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Abstract. Cell growth and division are highly regulated processes, although a notable exception is provided by the cancer cell, which arises as a variant that has lost the usual proliferation control pathways. Consequently, there is growing interest in the search for antitumoral substances with high efficacy, low toxicity, and minimum side effects. In this sense, we synthesize eight diindolylmethane derivatives and the in vitro antitumor activity against murine L5178Y lymphoma cells was assessed. The preliminary results showed that the substituent and its position on the phenyl group were important for its potency against the lymphoma cells tested. Compound **3a** was the most active compound with 93 % cell grown inhibition and 71.04% of apoptosis.
Keywords: DIM derivatives, apoptosis, cytotoxic effects, L5178Y cells.

Resumen. El crecimiento celular así como la división celular son procesos altamente regulados, sin embargo, una notable excepción son las células cancerosas, las cuales han perdido sus mecanismos de control y regulación. En consecuencia el creciente interés por la búsqueda de sustancias antitumorales con alta eficiencia, baja toxicidad y la menor cantidad de reacciones adversas, nos condujo a sintetizar una familia de ocho diindolimetanos, los cuales fueron evaluados como inductores de apoptosis. Los resultados preliminares mostraron que el sustituyente y la posición en el grupo fenilo son importantes para esta actividad. El compuesto **3a** de la serie estudiada mostró ser el más activo con un 93 % de la inhibición del crecimiento celular y una inducción de apoptosis de 71.04 %.
Palabras clave: Derivados del DIM, apoptosis, efecto citotóxico, Células L5178Y.

Introduction

Cancer is one of the most serious human health concerns world-wide. Consequently, there is growing interest in the search for anti-cancer substances with high efficacy, low toxicity, and minimum side effects. Both in vivo and in vitro 3,3′-diindolylmethane (DIM) is an acid-catalyzed condensation product of indole-3-carbinol, which is a constituent of cruciferous vegetables such as broccoli, cauliflower, cabbage and brussel sprouts [1-4]. Previous studies have shown that DIM is a promising antitumor agent; related compounds inhibit cancer in multiple target organs, such as mammary tissue, liver, endometrium, lung and colon in animal models [5-7] and also kills cancer cell lines [8-10].

Apoptosis is a normal and very important event in the life cycle of most cells. It is characterized by morphological and structural features involving mitochondrial swelling, release of cytochrome C, cytoplasmic membrane blebbing, chromatin condensation, caspase activation, DNA fragmentation, and cell fragmentation [11-13]. Another important event is the G_0/G_1 phase arrest, which is a crucial DNA damage checkpoint and acts as an important safeguard for genomic stability [14]. Neighboring cells and macrophages rapidly digest these cellular fragments or apoptotic bodies without inducing either inflammation or cell damage. Some evidence indicates that the regulation of the tightly controlled apoptotic process contributes to the pathogenesis of a number of human diseases. Several reports have shown that DIM inhibits cancer cell proliferation by inducing cell cycle arrest, translocation of cytochrome C from the mitochondria to the cytosol, activation of initiator caspase 9, effector of caspases 3 and 6 and induction of apoptosis [15-18].

On the other hand, recently we reported a new method to obtain several DIM derivatives by ecological friendly synthesis in presence of bentonitic clay as catalyst, reagent support and reaction media, using infrared light as energy source in short reaction times [19].

Based on our previous investigation dealing with the synthesis of antineoplastic compounds and their activity studies [20, 21], in this paper we synthesized a series of diindolylmethane derivatives with a substituted phenyl group on the methane carbon (Figure 1), and the in vitro antitumor activity against murine L5178Y lymphoma cells was assessed.

Results and discussion

Figure 2 summarizes the procedure that was used to prepare the new DIM derivatives. Table 1 shows the substituent groups and their position on the phenyl group as well as the corresponding

yields and the melting points. The spectroscopic data (^{1}HNMR, ^{13}CNMR, EI-MS) for each DIM derivative **3a-3h** in order to identify them were obtained. Is important to note that compounds **3b**, **3c** and **3e** were previously reported [22], and to our knowledge the other title compounds have not been reported.

Cell viability as an indicator of cytotoxicity was determined by measuring the capacity of L5178Y cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
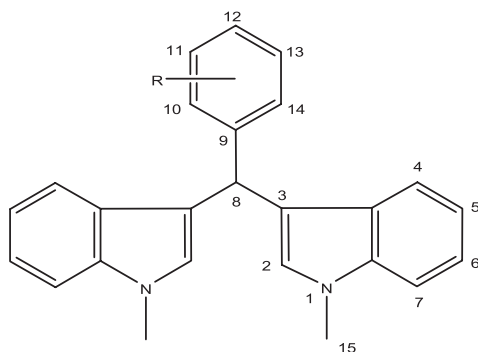

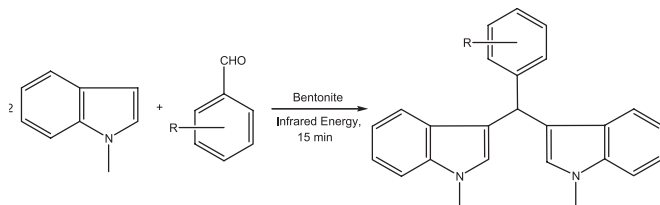**Fig. 1.** General structure of synthesized DIM derivatives.

Fig. 2. General procedure for the synthesis of compounds **3a-h**.

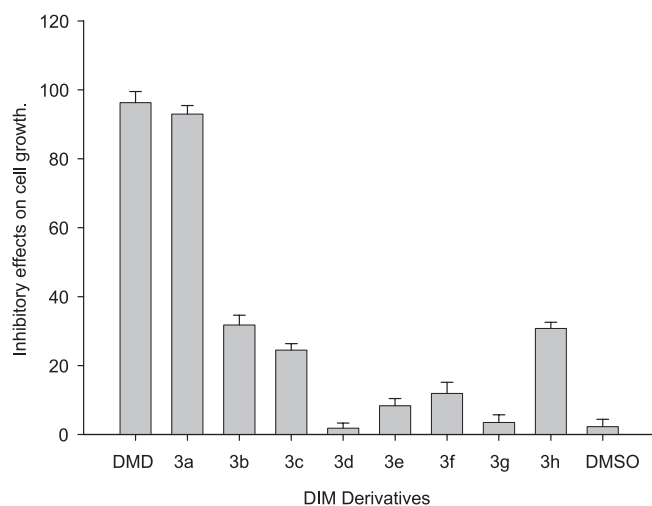**Fig. 3.** Inhibitory effects on the growth of murine L5178Y lymphoma cells with 100μM of each probed compound, determined by MTT assay at 24 h, as described previously. Data are represented as the mean ± SD, n = 3.

bromide) to formazan. MTT is reduced to the blue-colored formazan by the mitochondrial enzyme succinate dehydrogenase, which is considered as a reliable and sensitive measure of mitochondrial function [18].

The results of the cytotoxicity studies against the L5178Y cells of all tested compounds are summarized in Figure 3. These results show that compound **3a** (*m*-OH) had similar potent cytotoxicity as azodicarboxylic acid bis[dimethylamide]. Compared with the untreated cells, only 7 % of them were viable with this compound. The molecules containing *m*-OMe and *p*-OMe, as well as *m*-NO$_2$ and *p*-NO$_2$ did not affect the metabolic function of L5178Y cells. When the dimethylamino, methyl or formyl groups were introduced on the phenyl group the resulting **3b**, **3c** and **3h** compounds were more active than the **3d**, **3e**, **3f** and **3g** products.

In general, we observed that when the substituent group ability to achieve an hydrogen bond decreases the S phase of cell cycle is more arrested. In addition, this behavior is enhanced with substituent groups in *meta* position on phenyl group. However, with these results a structure-activity relationship is not very clear, since for **3d-3g** compounds the substituent group electronic nature are both electron releasing and electron withdrawing. In this way we suggest that the substituent group availability to form hydrogen bonds assists the inhibition of the enzyme succinate dehydrogenase, with the consequently loss of mitochondrial function.

As cancer cells are prone to have inactivated cell cycle checkpoints and/or apoptotic progress, regulation of cycle progression and induction of apoptosis have been regarded as novel strategies for the controlling cancer cells proliferation; thus, the effect of the compounds on cell cycle progression was analyzed by flow cytometry.

The induction of cell death was monitored by the appearance of the sub-G$_0$/G$_1$ peak in the cell cycle analysis (Table 2). The cell shrinkage characteristic of apoptotic cells was also evident in the FSC vs SSC dot plots generated by **3a**. This cell shrinkage was observed as a reduction in the FSC Figure 4. Compared to control cells, those exposed to DIM derivatives **3b-3h** were strongly arrested at the S phase of the cell cycle for up to 24h. When the murine L5178Y lymphoma cells were treat-

Table 1. Synthesized DIM derivatives^a

Compound	R	Yield (%)	Mp (^{o}C)
3a	*m*-OH	90	197-189
3b	*p*-NMe$_2$	90	165-167
3c	*p*-Me	85	196-198
3d	*m*-OMe	85	179-181
3e	*p*-OMe	90	211-213
3f	*m*-NO$_2$	70	161-163
3g	*p*-NO$_2$	70	211-213
3h	*p*-CHO	80	231-232

^{a}All the compounds were characterized by their spectroscopic data (^{1}H NMR, ^{13}C NMR, EIMS).

ed with 100 µM of **3a** during 24h, the cells population in the sub G_0/G_1 phase was drastically increased to 71.04% Figure 5. An augment of cells in the sub G_0/G_1 phase generally indicates an increase of apoptotic cell death; therefore, our data showed that **3a** is an effective apoptotic inducer agent on these cells.

Conclusion

In conclusion, this study provides experimental evidence that DIM derivative **3a** (*m*-OH) induces strong apoptosis in murine L5178Y lymphoma cells and DIM derivatives **3b-3h** stop the cell replication by arresting cells in the S phase of the cell cycle. We are currently conducting further studies to compare the results here presented with those that will be obtained with DIM and also to know the involved molecular mechanisms that promote apoptosis in human cancer cells with the use of the reported compounds. This could lead to the development of new and more effective strategies in cancer prevention and treatment.

Experimental

Chemical Section.

All reagents were used as obtained from commercial suppliers (Sigma-Aldrich) without further purification. Melting points of **3a-3h**, uncorrected, were determined on a Fisher-Johns apparatus. The synthesized compounds were routinely checked by ^{1}HNMR and ^{13}CNMR and mass spectrometry. ^{1}HNMR and ^{13}CNMR spectra were recorded at ambient temperature using a Varian Mercury-300 spectrometer at 300 MHz and 75 MHz for hydrogen and carbon, respectively; the solvents were $CDCl_3$ or DMSO-d_6, and TMS was employed as internal reference. The EIMS measurements were determined using JEOL (JMS-SX102-A and JMS-AX505-HA) mass spectrometers.

Table 2. Effect of diindolylmethanes **3a-h** on the cell cycle of murine L5178Y lymphoma cells.[a]

Compound	Content of cell cycle (%)			
	Sub G_0/G_1	G_0/G_1	S	G_2/M
DMSO	1.32	64.53	21.62	12.53
3a	71.04	16.45	9.73	2.78
3b	1.90	53.22	42.88	2.01
3c	2.06	44.67	51.18	2.08
3d	1.88	52.49	44.26	1.37
3e	2.60	61.68	34.60	1.12
3f	1.79	62.18	35.28	0.75
3g	1.88	66.03	31.35	0.74
3h	2.33	63.93	31.76	1.98
DMD	42.03	23.43	27.24	7.30

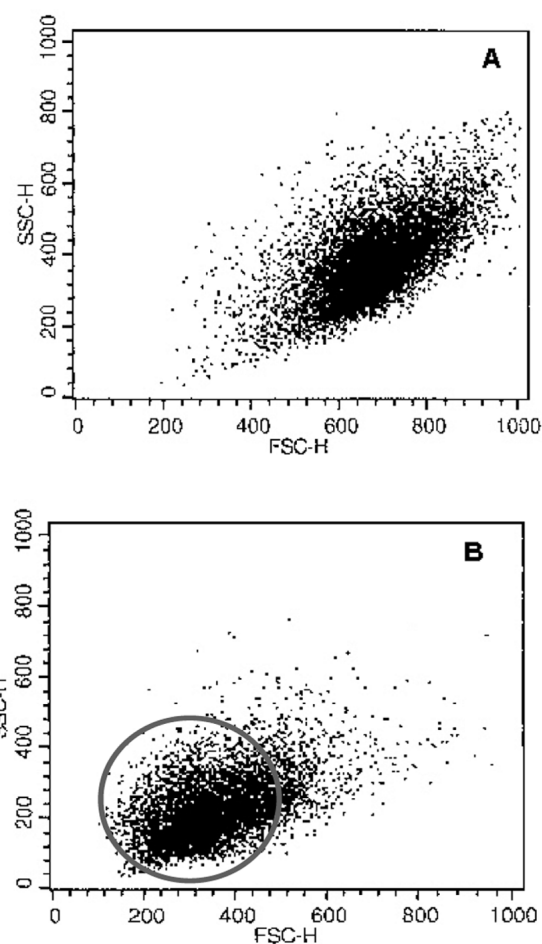[a] The used concentration for each compound was 100 µM.

Fig. 4. Effect of **3a** on the cellular volume. FSC vs SSC dot plot of L5178Y cells incubated for 24 h. A) Untreated and B) treated with **3a** 100µM. The cells indicated inside the circle are those with reduced cellular volume. One of three representative experiments is shown.
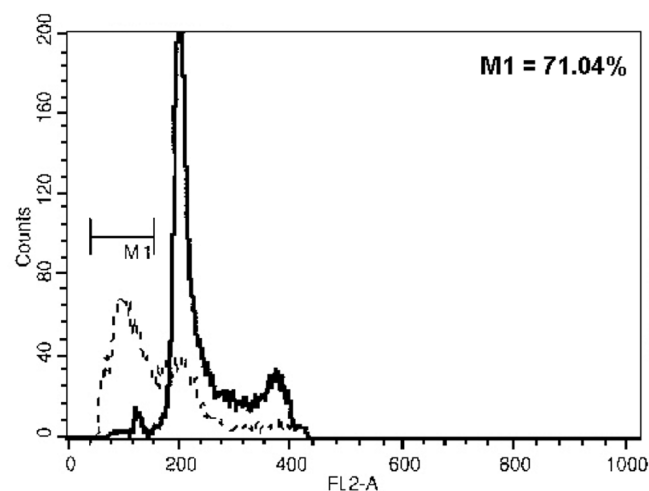

Fig. 5. Effect of **3a** on DNA distribution. DNA histograms of L5178Y cells: (—) untreated and (⋯⋯) treated with **3a** 100 µM. Results are expressed as a percentage of the Sub G_0/G_1.

General method for the synthesis
of Diindolylmethanes 3a-3h.

A typical experimental procedure is as follows. To a mixture of indole (1.0 g, 8.54 mmol) and the corresponding benzaldehyde (0.45 g, 4.27 mmol), a bentonitic clay (4g) was added. The reaction mixture was IR irradiated with a commercial IR lamp (250 W) for 15 minutes, according to the methodology reported by Pool and Teuben [23] (the temperature reached during each reaction was 180 °C). After the reaction time no changes were detected by thin layer chromatography. Then, to the produced reaction mixture a 1:1 water-methanol mixture was added for recrystallization purpose to give the corresponding diindolylmethane derivative.

(*m*-Hydroxyphenyl)-3,3'-diindolylmethane (**3a**): ^{1}H NMR (Acetone-d_6 300 MHz) δ 3.71 (6H, s, H-15), 5.81 (1H, s, H-8), 6.66 (1H, ddd, J_o = 7.8 Hz, J_m = 3.9 Hz, J_p= 1.0 Hz, H-12), 6.70 (2H, d, J = 1.0 Hz, H$_2$), 6.83 (1H, t, J_m = 4.2 Hz, J_p = 2.1 Hz, H-14), 6.86 (1H, m, H-10), 6.91 (2H, td, J_o = 15 Hz, J_m = 7.8 Hz, J_p = 1.0 Hz, H-7), 7.09 (1H, m, H-11), 7.11 (2H, td, J_o = 15.4 Hz, J_m = 7.8 Hz, J_p = 1.0 Hz, H-4), 7.34 (4H, m, H-5 and H-6), 8.15 (1H, br s, O*H*); ^{13}C NMR (Acetone- d_6, 50 MHz) δ 158.1 (C-13), 147.5 (C-9), 138.4 (C-7a), 129.8 (C-11), 128.8 (C-2), 128.4 (C-3a), 122.0 (C-4), 120.7 (C-10), 120.4 (C-6), 119.2 (C-7), 118.8 (C-3), 116.3 (C-14), 113.7 (C-14), 110.0 (C-5), 40.7 (C-8), 32.7 (C-15); EIMS *m/z* (rel. int.): 366 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{25}H_{22}N_2O$; *m/z* 273 [M$^+$-C$_6$H$_5$O].

(*p*-Dimethylaminophenyl)-3,3'-diindolylmethane (**3b**): ^{1}H NMR (Acetone-d_6 300 MHz) δ 2.88 (6H, s, N(C*H$_3$*)$_2$), 3.73 (6H, s, H-15), 5.77 (1H, s, H-8), 6.66 (2H, d, J = 0.9 Hz, H-2), 6.67 (2H, d, J_o = 8.7 Hz, H-11), 6.90 (2H, td, J_o = 15.0 Hz, J_m = 7.7 Hz, J_p = 1.0 Hz, H-7), 7.12 (2H, td, J_o = 15.0 Hz, J_m = 7.7 Hz, J_p = 1.2 Hz, H-4), 7.19 (2H, d, J_o = 8.8 Hz, H-10), 7.34 (4H, m, H-5 and H-6); ^{13}C NMR (Acetone- d_6, 50 MHz) δ 150.0 (C-12), 138.4 (C-7a), 133.7 (C-9), 129.8 (C-10), 128.7 (C-4), 128.5 (C-3a), 121.9 (C-6), 120.5 (C-11), 119.7 (C-3), 119.0 (C-7), 113.2 (C-2), 110.0 (C-5), 40.8 (N(C*H$_3$*)2), 39.9 (C-8), 32.6 (C-15); EIMS *m/z* (rel. int.) 393 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{27}H_{27}N_3$; *m/z* 273 [M$^+$-C$_6$H$_4$NMe$_2$].

(*p*-Methylphenyl)-3,3'-diindolylmethane (**3c**): ^{1}H NMR (Acetone-d_6 300 MHz) δ 2.28 (3H, s, C-C*H$_3$*), 3.73 (6H, s, H-15), 5.85 (1H, s, H-8), 6.68 (2H, d, J = 0.9 Hz H-2), 6.90 (2H, td, J_o = 14.7 Hz, J_m = 7.5 Hz, J_p = 1.2 Hz, H-7), 7.07 (2H, d, J_o = 7.8 Hz, H-11), 7.12 (2H, td, J_o = 15.8 Hz, J_m = 7.7 Hz, J_p = 1.2 Hz, H-4), 7.25 (2H, d, J_o = 7.8 Hz, H-10); ^{13}C NMR (Acetone- d_6, 50 MHz) δ 142.8 (C-9), 138.4 (C-7a), 135.9 (C-12), 129.5 (C-10), 129.3 (C-11), 128.8 (C-2), 128.4 (C-3a), 122.0 (C-4), 120.4 (C-6), 119.1 (C-3), 119.0 (C-7), 110.0 (C-5), 32.6 (C-15), 21.0 (C-C*H$_3$*); EIMS *m/z* (rel. int.): 364 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{26}H_{24}N_2$; *m/z* 273 [M$^+$-C$_6$H$_4$Me].

(*m*-Methoxyphenyl)-3,3'-diindolylmethane (**3d**): ^{1}H NMR (Acetone-d_6, 300 MHz) δ 3.71 (3H, s, O-C*H$_3$*), 3.74 (6H, s, H-15), 5.87 (1H, s, H-8), 6.73 (2H, d, J = 0.9 Hz, H-2), 6.76 (1H, m, H-12), 6.92 (2H, td, J_o = 15 Hz, J_m = 7.7 Hz, J_p = 0.9 Hz, H-7), 6.96 (2H, m, H-10 and H-14), 7.12 (2H, td, J_o = 15.1 Hz, J_m = 7.5 Hz, J_p = 1.2 Hz, H-4), 7.19 (1H, dd, J_o = 7.0 Hz, J_p = 1.0 Hz, H-11), 7.35 (4H, m, H-5 and H-6); ^{13}C NMR (Acetone-d_6, 50 MHz) δ 160.6 (C-13), 147.5 (C-9), 138.4 (C-7a), 129.8 (C-11), 128.8 (C-2), 128.4 (C-3a), 122.0 (C-4), 121.8 (C-10), 120.4 (C-6), 119.2 (C-7), 118.8 (C-3), 115.6 (C-14), 111.5 (C-12), 110.1 (C-5), 55.3 (O-*C*H3), 40.8 (C-8), 32.7 (C-15); EIMS *m/z* (rel. int.): 380 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{26}H_{24}N_2O$; *m/z* 273 [M$^+$-C$_6$H$_4$OMe].

(*p*-Methoxyphenyl)-3,3'-diindolylmethane (**3e**): ^{1}H NMR (Acetone-d_6, 300 MHz) δ 3.74 (6H, s, H-15), 3.75 (3H, s, O-C*H$_3$*), 5.84 (1H, s, H-8), 6.68 (2H, d, J = 0.9 Hz, H$_2$), 6.83 (2H, dd, J_o = 9.0 Hz, J_m = 2.4 Hz, H-11), 6.91 (2H, td, J_o = 15.0 Hz, J_m = 7.4 Hz, J_p = 0.9 Hz, H-7), 7.12 (2H, td, J_o = 15.3 Hz, J_m = 7.2 Hz, J_p = 1.2 Hz, H-4), 7.28 (2H, ddd, J_o = 8.9 Hz, J_m = 7.4 Hz, J_p = 0.6 Hz, H-10), 7.33 (2H, m, H-5), 7.35 (2H, m, H-6); ^{13}C NMR (Acetone-d_6, 50 MHz) δ 158.9 (C-12), 138.4 (C-9), 137.8 (C-7a), 130.2 (C-10), 128.8 (C-2), 128.4 (C-3a), 122.0 (C-4), 120.4 (C-5), 119.2 (C-3), 119.1 (C-7), 114.2 (C-11), 110.1 (C-6), 55.3 (O-*C*H3), 39.9 (C-8), 32.7 (C-15); EIMS *m/z* (rel. int.): 380 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{26}H_{24}N_2O$; *m/z* 273 [M$^+$-C6H4OMe].

(*m*-Nitrophenyl)-3,3'-diindolylmethane (**3f**): ^{1}H NMR (Acetone-d_6, 300 MHz) δ 3.74 (6H, s, H-15), 6.11 (1H, s, H-8), 6.80 (2H, d, J = 0.9 Hz H-2), 6.94 (2H, td, J_o = 15 Hz, J_m = 7.4 Hz, J_p = 0.9 Hz H-7), 7.15 (2H, td, J_o = 15.4 Hz, J_m = 7.9 Hz, J_p = 1.2 Hz H-4), 7.35 (2H, m, H-5), 7.38 (2H, m, H-6), 7.56 (1H, dd, J_o = 15.9 Hz, J_p = 7.8 Hz, H-11), 7.84 (1H, d, J_m = 7.8 Hz, H-14), 8.10 (1H, ddd, J_o = 8.1 Hz, J_m = 2.4 Hz, J_p = 1.0 Hz, H-12), 8.24 (1H, t, J_o = 4.2 Hz, J_m = 2.1 Hz, H-10); ^{13}C NMR (Acetone-d_6, 50 MHz) δ 149.2 (C-13), 148.4 (C-9), 138.4 (C-7a), 135.9 (C-10), 130.2 (C-11), 129.1 (C-2), 128.0 (C-3a), 123.8 (C-14), 122.3 (C-4), 121.9 (C-12), 120.1 (C-5), 119.5 (C-7), 117.3 (C-3), 110.3 (C-6), 40.4 (C-8), 32.7 (C-15); EIMS *m/z* (rel. int.): 395 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{25}H_{21}N_3O_2$; *m/z* 273 [M$^+$-C$_6$H$_4$NO$_2$].

(*p*-Nitrophenyl)-3,3'-diindolylmethane (**3g**): ^{1}H NMR (Acetone-d_6, 300 MHz) δ 3.76 (6H, s, H-15), 6.10 (1H, s, H-8), 6.81 (2H, d, J = 0.9 Hz, H-2), 6.94 (2H, td, J_o = 15.0 Hz, J_m = 6.9 Hz, J_p = 0.9 Hz, H-7), 7.16 (2H, td, J_o = 15.2 Hz, J_m = 6.9 Hz, J_p = 1.2 Hz, H-4), 7.37 (4H, m, H-5 and H-6), 7.65 (2H, dd, J_o = 8.7 Hz, J_p = 0.6 Hz, H-10), 8.16 (2H, d, J_o = 9.0 Hz, H-11); ^{13}C NMR (Acetone-d_6, 50 MHz) δ 153.9 (C-12), 147.3 (C-9), 138.5 (C-7a), 130.5 (C-10), 129.1 (C-2), 128.0 (C-3a), 124.2 (C-11), 122.3 (C-4), 120.2 (C-5), 119.5 (C-7), 117.7 (C-3), 110.3 (C-6), 40.7 (C-8), 32.8 (C-15); EIMS *m/z* (rel. int.): 95 [M]$^{+\cdot}$ and base peak, corresponding to molecular formula $C_{25}H_{21}N_3O_2$; *m/z* 273 [M$^+$-C$_6$H$_4$NO$_2$].

(*p*-**Formylphenyl)-3,3'-diindolylmethane** (**3h**): ^{1}H NMR (Acetone-d_6, 300 MHz) δ 3.75 (6H, s, H-15), 6.02 (1H, s, H-8), 6.77 (2H, d, J = 0.9 Hz, H-2), 6.93 (2H, td, J_o = 15.0 Hz, J_m = 7.3 Hz, J_p = 0.9 Hz, H-7), 7.14 (2H, td, J_o = 15.5 Hz, J_m = 7.5 Hz, J_p = 0.9 Hz, H-4), 7.38 (4H, m, H-5 and H-6), 7.60 (2H, d, J_o = 8.4 Hz, H-10), 7.84 (2H, d, J_o = 8.4 Hz, H-11), 9.99 (1H, s, C*H*O); ^{13}C NMR (Acetone-d_6, 50 MHz) δ 192.5 (*C*HO), 153.0 (C-12), 138.4 (C-7a), 135.9 (C-9), 130.3 (C-11), 130.1 (C-10), 129.0 (C-2), 128.2 (C-3a), 122.2 (C-4), 120.2 (C-5), 119.4 (C-7), 117.8 (C-3), 110.2 (C-6), 40.9 (C-8), 32.7 (C-15); EIMS *m/z* (rel. int.): 378 [M][+.] and base peak, corresponding to molecular formula $C_{26}H_{22}N_2O$; *m/z* 273 [M[+]-C_6H_4CHO].

Biological Section.

Cell line and culture maintenance.

For the experiments, 2 x 10^{6} L5178Y tumor cells were implanted into the peritoneal cavity of 8- to 10- week-old BALB/C mice. On day 12 after tumor implantation, the mice were euthanized and the tumors were excised under sterile conditions and washed three times with PBS 1X. The cells were maintained in an RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 1% non-essential amino acids at 37 ºC under a 5% CO_2 atmosphere with 95% humidity.

In vitro cytotoxicity in murine L5178Y lymphoma cells using the MTT assay.

The L5178Y cells were suspended at a final concentration of 5x10^{4}/mL, seeded in 96-well microtiter plates and treated with 100 µM of each of the DIM derivatives **3a-h** dissolved in DMSO. The final concentration of solvent in the cell culture medium was less than 0.1%. The cells were then incubated at 37 ºC for 24 h, followed by incubation with MTT (0.5 mg/mL) for 4 h. MTT is reduced by the mitochondrial dehydrogenases of viable cells to a purple formazan product. The MTT/formazan product was dissolved in 100 µL of DMSO, and the corresponding absorbance, at 560 nm was measured with a microplate reader. Azodicarboxylic acid bis[dimethylamide] (DMD) was used with the same 100 µM concentration as a positive control anticancer agent. At each point, cell survival was calculated as the fraction of cells alive relative to control.

Flow cytometry analysis for DNA distribution.

Cell suspensions (1 x 10^{6}) were pelleted, resuspended in 500 µL of phosphate-buffered saline (PBS) and fixed for at least 24 h at -20ºC in 70% ethanol. Fixed cells were washed twice with PBS 1X and permeabilized with phosphate-citrate buffer at pH 7.8 for 3 min, after the cells were washed twice with PBS 1X and stained with 300 µL of 20 µg/mL PI (Sigma), and incubated for at least 30 min at room temperature. The stained cells were analyzed using a BD Biosciences (San Jose, CA)

FACScan flow cytometer and CellQuest software for relative DNA distribution. We employed DMD as positive control.

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