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Synthesis of a new bis(indolyl)methane that inhibits growth and induces apoptosis in human prostate cancer cells

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The synthesis and the antiproliferative activity against the human breast MCF-7, SkBr3 and the prostate LNCaP cancer cell lines of a series of bis(indolyl)methane derivatives are reported. The synthesis of new compounds was first accomplished by the reaction of different indoles with trimethoxyacetophenone in the presence of catalytic amounts of hydrochloric acid. A second procedure involving the use of oxalic acid dihydrate [$(CO_2H)_2{\cdot}2H_2O$] and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide in water was carried out and led to better yields. Compound **5b** significantly reduced LNCaP prostate cancer cell viability in a dose-dependent manner, with an IC_{50} of $0.64 \pm 0.09\ \mu M$. To determine whether the growth inhibition was associated with the induction of apoptosis, treated cells were stained using DAPI. LNCaP cells treated with $1\ \mu M$ of **5b** showed the morphological changes characteristic of apoptosis after 24 h of incubation.

Keywords: antiproliferative activity; apoptosis; bis(indolyl)methane; indole

1. Introduction

Breast and prostate cancers are frequently diagnosed malignancies in Western countries (Ferlay et al. 2007). Although many anticancer drugs have been developed and used clinically for controlling breast and prostate cancers, more specific and efficacious anticancer and/or chemopreventive agents are still needed. Bis(indolyl)methanes and their derivatives constitute an important class of heterocyclic compounds used in the pharmaceutical industry. These molecules can be found in plants belonging to Cruciferae family and they are known to promote oestrogen metabolism and to induce apoptosis in human tumour cells. Bis(3-indolyl)methane (3,3′-diindolylmethane, DIM, **1**) is the principal compound derived from the metabolism of the indole-3-carbinole (I3C, **2**), an autolysis product of the glucosinolate glucobrassicin found in vegetables such as broccoli, Brussels sprouts cabbage and kale (Figure 1). The diindolylmethane is a promising antitumour agent (Hong et al. 2002). On the basis of the structure of previously reported bis(indolyl)methanes, a novel series of derivatives were designed and synthesised as anticancer agents. The cytotoxicity of the new compounds was determined towards different cancer cell lines: MCF-7 and SkBr3 as models for breast cancer and LNCaP as a model for prostate cancer.

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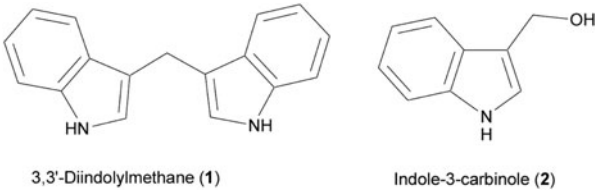


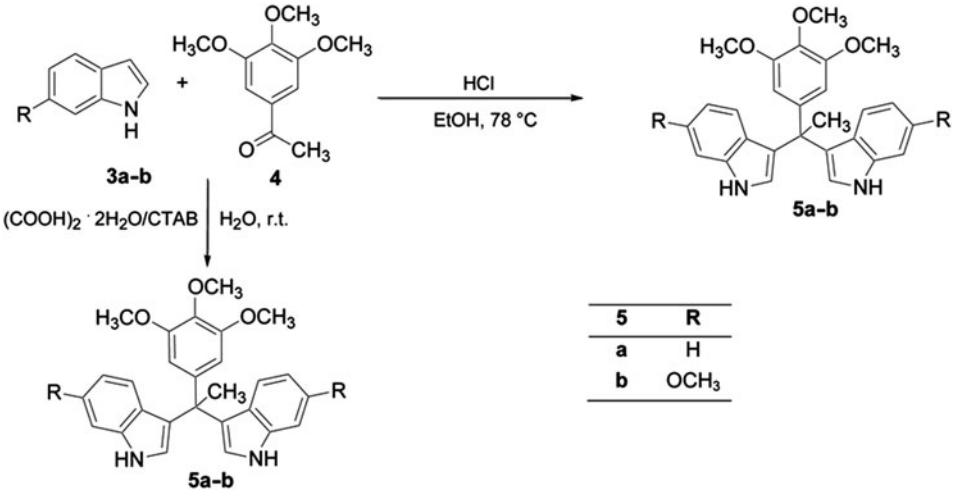
Figure 1. Chemical structure of 3,3′-diindolylmethane and its precursor, I3C.

2. Results and discussion

2.1 *Synthesis of bis(indolyl)methanes*

The synthesis of bis(indolyl)methanes is based on the indole reaction with aldehydes or ketones, which leads to the formation of an intermediate that reacts with a second molecule of indole to form bis(indol-3-yl)methanes (Ghorbani-Vaghei et al. 2010). Several methods, based on the use of different promoters, were reported in previous years (Deb & Bhuyan 2005; Zhang & Du 2009; Epifano et al. 2011). The new derivative **5a** was obtained from 1H-indole **3a** and 3′,4′,5′-trimethoxyacetophenone **4** in the presence of catalytic amounts of hydrochloric acid (Scheme 1). This procedure provided a yield of 26%. We wanted to assess whether the procedure could also be applied to the synthesis of substituted compounds. To this end, the 6-methoxy-indole was used in place of 1H-indole. The substituted bis(indolyl)methane **5b** was obtained with a yield of 9%. Our goal then was to improve the strategy of synthesis of compounds **5a** and **5b** in order to increase the yield of reactions. The analogues were obtained using a more appropriate procedure described by Ghorbani-Vaghei et al. (2010). The reaction is described in Scheme 1: the indole reacts with trimethoxyacetophenone in the presence of oxalic acid dihydrate [$(CO_2H)_2{\cdot}2H_2O$] and *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) in water. This procedure gave increased yields compared with previous attempts.

Compound **5a** was obtained with a yield of 62%, whereas the substituted derivative **5b** was synthesised with a yield of 42%. The synthesised compounds were characterised by ^{1}H NMR, ^{13}C NMR and mass spectral data.



Scheme 1. Synthesis of bis(indolyl)methanes **5a** and **5b**. **3a**: R = —H; **3b**: R = —OCH_3. Reagents and conditions: (a) HCl, EtOH, 78°C, 19 and 22 h for **5a** and **5b**, respectively. (b) $(COOH)_2{\cdot}2H_2O$/CTAB, H_2O, room temperature, 7 and 10 h for **5a** and **5b**, respectively.

2.2 *Antiproliferative activity of new analogues*

The effects of synthesised compounds on viability of human cancer cell lines (MCF-7, SkBR3 and LNCaP) were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a simple bioassay used for the primary screening of compounds. For each cell line used in this study, there was a linear relationship between cell number and absorbance, measured at 570 nm in both control and drug-treated wells. Cisplatin, doxorubicin and paclitaxel were used as positive controls. The antiproliferative activity of synthesised compounds was determined after 48 h of treatment. Effects on proliferation of MCF-7, SkBr3 and LNCaP cell lines are shown in Table 1. Compound **5b** significantly reduced LNCaP prostate cancer cell viability in a dose-dependent manner compared with their respective vehicle (0.1% dimethyl sulfoxide (DMSO))-treated control cells, with a 50% inhibition (IC_{50}) value of 0.64 ± 0.09 μM. Between the other two cell lines, MCF-7 and SkBr3, the latter showed a better inhibition on its proliferation rate in response to compound **5b**, with IC_{50} values of 20.03 ± 2.18 and 9.6 ± 1.36 μM, respectively. On the contrary, compound **5a** showed no activity.

Obtained data show that the presence of a methoxy group in position 6 of the indole leads to a substantial improvement in cytotoxic activity. Some 6,6′-methoxy-diindolylmethanes and derivatives were already synthesised by Benabadji et al. (2004). These molecules showed good antioxidant activity, which was related to the presence of the methoxy group, a strong electron-donating group which increases the stability of the benzene ring and amplifies the radical scavenging activity of compounds. Previous studies about a series of 3-formyl-2-phenylindoles demonstrated as well that the presence of a methoxy group at *para* position of the 2-phenyl ring led to more active compounds against human breast cancer cells, a finding which was correlated with a different binding mode/site (Gastpar et al. 1998). Here, the presence of a methoxy groups clearly influenced the antiproliferative activity of tested compounds. Further studies will, of course, be needed to confirm these observations and evaluate their significance.

2.3 *Evaluation of the mechanism of action*

Observation of LNCaP cells under a microscope revealed that cells treated for 48 h with vehicle exhibited normal features with typical adherent, membrane intact morphology. On the contrary, treatment with different concentrations of **5b** (from 0.0156 to 100 μM) for 48 h revealed changes in morphology for the majority of LNCaP cells (Figure 2, bright field). To assess if morphological changes and growth inhibition were associated with an apoptotic mechanism, nuclei of cell monolayers were stained with a fluorescent dye, DAPI. Cells were left untreated (control) or treated for 24 h with compound **5b** before being stained with DAPI (Sigma-Aldrich, Milan, Italy). This method showed that LNCaP cells treated with compound **5b** had brighter blue nuclei compared with control, untreated cells (Figure 2, DAPI). This is consistent with more condensed chromatin which is a feature of apoptotic cells.

Table 1. Antiproliferative activity of bis(indolyl)methanes.

	IC_{50} (μM)		
Cell line	LNCaP	SkBr3	MCF-7
5a	ND	ND	ND
5b	0.64 ± 0.09^{a}	9.60 ± 1.36^{c}	20.03 ± 2.18^{e}
Cisplatin	2.01 ± 0.12^{b}	13.90 ± 0.90^{dc}	15.46 ± 1.08^{d}
Doxorubicin	28.41 ± 2.52^{f}	–	1.9 ± 0.03^{b}
Paclitaxel	–	23.94 ± 1.99^{e}	–

Notes: Data are expressed as mean ± SEM ($n = 6$). Different letters indicate statistically significant differences at $P < 0.05$ (Tukey's test). ND: not detectable.

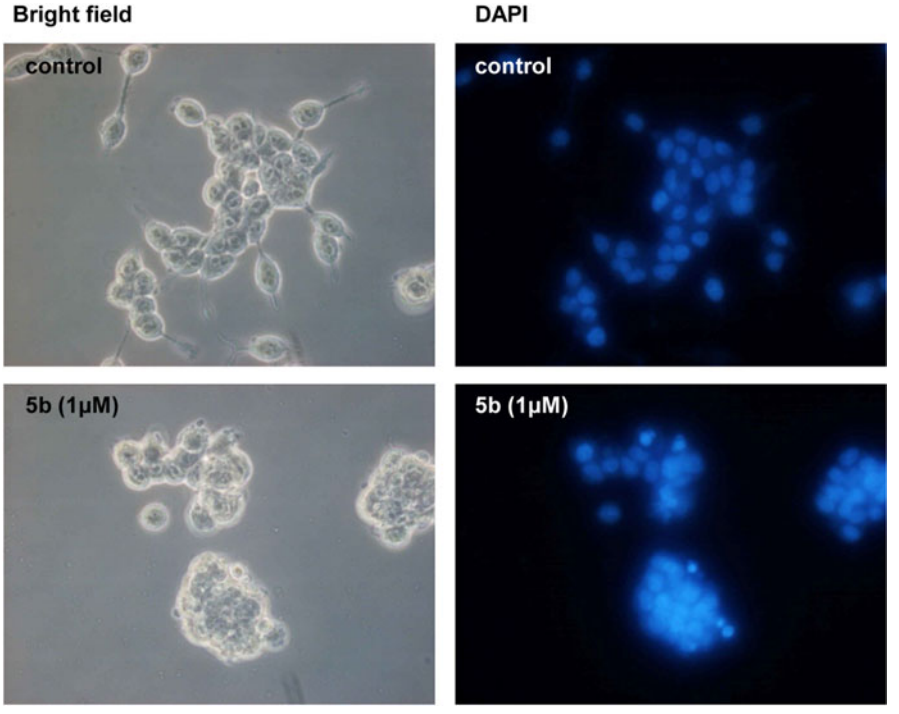


Figure 2. LNCaP cells were treated with compound **5b** (1 μM) for 24 h, fixed with paraformaldehyde, dyed with DAPI and observed under a fluorescent microscope (400 ×). Control, untreated LNCaP cells. Brightness indicates condensed nuclei. Data presented is a representative from three separate experiments.

3. Experimental

3.1 *Chemicals and instrumentation*

Reagents were obtained from Sigma-Aldrich S.p.A. (Milan, Italy), Merck (Milan, Italy) and Acros Organics (Milan, Italy) and used without further purification. Solvents were obtained from VWR International (Milan, Italy) and purified and stored according to standard procedures. Melting points were determined on a LEICA VM microscope equipped with a heating stage and are uncorrected. NMR spectra were recorded at 300 MHz (^{1}H) and at 75 MHz (^{13}C) with an AC 300 Bruker spectrometer (Milan, Italy). Chemical shifts are given in parts per million (ppm, δ) relative to solvent peaks as internal standards (δ: $CDCl_3$: 7.27 ppm (^{1}H), 77.0 ppm (^{13}C); acetone-d_6: 2.05 ppm (^{1}H), 29.8 and 206.0 ppm (^{13}C)); coupling constants are given in hertz (Hz, *J*). Mass spectra (MS) were measured with a Nermag R10-10C mass spectrometer (CI/NH_3) (Rueil-Malmaison, France) or with a ZQ 2000 Waters mass spectrometer (ESI) (Waters S.p.A., Milan, Italy). Flash column chromatography was carried out with silica gel (SDS 60 ACC 35–70μM). The reactions were monitored by thin-layer chromatography using Merck Kieselgel 60 F254 silica gel; zones were detected visually under ultraviolet irradiation (254 and 366 nm) and by spraying with sulphuric vanillin followed by heating.

3.2 *Synthesis of bis(indolyl)methanes*

3.2.1 *3-[1-(1H-Indol-3-yl)-1-(3′,4′,5′-trimethoxyphenyl)ethyl)-1H-indole (**5a**)*

(a) The indole **3a** (100 mg, 0.854 mmol) and the 3′,4′,5′-trimethoxyacetophenone **4** (89.8 mg, 0.427 mmol) were dissolved in absolute ethanol. Catalytic amounts (0 μL,

0.0085 mmol) of hydrochloric acid were added. The reaction mixture was heated at 78°C for 19 h. After cooling, the precipitate was recovered by vacuum filtration and washed with absolute ethanol, resulting in **5a**, obtained as a white solid (47.2 mg), with a yield of 26%.

(b) A solution of indole **3a** (100 mg, 0.854 mmol) and 3′,4′,5′-trimethoxyacetophenone **4** (89.8 mg, 0.427 mmol) in the presence of CTAB (5 mol%) and oxalic acid dihydrate [$(CO_2H)_2{\cdot}2H_2O$ (50 mol%)] was placed under agitation at room temperature in water. After about 7 h, the reaction was finished and the precipitated product was filtered and obtained by recrystallisation from a solution of EtOH–H_2O as a white solid (112.8 mg). Yield = 62%; m.p. = 285–287 °C; ^{1}H NMR (300 MHz, acetone-d_6): δ 2.33 (s, 3H); 3.59 (s), 3.71 (s, 3H), 6.77 (s, 2H), 6.83 (ddd, 2H, J = 8.1, 7.1, 1.1 Hz), 6.85 (s, 2H), 7.03 (ddd, 2H, J = 8.1, 7.1, 1.1 Hz), 7.28 (d, 2H, J = 8.1 Hz), 7.39 (d, 2H, J = 8.1 Hz), 9.99 (br s, 2H, D_2O exch, NH × 2); ^{13}C NMR (75 MHz, acetone-d_6): δ34.0, 43.9, 55.4 (× 2), 59.6, 106.4 (× 2), 111.4 (× 2), 118.1 (× 2), 120.8 (× 2), 121.6 (× 2), 123.5 (× 4), 126.6 (× 2), 136.5, 137.6 (× 2), 144.2, 152,6 (× 2); MS (ZQ2000/ESI +): m/z 449 $[M + Na]^+$, 465 $[M + K]^+$.

3.2.2 *3-[1-(6-Methoxy-1H-indol-3-yl)-1-(3′,4′,5′-trimethoxyphenyl)ethyl]-6-methoxy-1H-indole (**5b**)*

(a) The 6-methoxyindole **3b** (100 mg, 0.68 mmol) and the 3′,4′,5′-trimethoxyacetophenone **4** (71.4 mg, 0.34 mmol) were dissolved in absolute ethanol. Catalytic amounts of hydrochloric acid were added. The reaction mixture was heated at 78°C for 22 h. After purification carried out using flash chromatography (eluent: CH_2Cl_2), compound **5b** was obtained as a yellow amorphous solid (14.84 mg, 9% yield).

(b) A solution of 6-methoxyindole **3b** (50 mg, 0.34 mmol) and 3′,4′,5′-trimethoxyacetophenone **4** (35.7 mg, 0.17 mmol) in the presence of CTAB (5 mol%) and oxalic acid dihydrate[$(CO_2H)_2{\cdot}2H_2O$ (50 mol%)] was placed under agitation at room temperature in water. After about 10 h, the reaction was finished. After purification carried out using flash chromatography (eluent: CH_2Cl_2), compound **5b** was obtained as a yellow amorphous solid (34.7 mg, 42% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.62 (s, 3H), 3.78 (s, 9H), 3.79 (s, 6H), 6.19 (s, 4H), 6.66 (dd, J = 8.6, 2.3 Hz, 2H), 6.81 (d, J = 2.2 Hz, 2H), 7.24 (d, J = 8.6 Hz, 2H), 7.85 (s, 2H, NH × 2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.8, 37.5, 55.2 (× 2), 56.0, 55.5, 60.0, 104.5 (× 2), 109.0 (× 2), 119.1 (× 2), 119.5 (× 2), 119.9 (× 2), 120.4 (× 4), 121.4 (× 2), 142.6, 152.2, 152.9 (× 2), 156.4 (× 2); MS (ZQ2000/ESI +): m/z 509 $[M + Na]^+$.

3.3 *Assessment of cell proliferation*

The MTT assay was conducted as previously reported (Conforti et al. 2010) to detect the cell proliferation of the human cancer cell lines MCF-7, SkBr3 and LNCaP obtained from American Type Culture Collection (LGC Standards, Teddington, Middlesex, UK) (Nos HTB-22, HTB-30 and CRL-1740, respectively). MCF-7 cells were cultured in DMEM medium (Sigma) while SkBr3 and LNCaP cells were grown in RPMI-1640 medium, both supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin (complete medium). Cell monolayers were subcultured onto 96-well culture plates (2×10^4 cells/well) used for experiments 24 h later.

Cells were then treated with serial concentrations of the synthesised compounds. A total of 100 μL/well of each concentration was added and used in triplicates to obtain final dilutions

ranging from 0.0156 to 100 μM. Treatments never exceeded 0.5% of the solvent (DMSO), per cent of solvent that was used to treat control wells. The culture plates were kept at 37°C with 5% (v/v) CO_2. After 48 h of incubation, medium was removed from each well. Subsequently, 100 μL of 0.5% (w/v) MTT (Sigma), dissolved in phosphate-buffered saline (PBS), were added to each well and allowed to incubate for an additional 4 h. MTT was then removed and 100 μL of DMSO were added to each well to dissolve the formazan crystals. Absorbance values at 570 nm were measured using a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as IC_{50} which is the concentration giving 50% inhibition compared with the control (untreated cells).

3.4 *Determination of nuclear morphological changes*

Cells were cultured in complete medium for 24 h on microscope slides (1×10^5 cells) and then treated in serum-free medium for 24 h. Cells were washed with PBS and fixed in 4% formaldehyde for 10 min. Fixed cells were washed and incubated with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (0.2 μg/mL) for 10 min in a humidified chamber, protected from light, at 37°C. Cells were then washed three times with cold PBS and one drop of mounting solution was added. Cell nuclei were observed and imaged by an inverted fluorescence microscope with excitation at 350 nm and emission at 460 nm. The number of apoptotic nuclei was determined in at least six randomly selected areas from three coverslips of each experimental group. The results were expressed as apoptotic index as a percentage of apoptotic cells relative to the total number (1×10^5) of cells.

3.5 *Statistical analysis*

All experiments were carried out in sestuplicate. Data were expressed as mean value ± SEM. The concentration giving 50% inhibition (IC_{50}) was calculated by non-linear regression using the GraphPad Prism Software. Statistical significance was assessed using a one-way analysis of variance using the SigmaStat Software. The significant differences among the means were analysed using Tukey's multiple comparison test. Differences of $P < 0.05$ were considered significant.

4. Conclusions

Dietary consumption of cruciferous vegetables may exert a protective effect against some chronic diseases and specific cancers, because of the content of isothiocyanates and I3C, considered as the major chemopreventive and chemotherapeutic phytochemicals of these plants. I3C is highly unstable and gives a broad spectrum of products, but it is primarily converted into the dimeric product DIM. Several *in vivo* studies demonstrated the anti-tumourigenic activity of I3C. As a result of this discovery, a series of symmetrical DIM derivatives have been synthesised during the last years and some of them showed anti-carcinogenic activity (Safe et al. 2008). To further validate these previous observations, this study deals with the synthesis of some new DIM analogues and with the evaluation of their biological activity. Analogue **5b** showed the best antiproliferative activity against human prostate cancer cells LNCaP (IC_{50} of 0.64 ± 0.09 μM). LNCaP cells incubated with **5b** at a concentration of 1 μM showed morphological changes characteristic of apoptosis after an incubation period of 24 h compared with the control (untreated cells). Data indicating a more intense staining of nuclei of treated cells support the idea of an apoptotic mechanism of action for this compound. In conclusion, compound **5b** seems to be a promising compound potentially useful as anticancer agent, and further modifications of this molecule will be carried out in order to optimise the activity.

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