating cells with the investigated compounds at various concentrations (Figure 3a and Table 1).

Table 1. Cytotoxicities of investigated compounds in normal (Vero) and cancer (HEK 293T) cells.

Compd	LC_{50} [μM][a]		Compd	LC_{50} [μM][a]	
	Vero	HEK 293T		Vero	HEK 293T
5a	58±2	22±2	**6a**	56±2	25±2
5b	61±2	19±3	**6b**	54±2	28±2
5c	66±2	23±1	**6c**	52±2	18±2
5d	56±3	18±2	**6d**	58±2	23±2
5e	55±2	13±2	**6e**	58±2	14±2
DIM[b]	61±2	44±2			

[a] Lethal concentrations required to kill 50% of cells in culture were determined after incubation with test compounds for 48 h; data represent the mean ±SD of three independent experiments. [b] Diindolylmethane (DIM) was used as a reference agent.

No significant cell death was observed after 48 h exposure with tested compounds. DIM aminophosphonate (**5e**) and DIM urea (**6e**) derivatives were found to be the most potent antiproliferative agents, while showing poor cytotoxicities in normal cells after 48 h of treatment (Table 1). The cytotoxic activities of these agents (**5e** and **6e**), in comparison with parent DIM, were studied. Accordingly, assays were performed after exposure with varied concentrations of compounds **5e**, **6e**, and DIM. Cell viability decreased in a dose-dependent manner. In HEK 293T cells, the viability was significantly lower than in Vero cells. Interestingly, DIM aminophosphonate (**5e**) and DIM urea (**6e**) exhibited more potent antiproliferative activities, with LC_{50} values of 13 and 14 μM, respectively, compared with DIM (LC_{50} = 44 μM). In addition, these DIM derivatives and DIM itself exhibited similar cytotoxicities in normal Vero cells, and no LC_{50} values were observed, even after treatment with compounds at 50 μM concentrations.

To further confirm the antiproliferative activity of the investigated compounds in cancer cells, a clonogenic cell survival assay was performed. This assay is a long-term cell viability test that determines the ability of a single cell to proliferate indefinitely, thereby retaining its reproductive ability to form a colony or a clone. DIM, DIM aminophosphonate (**5e**), and DIM urea (**6e**) showed decreased colony formation in a concentration-dependent manner with LC_{50} (50% decrease in cultured colonies) values of 31, 7, and 8 μM, respectively (Figure 3b). With regard to the capacity to impede colony formation, the enhanced potency of DIM derivatives relative to DIM was observed and found to be consistent with MTT assay results. The

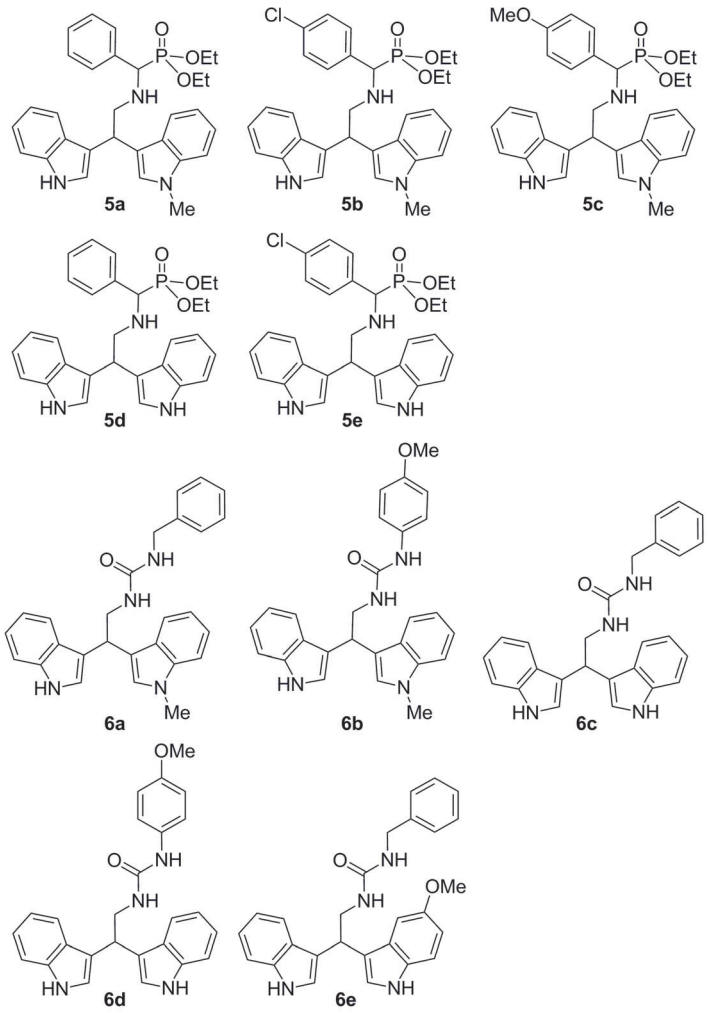


Figure 2. Synthesized DIM aminophosphonates **5a–e** and urea derivatives **6a–e**.

results of MTT and clonogenic assays indicate that DIM aminophosphonate and urea derivatives possess higher cytotoxic activities, relatively low toxicities toward normal cells, and higher potencies than DIM.

Comparison of DIM derivatives and etoposide in colon cancer cells

Once cytotoxic activities of DIM aminophosphonate and urea derivatives were established in a kidney cancer cell line, their cytotoxic potential in another cancer cell line, HCT-116 (colon cancer cells), was evaluated. The activities of investigated compounds were compared with that of etoposide, a clinically used anticancer drug. Accordingly, an MTT assay using HCT-116 cells was performed (Figure 4) in which the cells were treated with increasing concentrations of etoposide and the potent compounds **5e** and **6e**. After 48 h of treatment, cell viabilities were measured. The LC_{50} values for both **5e** and **6e** were 15 μM, whereas etoposide showed an LC_{50} value of 22 μM (Figure 4). Thus, the results showed that DIM derivatives had greater cytotoxicity in cancer cells than etoposide. This also reveals that DIM derivatives not only inhibit kidney cancer cells but also cause cytotoxic effects in colon cancer cells in a similar manner.

Anti-migratory activities in HEK 293T cells

The phenomena of cancer cell growth relates to its rapid and uncontrolled proliferation and migration. To examine the inhibitory effect of DIM aminophosphonate (**5e**) and DIM urea (**6e**) on anchorage-dependent cell migration, a wound healing assay using HEK 293T cells was performed. After making a wound, cancer cells were treated with compounds **5e** and **6e** at their LC_{50} concentrations, 13 μM and 14 μM, respectively. The wound was inspected microscopically over time. In untreated cells, migration occurred, and the wound healed in 48 h (Figure 5). However, in treated cells, migration was inhibited, and after prolonged time period of treatment, widespread apparent cell death was observed (Figure 5). Therefore, this assay, in conjunction with the assays as discussed above (clonogenic and MTT cell survival), demonstrates that DIM aminophosphonate (**5e**) and DIM urea (**6e**) possess potent inhibitory activities toward colony formation, viability, and migration of cancer cells.

Apoptosis studies

To determine the effect of DIM aminophosphonate (**5e**) and DIM urea (**6e**) on apoptosis in cancer cells, the formation of chromatin condensation and nuclear fragmentation in HEK 293T cells was analyzed using a DAPI nuclear staining experimental method (Figure 6a). Cells at 60–70% confluence were treated with investigated compounds at their LC_{50} concentrations for 48 h, followed by nuclear staining. Increased chromatin condensation was observed, as well as nuclear fragmentation and shrunken (apoptotic) nuclei in treated HEK 293T cells relative to untreated cells (Figure 6a). The relative number of apoptotic/non-apoptotic nuclei are presented in a bar diagram (Figure 6b, $*p < 0.05$). To confirm the apoptosis caused by compounds **5e** and **6e**, fluorescence-activated cell sorting (FACS) analysis was performed. The kidney cancer cells were treated with the investigated compounds at their respective LC_{50} concentrations for 48 h and then analyzed using FACS by staining with propidium iodide (Figure 6c,d). DNA content of the cells at various phases of the cell cycle was measured by Cell Quest software (Becton and Dickinson, CA). A high increase in apoptosis was observed for treated cells, with ~60 and 61% apoptosis observed for compounds **5e** and **6e**, respectively, whereas, little (~1%) apoptosis was observed for untreated cells (Figure 6c). A histogram of the cell cycle profile is presented in Figure 6d. Therefore, these results indicate that HEK 293T cells, after treatment with investigated DIM aminophosphonate and urea derivatives, undergo significant apoptosis.

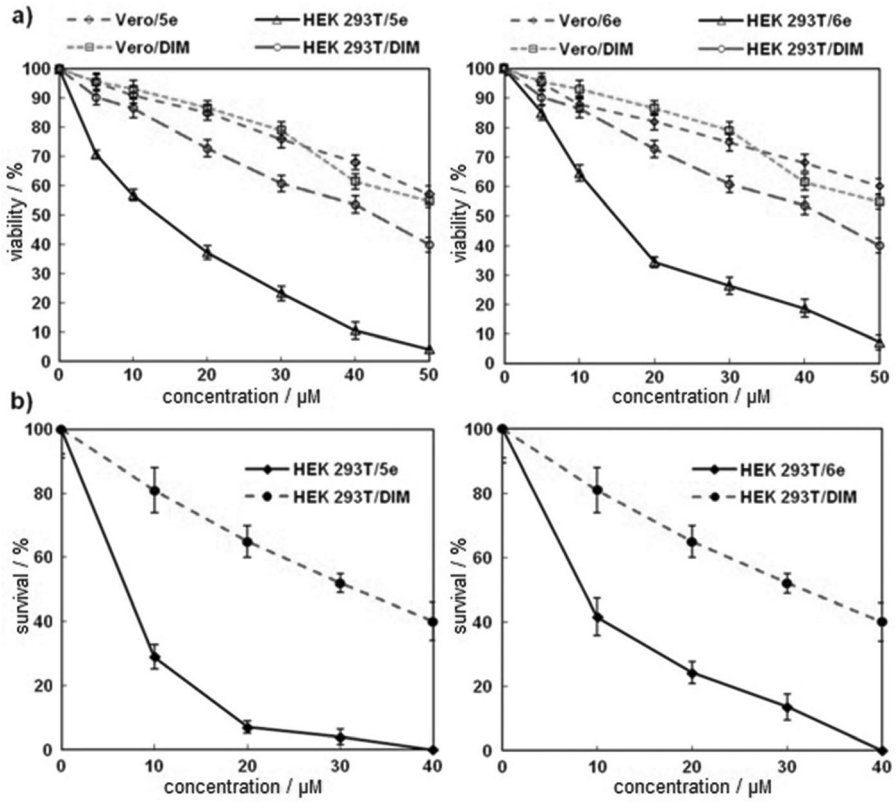


Figure 3. Antiproliferative activities of DIM, DIM aminophosphonate (**5e**) and DIM urea (**6e**); the activities for the two most potent compounds are presented. a) MTT cell viability assay: Cells (HEK 293T and Vero) were treated with DIM and investigated compounds at various concentrations for 48 h as described in the Experimental Section. b) Clonogenic cell survival assay: HEK 293T cells were treated with DIM and the investigated compounds **5e** and **6e**, according to a protocol described in the Experimental Section. Data shown are the mean ± SD of three different experiments.

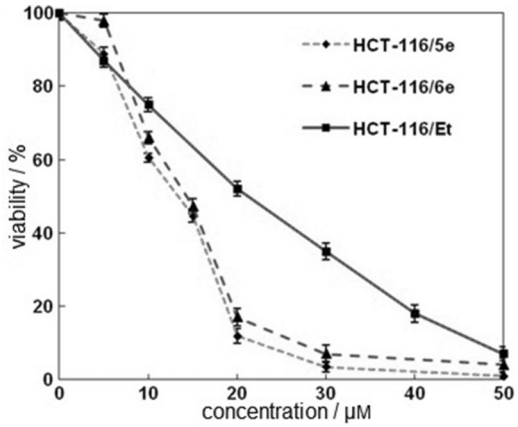


Figure 4. Effects of DIM aminophosphonate (**5e**) and DIM urea (**6e**) on cell survival in an anchorage-dependent manner. Comparison of anti-cell viability (HCT-116 cells) for potent investigated compounds with etoposide (Et). Data are the mean ± SD of three different experiments.

For further studies of apoptosis, immunocytochemistry and western blot analyses using representative apoptosis markers were also performed. Overexpression of caspase-3 and cleaved product of poly(ADP-ribose) polymerase (PARP), and down-regulation of NF-κB expression are the majorly observed phenomena for the apoptotic activities of anticancer agents.[29] The expression of caspase-3 was determined by immunocytochemistry in HEK 293T cells after exposure to test compounds at their respective LC_{50} concentrations for 48 h. Increased accumulation of caspase-3 expression was observed in treated cells relative to control (Figure 7). In comparison with untreated cells, after exposure to DIM derivatives in kidney cancer cells, the expressions of tumor suppressor gene p53 and caspase-3 increased, cleaved products of PARP appeared, and the expression of NF-κB decreased (Figure 8a). Together, these results reveal that investigated DIM aminophosphonate and urea derivatives possess apoptotic activities in kidney cancer cells.

Potential role of NF-κB (p65) transcription factor in anticancer activities

The parent natural product, DIM, is known to exhibit anticancer activity via NF-κB down-regulation.[30,31] In our study of apoptosis using western blot analysis with protein markers for DIM aminophosphonate and urea derivatives, NF-κB was found to be expressed at relatively lower levels (Figure 8a). Therefore, we speculated that NF-κB could play an important role in anticancer activities exhibited by these investigated compounds. Among all isoforms of NF-κB, p65 plays crucial role in cancer.[32] Accordingly, a luciferase-based promoter assay was performed using NF-κB (p65). The direct transcription factor activity of NF-κB (p65) was measured after treatment of HEK 293T cells with compounds **5e** and **6e** for 48 h following the reported method.[29] The parent compound, DIM, was also used in the assay for comparison of its activity with that of investigated DIM aminophosphonate and urea derivatives. The results are presented in Figure 8b. Compounds **5e** and **6e** were found to induce ~4.0- and 3.8-fold decreases in luciferase activities, respectively, while DIM caused a 2.0-fold decrease. These results signify that the structurally elaborated DIM aminophosphonate and urea derivatives are more potent NF-κB (p65) down-regulating agents than parent DIM and, moreover, they decreased not only the expression of NF-κB (p65) at transcriptional level (Figure 8b) but also at translational level (Figure 8a). These observations indicate that

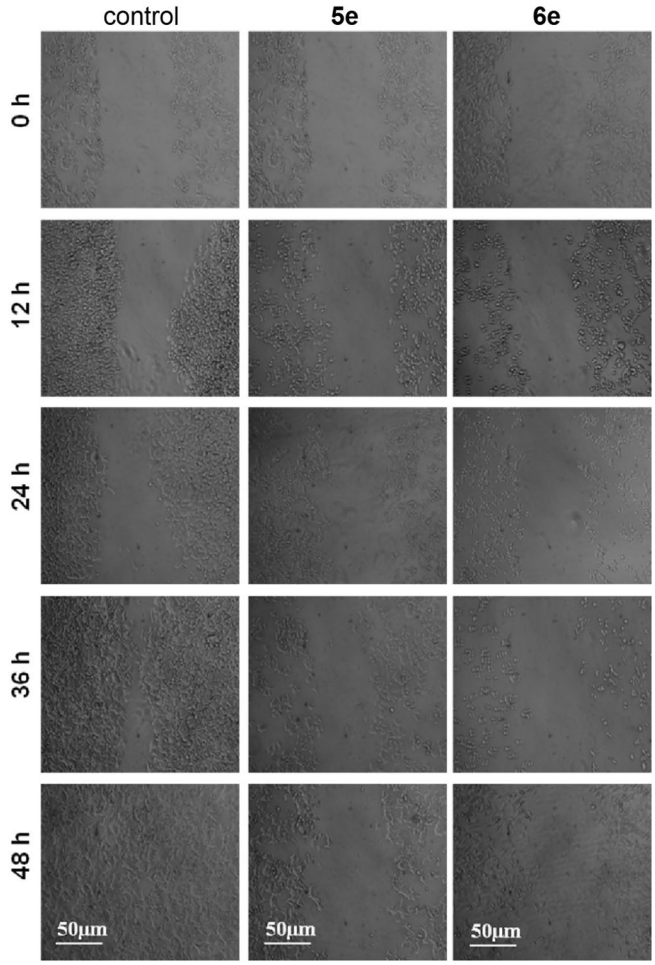


Figure 5. Anchorage-dependent cell migration of HEK 293T cells. After wound formation, cells were treated with DIM aminophosphonate (**5e**) and DIM urea (**6e**) at their LC_{50} concentrations. Micrographs were taken at intervals of 0, 12, 24, 36, and 48 h to evaluate the healing of the wound at 40× magnification. Images shown are representative of three independent experiments.

the down-regulation of NF-κB (p65) can be an important mode of action for anticancer activities exhibited by the investigated DIM-aminophosphonate and urea derivatives.

Induction of DNA damage by DIM derivatives

To further explore the possible involvement of DNA damage repair as a mechanism of action for the anticancer activities of DIM aminophosphonate and urea derivatives, a comet assay was carried out. In HEK 293T cells treated with compounds **5e** and **6e**, the average comet length was found to be greater (6- to 8-fold) relative to untreated cells (Figure 9A). This result indicates the DNA damaging efficacy of the investigated compounds.

Conclusions

In conclusion, rational structural elaboration of DIM, a biologically active natural product, led to the discovery of novel DIM aminophosphonate and urea derivatives as potent apoptotic anticancer agents. A four-step method was established for their synthesis. DIM aminophosphonate (**5e**) and DIM urea (**6e**) were found to be the most potent anticancer agents and exhibited higher anticancer activities than parent natural product DIM and etoposide (an anticancer drug). These compounds showed antiproliferative activities in kidney (HEK 293T) and colon (HCT-116) cancer cells and anti-migratory activities in kidney (HEK 293T) cancer cells while exhibiting low toxicity to normal cells (Vero). Relevant biophysical and immunological techniques, including DAPI nuclear staining, western blot analysis with apoptotic protein markers, flow cytometry, immunocytochemistry, and comet assays revealed good efficiencies for potent compounds **5e** and **6e** in apoptosis and DNA damage. It was found that down-regulation of NF-κB could play an important role in apoptosis, and compounds **5e** and **6e** were more potent than parent natural product DIM. The results of the present study represent the importance of structural elaboration of bioactive natural product DIM with rational incorporation of aminophosphonate and urea moieties toward the discovery of potent NF-κB-down-regulating apoptotic, anticancer, and anti-metastatic agents. This should motivate researchers to consider DIM or any other structurally simple bioactive natural product as important natural scaffolds for rational structural elaboration in drug discovery research.

Experimental Section

Chemistry

General: All reactants and reagents were obtained from commercial sources and used without further purification unless otherwise noted. *N*-Bromosuccinimide was used after recrystallization. NMR spectra were recorded on a Bruker Avance DPX 400 MHz spectrometer in $CDCl_3$/[D_6]DMSO/CD_3OD solvents using TMS as an internal standard. *J* values are given in Hz. Mass spectra were recorded using an LC–MS-Finnigan MAT-LCQ (for ESI and APCI), Bruker-Maxis (for HRMS) mass spectrometers. IR spectra were recorded on a Nicolet FT-IR Impact 410 instrument as thin film (neat). The reactions were monitored by TLC (Merck, silica gel 60 F_{254}). The purity of the synthesized compounds was analyzed by HPLC (Shimadzu LC-6AD system), using a Phenomenex RP-C18 column (250×4.60 mm), particle size 5 μm, flow rate 1 mL min^{-1}, using H_2O/CH_3CN (60:40) for compounds **5a**–**e**, and H_2O/CH_3CN (50:50) for compounds **6a**–**e** detection at 220 nm and 254 nm. The purity of tested compounds was found to be >95%. The investigated compounds were synthesized as racemic mixtures.

3-(2-Nitrovinyl)indole (2): Nitromethane (3 mmol, 0.16 mL) was added dropwise to a solution of indole-3-carboxaldehyde (**1**) (1 mmol, 145 mg) and CH_3COONH_4 (1 mmol, 77 mg) in CH_3COOH (0.75 mL). The mixture was stirred at reflux for 4 h and then cooled to room temperature. The resulting mixture was extracted with EtOAc (2×25 mL), and the solution was washed with H_2O (3× 20 mL) and dried with anhydrous Na_2SO_4. The solvent was evaporated under vacuum to provide NMR-pure 3-(2-nitrovinyl)-indole in 91% yield (164 mg). The compound was used in the subsequent reaction without further chromatographic purification.

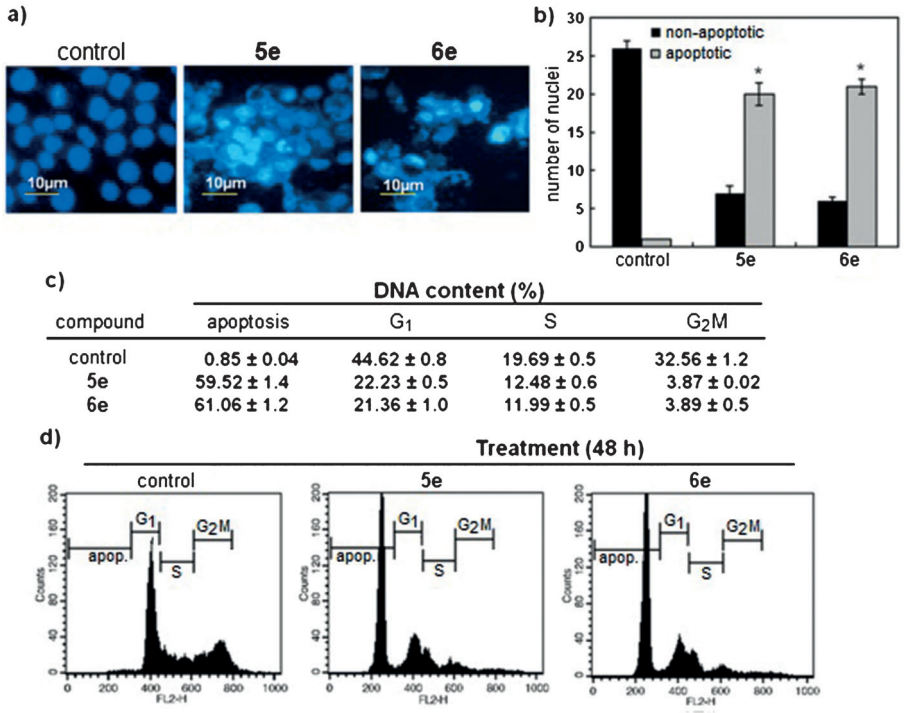


Figure 6. Apoptosis induced by DIM aminophosphonate (**5e**) and DIM urea (**6e**) in HEK 293T cells. a) DAPI nuclear staining: HEK 293T cells were treated with investigated compounds, and DAPI nuclear staining was performed according to the protocol described in the Experimental Section. Micrographs were taken at 40× magnification. b) Bar diagram represents normal (black) and apoptotic (grey) cells. Data represent the best of three individual experiments. Statistical significance was determined by Student's paired *t* test; $*p < 0.05$. c) Quantitative analysis of percentages of DNA in each phase of the cell cycle was done using Cell Quest software (Becton-Dickinson). d) Histograms of HEK 293T cell populations were measured by FACS using Cell Quest software. Apoptosis, G_1, S, and G_2M phases are indicated; data are the mean ± SD of three different experiments.

Representative experimental procedures

2,2-Di(indol-3-yl)-1-nitroethane (3a): A mixture of 3-(2-nitrovinyl)-indole (**2**), (1 mmol, 188 mg), indole (1.3 equiv, 152 mg), and NBS (10 mol%, 17.7 mg) in CH_2Cl_2 (2 mL) was stirred at 40 °C for 16 h. After completion of the reaction as indicated by TLC, the resulting mixture was extracted with EtOAc (2×25 mL). The combined organic solution was washed with H_2O (2×10 mL) and brine (10 mL), dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. Column chromatographic purification of the crude mixture on silica gel, eluting with EtOAc/hexane provided **3a** in 67% isolated yield (203 mg). Compounds **3b** and **3c** were also prepared following this procedure.

2,2-Di(indol-3-yl)ethylamine (4a): 2,2-Di(indol-3-yl)-1-nitroethane (**3a**) (1 mmol, 305 mg) was dissolved in EtOH (6 mL) to which 6 N HCl (3 mL) and Zn dust (10 equiv, 650 mg) were added. After stirring for 1 h at room temperature (25–26 °C), the resulting mixture was filtered. The filtrate was neutralized by dropwise addition of saturated aqueous $NaHCO_3$ solution and then extracted with EtOAc (4×30 mL). The organic solution was washed with H_2O (10 mL) and dried with anhydrous Na_2SO_4. The evaporation of solvent under reduced pressure provided the crude residue, which was directly used in the next reaction without column chromatography. Compounds **4b** and **4c** were also prepared following this procedure.

Diethyl (2,2-di(indol-3-yl)ethylaminophenmethylphosphonate (5d): A mixture of 2,2-di(indol-3-yl)ethylamine (0.5 mmol, 137.5 mg) (**4a**), benzaldehyde (0.5 mmol, 0.05 mL), diethyl phosphite (0.6 mmol, 0.07 mL), and $Mg(ClO_4)_2$ (10 mol%, 11 mg) in EtOH (2 mL) was stirred at 50 °C. After completion of the reaction as indicated by TLC, the resulting mixture was extracted with EtOAc (2×25 mL). The combined organic solution was washed with H_2O (2×10 mL) and brine (10 mL), dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. Column chromatographic purification of crude mixture on silica gel, eluting with EtOAc/hexane, provided **5d** in 52% isolated yield (260 mg). DIM aminophosphonate derivatives **5a**, **5b**, **5c**, and **5e** were also prepared following this procedure.

Diethyl 2-(3-indolyl)-2-(1-methylindol-3-yl)ethylaminophenmethylphosphonate (5a): Light-brown semisolid (288 mg, 56%): $R_f = 0.45$ (EtOAc/*n*-hexane, 75:25); IR (neat): $\tilde{\nu}_{max} = 3269, 2981, 1613, 1228, 1026, 969\ cm^{-1}$; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.19$ (br s, NH), 7.48–7.42 (m, 2H), 7.29–7.21 (m, 7H), 7.18–7.12 (m, 2H) 7.00–6.93 (m, 3H), 6.81–6.75 (m, 1H), 4.73–4.70 (m, 1H), 4.14–4.10 (m, 2H), 3.95–3.88 (m, 2H), 3.81 (br s, NH), 3.68–3.61 (m, 4H), 3.27–3.24 (m, 2H), 1.13 (t, $J = 8.0$ Hz, 3H), 0.98 ppm (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 137.2, 136.6, 135.8, 128.7, 128.3$ (2CH), 127.8, 127.2, 126.8 (2CH), 122.2, 121.8, 121.4, 119.6, 119.5, 119.0, 118.6, 117.2, 116.3, 115.8, 111.1, 109.1, 62.8, 62.7, 61.8 ($J = 152$ Hz), 52.5, 34.1, 32.6, 16.3, 16.1 ppm; MS (APCI): *m/z* (%): 516 (12) $[M+H]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{30}H_{34}N_3NaO_3P$: 538.2230, found: 538.2234.

Diethyl 2-(3-indolyl)-2-(1-methylindol-3-yl)ethylamino-4-chlorophenmethylphosphonate (5b): Light-brown semisolid (263 mg, 48%): $R_f = 0.47$ (EtOAc/*n*-hexane, 75:25); IR (neat): $\tilde{\nu}_{max} = 3271, 2982, 1614, 1228, 1023, 971\ cm^{-1}$; ^1H NMR (400 MHz, $CDCl_3$): $\delta =$ 8.06–8.02 (m,1H), 7.61–7.01 (m, 12H), 6.84–6.78 (m, 1H), 4.71 (s,1H), 4.06–3.84 (m, 4H), 3.69–3.66 (m, 4H), 3.24–3.22 (m, 2H), 1.15 (s, 3H), 1.01 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 137.3, 136.5, 133.4, 129.9, 129.8$ (2CH), 128.4 (2CH), 126.8, 122.0, 121.9, 121.5, 119.5, 119.4, 119.2, 118.6, 117.7, 117.3, 116.0, 115.7, 111.1, 109.1, 62.9, 62.7, 61.4 ($J = 158$ Hz), 52.9, 34.2, 32.7, 16.3, 16.1 ppm; MS (APCI): *m/z* (%): 550 (3) $[M+H]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{30}H_{33}ClN_3NaO_3P$: 572.1840, found: 572.1841.

Diethyl 2-(3-indolyl)-2-(1-methylindol-3-yl)ethylamino-4-methoxyphenmethylphosphonate (5c): Dark-brown semisolid (250 mg, 46%): $R_f = 0.42$ (EtOAc/*n*-hexane, 75:20); IR (neat): $\tilde{\nu}_{max} = 3245, 2978, 1248, 1028, 969\ cm^{-1}$; 1H NMR (400 MHz, $CDCl_3$): $\delta = 7.49–6.99$ (m, 9H), 6.89 (d, $J = 8.4$ Hz, 2H), 6.85–6.77 (m, 3H), 4.72 (t, $J =$ 7.4 Hz, 1H), 4.09–3.90 (m, 7H), 3.80 (s, 3H), 3.71–3.65 (m, 1H),

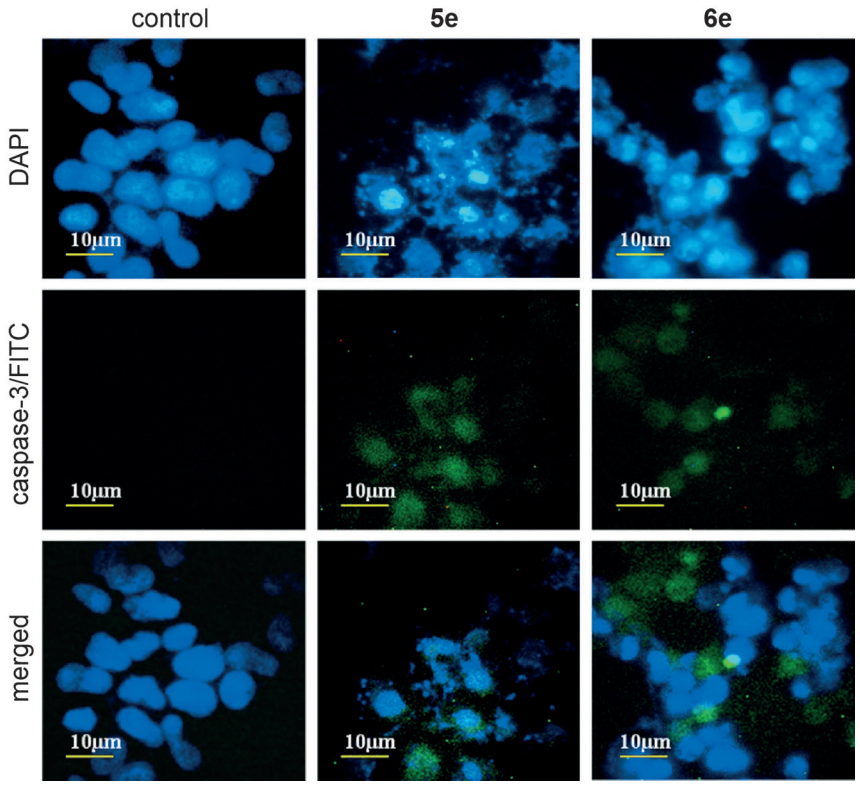


Figure 7. Caspase-3 expression: HEK 293T cells were treated with DIM aminophosphonate (**5e**) and DIM urea (**6e**), and staining was performed with anti-caspase-3 antibody and DAPI as described in the Experimental Section. Micrographs were taken at 40× magnification. The fields of DAPI-stained nuclei were merged with respective fields of caspase-3 staining with the help of IMAGE J software. Micrographs shown are the best of three individual experiments.

3.34–3.23 (m, 2H), 1.28 (t, J=7.0 Hz, 3H), 1.22 ppm (t, J=7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=158.6, 138.7, 137.2, 136.6, 128.7 (2CH), 128.4, 127.8, 127.2, 126.9, 123.0, 121.8, 121.4, 119.7, 119.5, 119.2, 117.2, 116.3, 115.8, 113.8 (2CH), 109.2, 62.8, 62.6, 61.8 (J= 154 Hz), 54.7, 52.8, 34.1, 32.7, 16.4, 16.4 ppm; MS (APCI): m/z (%): 546 (2) $[M+H]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{31}H_{36}N_3NaO_4P$: 568.2336, found: 568.2339.

Diethyl 2,2-di(3-indolyl)ethylaminophenmethylphosphonate (5d): Light-brown semisolid (260 mg, 52%): R_f=0.50 (EtOAc/*n*-hexane, 80:20); IR (neat): $\tilde{\nu}_{max}$=3228, 2926, 1634, 1238, 1024, 969 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ=7.32–7.30 (m, 1H), 7.26–7.19 (m, 8H), 6.98–6.88 (m, 4H), 6.80–6.77 (m, 2H), 4.60 (t, J= 7.2 Hz, 1H), 4.09–4.04 (m, 1H), 3.79–3.75 (m, 2H), 3.68- 3.64 (m, 2H), 3.14 (d, J=7.2 Hz, 2H), 1.00 (t, J=7.0 Hz, 3H), 0.88 ppm (t, J= 6.9 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ=137.0 (2C), 134.9, 128.6 (2CH), 128.1 (2CH), 127.7, 126.8 (2C), 121.9 (2CH), 120.8 (2CH), 118.6 (2CH), 118.0 (2CH), 116.5 (2C), 110.8 (2CH), 62.9, 62.7, 60.5 (J= 153 Hz), 51.7, 33.7, 15.1, 15.0 ppm; MS (APCI): m/z (%): 502 (30) $[M+H]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{29}H_{32}N_3NaO_3P$: 524.2073, found: 524.2077.

Diethyl 2,2-di(3-indolyl)ethylamino-4-chlorophenmethylphosphonate (5e): Light-brown semisolid (272 mg, 51%): R_f=0.52 (EtOAc/*n*-hexane, 80:20); IR (neat): $\tilde{\nu}_{max}$=3243, 2956, 1625, 1238, 972 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ=7.38 (t, J=7.7 Hz, 2H), 7.30–7.28 (m, 2H), 7.22–7.14 (m, 4H), 7.07–7.01 (m, 3H), 6.98 (s, 1H), 6.78 (t, J=7.6 Hz, 2H), 4.68 (t, J=7.2 Hz, 1H), 4.09–4.00 (m, 2H), 3.93–3.86 (m, 2H), 3.72–3.68 (m, 1H), 3.23 (d, J=7.2 Hz, 2H), 1.08 (t, J=7.0 Hz, 3H), 0.98 ppm (t, J=7.0 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD): 137.0 (2C), 134.0, 130.3 (2CH), 128.7, 128.0 (2CH), 126.7 (2C), 122.0 (2CH), 120.9 (2CH), 118.8 (2CH), 118.1 (2CH), 116.4 (2C), 110.9 (2CH), 63.3, 63.1, 60.1 (d, J=153 Hz), 51.7, 34.0, 31.3, 15.4, 15.2 ppm; MS (APCI): m/z (%): 536 (31) $[M+H]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{29}H_{31}ClN_3NaO_3P$: 558.1684, found: 558.1687.

1-Benzyl-3-(2,2-di(indol-3-yl)-ethyl)urea (6c): Benzyl isocyanate (0.5 mmol, 0.062 mL) was added to a solution of 2,2-di(indol-3-yl)ethylamine (**4a**) (0.5 mmol, 135.5 mg) in anhydrous CH_2Cl_2 (2 mL). The mixture was stirred at 25–26 °C for 1 h. After completion of the reaction as indicated by TLC, the resulting mixture was extracted with EtOAc (2×25 mL). The combined organic solution was washed with H_2O (2× 10 mL) and brine (10 mL), dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. Column chromatographic purification of the crude mixture on silica gel, eluting with EtOAc/hexane, provided **6c** in 51% isolated yield (208 mg). DIM urea derivatives **6a**, **6b**, **6d**, and **6e** were also prepared following this procedure.

1-(2-(Indol-3-yl)-2-(1-methylindol-3-yl)ethyl)-3-benzylurea (6a): Brown semisolid (248 mg, 59%): R_f=0.45 (EtOAc/*n*-hexane, 60:40); IR (neat): $\tilde{\nu}_{max}$=3401, 2976, 1628, 1275, 1051 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.32 (br s, NH), 7.57–7.50 (m, 2H), 7.21–7.16 (m, 4H), 7.08–6.96 (m, 7H), 6.82 (s, 1H), 6.65 (s, 1H), 4.73 (br s, NH), 4.65–4.61 (m, 1NH+ 1CH), 4.08 (s, 2H), 3.85–3.81 (m, 2H), 3.53 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=157.6, 139.0, 137.2, 136.5, 128.5 (2CH), 128.1, 127.3 (2CH), 127.2, 126.9, 126.5, 122.1, 122.0, 121.6, 119.8, 119.6, 119.3, 118.8, 117.0, 115.4, 111.2, 109.2, 44.9, 44.5, 34.6, 32.6 ppm; MS (APCI): m/z (%): 423 (2) $[M+H]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{27}H_{26}N_4NaO$: 445.1999, found: 445.1999.

1-(2-(Indol-3-yl)-2-(1-methylindol-3-yl)ethyl)-3-(4-methoxyphenyl)urea (6b): Pale-brown semisolid; (236 mg, 54%): R_f=0.42 (EtOAc/*n*-hexane, 60:40); IR (neat): $\tilde{\nu}_{max}$=3435, 2913, 1632, 1212 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=8.35 (br s, NH), 7.57–7.54 (m, 2H), 7.25–6.62 (m, 12H), 6.25 (br s, NH), 4.84 (br s, NH), 4.68 (m, 1H), 3.95–3.93 (m, 2H), 3.70 (s, 3H), 3.60 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=156.6, 154.9, 137.2, 136.5, 130.8, 127.3, 126.9, 124.2, 122.1, 121.6, 120.3, 119.6, 119.3 (2CH), 119.2, 118.9, 117.0, 115.8, 115.5, 114.3 (2CH), 111.2, 109.2, 55.4, 44.7, 34.4, 32.6 ppm; MS (APCI): m/z (%): 439 (1) $[M+H]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{27}H_{26}N_4NaO_2$: 461.1948, found: 461.1948.

1-Benzyl-3-(2,2-di(indol-3-yl)ethyl)urea (6c): Brown semisolid; (208 mg, 51%): R_f=0.52 (EtOAc/*n*-hexane, 70:30); IR (neat): $\tilde{\nu}_{max}$= 3403, 2927, 1642, 1548, 1241, 1093 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ=7.54 (d, J=7.7 Hz, 2H), 7.32–7.02 (m, 11H), 6.91 (t, J= 7.0 Hz, 2H), 4.70 (t, J=6.9 Hz, 1H), 4.24 (s, 2H), 3.91 ppm (d, J=

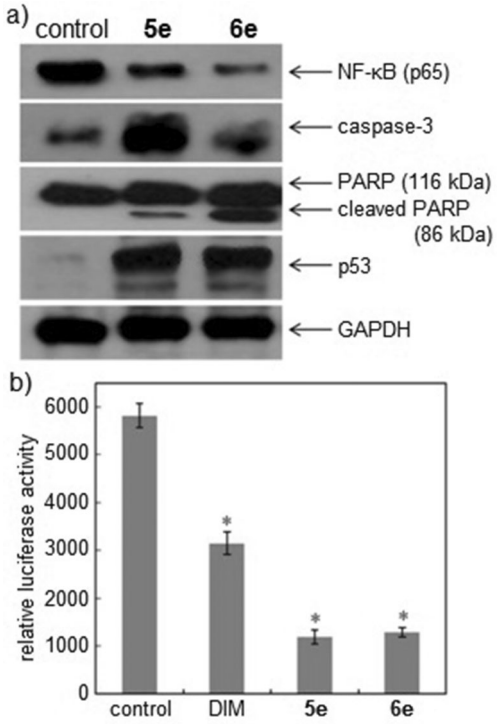


Figure 8. a) Western blot analysis of HEK 293T whole-cell lysate after treatment with DIM aminophosphonate (**5e**) and DIM urea (**6e**). Cells were treated with compounds at their LC_{50} concentrations (**5e**: 13 μM and **6e**: 14 μM) for 48 h prior to western blot analysis. GAPDH served as a protein loading control. Images shown are representative of three independent experiments. b) Relative luciferase activity of NF-κB (p65; p5XIP10B plasmid-containing NF-κB (p65) constructs) measured in HEK 293T cells is plotted as the mean ± SD of three different experiments. Statistical significance was determined by the paired *t* test, $*p < 0.05$.

6.9 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 159.8$, 139.8, 136.9, 128.1 (2CH), 127.0 (2C), 126.8 (2CH), 126.5 (2C), 122.1 (2CH), 120.8 (2CH), 118.7 (2CH), 118.1 (2CH), 116.4 (2C), 110.8 (2CH), 44.68, 43.27, 29.29 ppm; MS (APCI): *m/z* (%): 409 (2) $[M+H]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{24}N_4NaO$: 431.1842, found: 431.1842.

1-(2,2-Di(indol-3-yl)ethyl)-3-(4-methoxyphenyl)urea (6d): White semisolid; (233 mg, 55%): $R_f = 0.48$ (EtOAc/*n*-hexane, 70:30); IR (neat): $\tilde{\nu}_{max} = 3410$, 2920, 1632, 1450, 1275 cm^{-1}; ^{1}H NMR (400 MHz, $[D_6]$DMSO): $\delta = 8.27$ (br s, NH), 7.56 (d, $J = 7.6$ Hz, 2H), 7.32 (d, $J = 7.8$ Hz, 2H), 7.25–7.21 (m, 4H), 7.02 (t, $J = 6.7$ Hz, 2H), 6.90 (t, $J = 6.9$ Hz, 2H), 6.78 (d, $J = 7.7$ Hz, 2H), 5.95 (br s, NH), 4.59 (m, 1H), 3.86 (m, 2H), 3.67 ppm (s, 3H); ^{13}C NMR (100 MHz, $[D_6]$DMSO): $\delta =$ 155.9, 154.2, 136.9 (2CH), 134.1, 127.1 (2CH), 122.9 (2CH), 121.2 (2C), 119.5 (2CH), 119.4 (2C), 118.5 (2CH), 116.7 (2CH), 114.3 (2CH), 111.8 (2C), 55.5, 44.1, 35.1 ppm; MS (APCI): *m/z* (%): 425 (5) $[M+H]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{26}H_{24}N_4NaO_2$: 447.1791, found: 447.1791.

1-(2-(Indol-3-yl)-2-(5-methoxyindol-3-yl)ethyl)-3-benzylurea (6e): Light-brown semisolid; (219 mg, 50%): $R_f = 0.46$ (EtOAc/*n*-hexane, 70:30); IR (neat): $\tilde{\nu}_{max} = 3432$, 2917, 1630, 1441, 1275 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): $\delta = 7.57$ (d, $J = 7.9$ Hz, 1H), 7.34 (d, $J = 8.1$ Hz, 1H) 7.27–7.17 (m, 6H), 7.08–7.04 (m, 4H), 6.94 (t, $J = 7.5$ Hz, 1H), 6.45 (dd, $J = 8.7$, 2.3 Hz, 1H), 4.68 (t, $J = 7.4$ Hz, 1H), 4.27 (s, 2H), 3.92 (d, $J = 7.4$ Hz, 2H), 3.71 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 159.8$, 153.2, 136.9, 132.1, 128.0 (2CH), 127.3, 127.2, 126.7 (2CH), 126.5 (2CH), 122.7, 121.9, 120.8, 118.8, 118.0, 116.4, 116.2, 111.4, 111.1(2C), 110.8, 54.8, 44.5, 43.2, 34.7 ppm; MS (APCI): *m/z* (%): 439 (2) $[M+H]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{27}H_{26}N_4NaO_2$: 461.1948, found: 461.1948.

Biology

Maintenance and treatment of cell lines: HEK 293T (kidney), HCT-116 (colon), and Vero (normal) cell lines were cultured in monolayers and maintained in DMEM (Dulbecco's modified eagle medium) with 10% fetal bovine serum (FBS), 100 U mL^{-1} penicillin–streptomycin and 1.5 mM glutamine.[30] All other cell culture reagents were purchased from HIMEDIA (India). Cells were allowed to grow in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching 70–80% confluence, cells were treated with the indicated concentrations of different compounds dissolved in sterile DMSO. MTT and etoposide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cells were treated at various time intervals (24, 48, and 72 h) with the addition of freshly prepared working concentrations of compounds as shown.

MTT assay: To investigate the cytotoxicity of investigated compounds in kidney cancer (HEK 293T), colon cancer (HCT-116), and normal kidney (Vero) cells, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed.[33] This is a colorimetric assay that measures the reduction of MTT (yellow) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria, where it is reduced to an insoluble, dark purple formazan product. The cells are then solubilized with an organic solvent (e.g., isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. As reduction of MTT can only occur in metabolically active cells, the level of activity is thus a measure of the viability of the cells. For our study, cells were seeded in triplicate for different time periods (24, 48, and 72 h) and then treated with investigated compounds at concentrations ranging from 0 μM to 100 μM. After treatment, MTT was added, and cells were incubated for 4–6 h for the formation of formazan crystals. The resulting formazan crystals were then dissolved in organic solvent, leading to formation of purple color, which was measured spectrophotometrically at a wavelength of 570 nm using an ELISA reader (Tristar, Berthold Technologies, Germany). This experiment was performed in triplicate.

Clonogenic assay: The clonogenic cell survival assay is a long-term cell viability assay that determines the ability of a single cell to proliferate indefinitely, thereby retaining its reproductive ability to form a colony or a clone. This assay was performed to examine the anti-colony-forming potential of investigated DIM derivatives in kidney cancer (HEK 293T) cell lines, according to the protocol described previously.[29] Approximately 2000–2500 HEK 293T cells were seeded in 24-well plates, allowed to grow for 24 h, and then treated with various investigated compounds at their LC_{50} concentrations for the next 48 h. The media was then aspirated, and cells were allowed to grow in fresh medium for another five to six doublings; afterward, media was removed from the wells, and the plate was air-dried and stained with 0.2% crystal violet. The wells were washed twice with distilled water, and colonies were counted using a gel documentation system (UVP, Germany).

DAPI nuclear staining: Chromatin condensation, nuclear fragmentation, and bubble-shaped nuclei are the major phenotypically observed phenomena for apoptotic cells. Apoptosis can be detected by staining the cells with 4,6-diamidino-2-phenylindole (DAPI), according to the protocol described previously.[34] Briefly, HEK 293T cells were treated after reaching 70–80% confluence with LC_{50} con-

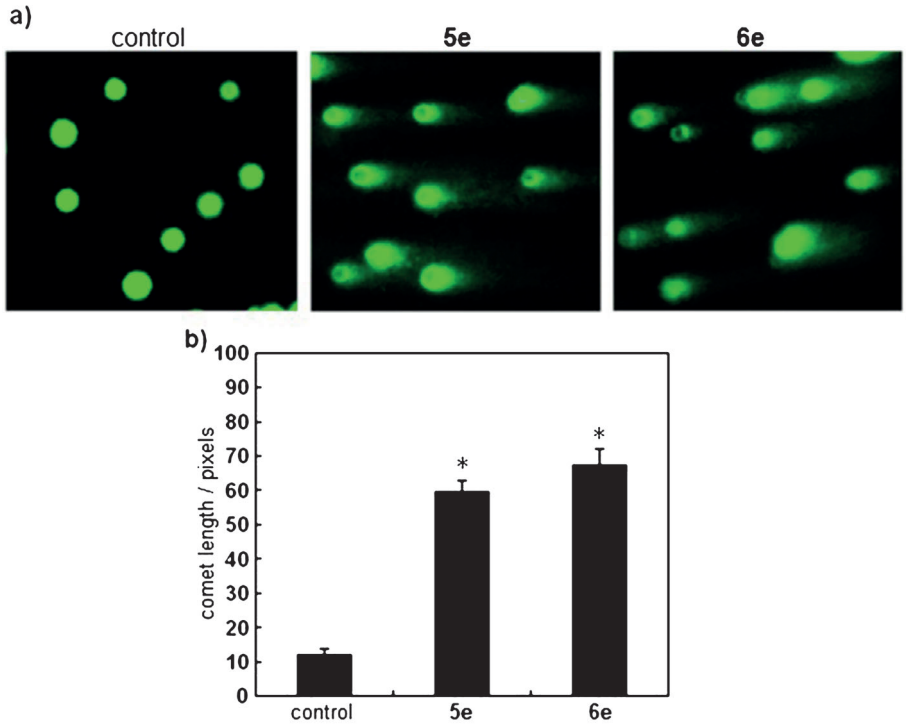


Figure 9. Comet assay: a) HEK 293T cells were treated with DIM aminophosphonates (**5e**) and DIM urea (**6e**), and a comet assay was performed as described in the Experimental Section. Images were captured using a fluorescence microscope (Nikon, Japan) at 10× magnification and represent one of three different experiments. b) The diagram shows average tail length of comet formation taken from images in panel a). The tail lengths of the comets were measured using commercially available TriTek CometScore software. Data are the mean ± SD of three different experiments; $*p < 0.05$.

centrations of DIM derivatives for 48 h. Cells were washed with ice-cold 1× PBS and fixed in an ice-cold acetone/MeOH (1:1) mixture for 10 min at 4 °C in the dark. Cells were then washed twice with ice-cold 1× PBS and stained with DAPI. The excess DAPI stain was removed with repeated washings with ice-cold 1× PBS, apoptotic cells were examined, and micrographs were taken at 40× magnification using a fluorescence microscope (Nikon, Japan).

Wound healing assay: A wound healing assay[29] was carried out to assess the anti-migratory activity of cancerous cells in the presence of investigated compounds. For this, HEK 293T cells were seeded in 35 mm tissue culture plates. After reaching 90% confluence, a sharp wound was made on the monolayer by scratching cells at an angle of 37° from the plane by using a sterile 200 μL microtip. Cells were treated with DIM derivatives at their LC_{50} concentrations and allowed to proliferate for 48 h with respect to the control. Micrographs were taken at different time intervals (0, 12, 24, 36, and 48 h) at 40× magnification with an inverted microscope (Nikon, Japan) to determine the healing of the wound. Data presented here is the best of three independent experiments.

Comet assay: For evaluation of DNA damage capacity of the cells after treatment with DIM derivatives, a single cell gel electrophoresis (comet assay) was performed according to the protocol described earlier.[35] Briefly, HEK 293T cells were treated with DIM derivatives at their LC_{50} concentrations for 48 h. After treatment, cells were harvested by trypsinization, and slides were prepared in 0.5% low melting agarose and then stained with SYBR green in the dark at 4 °C. Images were captured using a fluorescence microscope (Nikon, Japan) at 10× magnification to observe the migration of fragmented DNA. Tail lengths of the comets were measured using commercially available TriTek CometScore software.

Cell cycle analysis: To investigate cell cycle regulation and to understand the mechanism of action of DIM derivatives on apoptosis of kidney cancer cells, FACS analysis was used; 70–80% confluent HEK 293T cells were treated by DIM derivatives at LC_{50} concentrations for 48 h and incubated. Cells were then harvested according to the protocol described previously.[29] For cell cycle analysis, signals of 10000 cells per sample were considered. Evaluation of DNA content in cells was carried out using FACS (Becton-Dickinson, CA) analysis. Calculation of the population of cells in different phases of the cell cycle was determined using Cell Quest Software (Becton-Dickinson). Data shown here is the best of three independent experiments.

Western blot analysis: HEK 293T cells were seeded in 100 mm tissue culture plates and incubated at 37 °C in a CO_2 incubator. At 80% confluence, cells were treated by DIM derivatives at their LC_{50} concentrations for 48 h. Cells were then harvested, and the lysate was prepared by adding RIPA lysis buffer containing protease inhibitors.[34] Protein concentrations were estimated using the Bradford method. Proteins (40–50 μg) were separated by 10% SDS-PAGE. For immunoblotting, antibodies for anti-caspase-3, anti-NF-κB (p65), anti-PARP, anti-p53, and GAPDH (loading control) were used. All antibodies were procured from Cell Signaling Technology (CA, USA).

Immunocytochemistry analysis of caspase-3 expression: HEK 293T cells were seeded in a 96-well plate, and the plate was kept inside a CO_2 incubator for 24 h. After confluence, the cells were treated at the LC_{50} value of the investigated compounds and were incubated for 24 h. After incubation, the media was removed and washed with 1× PBS. The cells were fixed by adding acetone/MeOH (1:1), and the plate was incubated for 20 min at −20 °C. The fixer was then removed, and the plate was blocked for 30 min by adding 2% BSA and triton X-100 in 1× PBS. After blocking, primary antibody against caspase-3 (cat. #9662, Cell Signaling) was added, and the plate was incubated for 2 h at 37 °C. The plate was then washed with PBS, and secondary antibody conjugated with FITC was added before incubating for 1 h at 37 °C. Finally, the plate was stained with DAPI nuclear stain, and the cells were observed under a fluorescent microscope (Nikon, Japan). Micrographs were taken at 40× magnification.

Luciferase assay: To measure the activity of transcription factor promoters, a luciferase-based assay was performed. According to the principle, the relative luciferase activity is proportional to the pro-

moter activity of the cells.[29] After reaching 70–80% confluence, the two plasmid constructs, NF-κB (p65) (2.0 μg) and β-galactosidase (1.0 μg), were transfected in HEK 293T cells and incubated for 6–8 h. The NF-κB luciferase construct p5XIP10B (containing five tandem copies of the NF-κB site from the IP10 gene) and β-galactosidase were transfected transiently with lipofectamine for 6–8 h. After incubation, existing media was replaced with fresh media and incubated again for the next 12 h. The cells were then treated with compounds according to their respective LC_{50} concentrations. Transfection efficiency was obtained by measuring the activity of β-galactosidase. Luciferase activity was measured using a microplate reader (Multimode ELISA reader, Tristar, Berthold Technologies, Germany).

Abbreviations

4′-6-Diamidino-2-phenylindole (DAPI); 3,3′-diindolylmethane (DIM); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); fluorescence-activated cell sorting (FACS); lethal concentration required to kill 50% of cells in culture (LC_{50}); nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB); poly(ADP-ribose) polymerase (PARP).

Acknowledgements

The authors thank Dr. George R. Stark (Lerner Research Institute, Cleveland Clinic Foundation, USA) for providing plasmid 5XIP10B. The authors also gratefully acknowledge financial support of this investigation from the Indian Council of Scientific & Industrial Research (CSIR), the Department of Biotechnology (DBT) of the Government of India and the Indian Council of Medical Research (ICMR). S.K. is thankful to the Department of Science and Technology (DST), India for his fellowship.

Keywords: 3,3′-diindolylmethanes · apoptosis · antitumor agents · cancer · indole alkaloids · NF-κB

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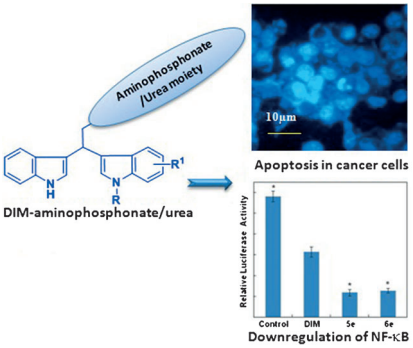
Received: June 19, 2013
Revised: July 23, 2013
Published online on ■■ ■■, 0000

DIM the lights: A rational approach toward structural elaboration of a bioactive natural product, diindolylmethane (DIM), led to our exploration of novel DIM aminophosphonate and urea derivatives as potent NF-κB p65-downregulating apoptotic anticancer agents that possess enhanced activities over the parent DIM.



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