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ORIGINAL ARTICLE

In-vitro cytotoxicity of synthesized phthalide-fused indoles and indolines against HL-60 and HepG2 cells

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Abstract Phthalide derivatives bearing indole or indoline moieties were successfully synthesized via eco-friendly method and were evaluated for their antiproliferative activity on HL-60 and HepG2 cell lines *in vitro*. At a final concentration of 100 μM, most of the compounds showed moderate potency on both the cell lines tested. Compound **3b** bearing 5-chloro substituted indoline had the best potency against HL-60 and HepG2 cell lines with IC_{50} values of 45.4 and 57.7 μM, respectively. It was also found that replacement of a conjugated indoline to indole moiety gave better antiproliferative activity on HL-60 cells by almost two-fold. Morphological observation demonstrated numerous fragmented nuclei which are indicative of apoptosis. Molecular docking studies predicted non-covalent interactions and H-bonding of selected compounds with the P2 binding hot spot of the anti-apoptotic protein, Bcl-2, formed by Asp108, Phe109, Met112, Leu134, Arg143, Ala146 and Val153. Overall, our work highlights the potential of synthesized phthalide-fused indoles or indolines as antitumor agents.

1. Introduction

We have previously synthesized phthalide-fused indolines linked by an exocyclic double bond (Sheryn et al., 2018). In this study, the synthesized 3-substituted phthalides were linked to an indole at C3 position of the lactone ring and possess a similar framework as noscapine, a phthalideisoquinoline alkaloid, by replacing the isoquinoline unit with an indole moiety (Fig. 1). Noscapine is a prominent antitussive drug with remarkable cytotoxicity towards a variety of cancer cell types while having no significant toxicity to normal cells (Ke et al., 2000; Porcù et al., 2014; Rida et al., 2015). Indole containing products are found naturally through marine organisms, particularly from the marine sponges (Mollica et al., 2012). Compounds containing the indolic system have been reported to give high cytotoxicity and established promising antitumor

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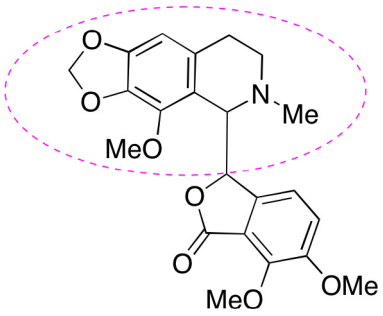


Fig. 1 The chemical structure of noscapine and its isoquinoline scaffold.

properties against various human tumor cell lines (Haider et al., 2007; Ma et al., 2015; Sri Ramya et al., 2017). Similarly, natural occurring and synthetic bisindolylmethanes (BIMs), have recently attracted much attention for their anticancer potency (Chen et al., 2017; Hatti et al., 2015; Kamal et al., 2010).

Microwave irradiation is becoming more prevalent in organic syntheses because of its advantages over the conventional method as well as environmental benefits. Microwave irradiation generally boosts chemical reactions by reducing reaction time and improved reaction yields (Penieres-Carrillo et al., 2017; Raunak et al., 2005; Solanki and Shekhawat, 2012; Srivastava et al., 2013). The conventional synthesis of 3-indolyl-substituted phthalide with the use of catalyst required a reaction time between 2 and 24 h, However, the reactions performed by microwave irradiation could be accelerated from 2 h to 5 min (Tang et al., 2012). In addition, a review displayed examples of specific reactions occurred by microwave irradiation which do not happen under conventional heating (Dallinger and Kappe, 2007).

In continuation of our interest in the syntheses of 3-substituted phthalide derivatives, herein we report synthesis utilizing eco-friendly method such as the use of microwave energy source for the activation of reactions, catalyst-free conditions and non-toxic solvent like water. All the derivatives were evaluated for their cytotoxic effect on human leukemia HL-60 and human hepatoma HepG2 cells.

2. Experimental

2.1. General methods

All chemicals and solvents were obtained from commercially available sources and used without further purification. All reactions were performed under conventional reflux and microwave irradiation methods. Microwave irradiation was performed using Monowave 50 (Anton Paar) employing sealed reaction vessels and an internal temperature probe and pressure sensor. MS spectra were obtained using the Waters UPLC-MS system (Aquity UPLC XevoQTof). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer at 400 MHz and 100 MHz, respectively using TMS as internal standard. IR spectra were recorded on a Perkin Elmer Spectrum 400 FT-IR/FT-FIR spectrometer equipped with a PIKE GladiATR. Column chromatography was performed on silica gel 60 (0.06–0.2 mm).

2.2. Cell culture

HepG2 cell line was kindly provided by the Division of Antioxidant Research (Life Science Research Center, Gifu University) and HL-60 cell line was obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). The HepG2 and HL-60 cell lines were cultured in Dulbecco's modified eagle medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and RPMI 1640 media (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. The cultured media were supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotics, penicillin-streptomycin (Gibco®, Life Technologies, Thermo Fisher Scientific Inc., MA, USA). Cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2.

2.3. In vitro CCK-8 assay

Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan) and noscapine hydrochloride hydrate, purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) was used as the positive control. The *in vitro* cytotoxic activities of compounds were evaluated against two human cancer cell lines (HL-60 and HepG2). The cells (100 μL, 2×10^4 cells/mL) were seeded in 96-well plates. After 24 h incubation, test compounds dissolved in DMSO were added to the cell culture at a final DMSO concentration of less than 1%. After 48 h of subsequent incubation, CCK solution (10 μL) was added and the plates were further incubated for 2 h. Visible absorption was measured at 436 nm using a microplate reader (E_{max} precision microplate reader, Molecular Devices).

2.4. Morphological changes

For the morphological assessment of apoptosis, compound (100 μM) was added to cultured HL-60 cells (1 mL, 2×10^5 - cells/mL) and incubated for 48 h. In addition, 10 μL of DMSO alone was added to another set of cells as the solvent control. The culture medium was stained with Hoechst 33342 solution (5 μL) and incubated for another 30 min. After centrifugation, the excess dye was removed and the collected cells were rinsed with PBS and then examined under a fluorescence microscope (Axiovert 40, Carl Zeiss).

2.5. Docking and modeling studies

The crystallographic coordinates of Bcl-2 co-crystallized with navitoclax (PDB ID: 4LVT (Souers et al., 2013)) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org) and was used in performing the docking studies. The 3D structure of Bcl-2 was first cleaned and the inhibitor binding site was accounted for further analysis. All compounds were drawn using Chem3D software and their conformations were optimized using Gaussian09 software. The geometry optimization of compounds was carried out using DFT methods at the B3LYP/3-21G level. All docking simulations were performed using AutoDock version 4.2 for 100 iterations each compound. Grid maps were prepared with $50 \times 62 \times 52$ points with a

point spacing of 0.375 Å. The grid box was allocated at the center of the protein using *x,y,z* coordinates of 6.021, −3.611, −5.598, respectively. The typical parameters set by the AutoDock 4.2 package for Lamarckian genetic algorithm were population size of 150 individuals, maximum 2.5×10^6 of energy evaluations and maximum 27,000 of generations per iteration. The best ranking pose of compounds toward Bcl-2 was visualized with Discovery Studio version 4.5.

2.6. Synthesis of phthalide-fused indoles and indolines

The preparations of compounds **4a–c** were preceded by microwave irradiation or reflux in water. The chemical structures of the synthesized compounds were confirmed by spectroscopic studies.

2.6.1. Conventional

A mixture of a phthalaldehydic acid derivative (1.1 mmol) and an indole derivative (1.0 mmol) was stirred in distilled water (5.0 mL) in reflux conditions for 24 h. The crude precipitate formed was filtered, washed with minimal amount of cold ethanol and acetone and finally dried in oven to give pure products (as monitored on TLC and spectral data) except for **4a**, which formed red colored sticky product, was purified by column chromatography (EtOAc/petroleum ether 1:2).

2.6.2. Microwave irradiation

A mixture of a phthalaldehydic acid derivative (1.1 mmol) and an indole derivative (1.0 mmol) was mixed in EtOH (0.5 mL) in a reaction vessel. The mixture was then irradiated in Monowave 50 at 160 °C for 5 min and while stirring at 500 rpm. The reaction mixture was evaporated in vacuo and the product obtained was washed with a minimal amount of cold EtOH and purified by recrystallization to give pure products (as monitored on TLC and spectral data).

2.6.3. 3-(1-Methylindol-3-yl)isobenzofuran-1(3H)-one (*4a*) (Noland and Johnson, 1960)

Compound **4a** was synthesized from starting materials 2-carboxybenzaldehyde (**1a**) and 1-methylindole (**2c**). This product was purified by recrystallization from acetone-EtOH to give yellow solid; IR (ATR, υ_{max}/cm^{-1}): 1736 (C═O), 1553, 1475, 1464 (C═C), 1284 (C—N), 1065 (C—O), 921, 745; ^{1}H NMR (400 MHz; $CDCl_3$) δ (ppm): 3.77 (s, 3H, N-C*H*$_3$), 6.79 (s, 1H, H-2), 7.05–7.09 (m, 2H, H-2′ and ArH-7′), 7.17 (dt, 1H, J = 8.0, 0.8 Hz, ArH-8′), 7.26 (ddd, 1H, J = 8.3, 7.1, 1.4 Hz, ArH-6′), 7.34 (dt, 1H, J = 8.0, 1.0 Hz, ArH-5′), 7.46 (dd, 1H, J = 7.4, 1.0 Hz, ArH-8), 7.62 (ddd, 1H, J = 7.6, 1.6, 0.8 Hz, ArH-6), 7.69 (td, 1H, J = 7.6, 1.2 Hz, ArH-7), 8.04 (dt, 1H, J = 7.6, 1.0 Hz, ArH-5); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 33.0 (N-*C*H$_3$), 77.7 (*C*-2), 109.6 (*C*-1′), 109.8 (Ar-*C*H), 119.1 (Ar-*C*H), 120.1 (Ar-*C*H), 122.5 (Ar-*C*H), 123.2 (Ar-*C*H), 125.6 (Ar-*C*H), 126.3 (Ar-*C*), 126.8 (Ar-*C*), 129.3 (*C*-2′), 129.3 (Ar-*C*H), 134.1 (Ar-*C*H), 137.5 (Ar-*C*), 149.3 (Ar-*C*), 170.7 (*C*═O); HRESITOFMS: *m/z* calcd for $C_{17}H_{13}NO_2Na$ $[M+Na]^+$ 286.0844, found 286.0844.

2.6.4. 3-(5-Bromo-1-methylindol-3-yl)isobenzofuran-1(3H)-one (*4b*)

Compound **4b** was synthesized from starting materials 2-carboxybenzaldehyde (**1a**) and 5-bromo-1-methylindole (**2d**). This product was purified by recrystallization from acetone-EtOH gave colorless needles; IR (ATR, υ_{max}/cm^{-1}): 1750 (C═O), 1475, 1463 (C═C), 1293 (C—N), 1063 (C—O), 936, 783 (C-Br), 710; ^{1}H NMR (400 MHz; DMSO d_6) δ (ppm): 3.78 (3H, s, N-C*H*$_3$), 7.02 (s, 1H, H-2), 7.12 (d, 1H, J = 1.6 Hz, ArH-5′), 7.30 (dd, 1H, J = 8.6, 1.8 Hz, ArH-7′), 7.47 (d, 1H, J = 8.8 Hz, ArH-8′), 7.50–7.53 (m, 2H, H-2′ and ArH-8), 7.70 (t, 1H, J = 7.4 Hz, ArH-6), 7.81 (td, 1H, J = 7.5, 0.9 Hz, ArH-7), 7.99 (d, 1H, J = 7.6 Hz, ArH-5); ^{13}C NMR (101 MHz, DMSO d_6) δ (ppm): 33.3 (N-*C*H$_3$), 77.4 (*C*-2), 109.1 (*C*-1′), 112.8 (Ar-*C*), 113.1 (Ar-*C*H), 121.0 (Ar-*C*H), 123.9 (Ar-*C*H), 124.9 (Ar-*C*H), 125.5 (Ar-*C*H), 126.1 (Ar-*C*), 128.0 (Ar-*C*Br), 130.1 (Ar-*C*H), 132.2 (*C*-2′), 135.2 (Ar-*C*H), 136.3 (Ar-*C*), 149.6 (Ar-*C*), 170.4 (*C*═O); HRESITOFMS: *m/z* calcd for $C_{17}H_{12}NO_2NaBr$ $[M+Na]^+$ 363.9949, found 363.9928.

2.6.5. 6,7-Dimethoxy-3-(5-bromo-1-methylindol-3-yl) isobenzofuran-1(3H)-one (*4c*)

Compound **4c** was synthesized from starting materials 6-formyl-2,3-dimethoxybenzoic acid (**1b**) and 5-bromo-1-methylindole (**2d**). White solid product was obtained; IR (ATR, υ_{max}/cm^{-1}): 1741 (C═O), 1500, 1475, 1425 (C═C), 1269 (C—N), 1046 (C—O), 939, 768 (C-Br), 706; ^{1}H NMR (400 MHz; $CDCl_3$) δ (ppm): 3.73 (s, 3H, N-C*H*$_3$), 3.95 (s, 3H, O-C*H*$_3$), 4.18 (s, 3H, O-C*H*$_3$), 6.57 (s, 1H, H-2), 7.01 (s, 1H, H-2′), 7.03 (dd, 1H, J = 8.4, 0.8 Hz, ArH-8), 7.17 (d, 1H, J = 8.4 Hz, ArH-8′), 7.24 (d, 1H, J = 8.4 Hz, ArH-7), 7.31 (dd, 1H, J = 8.6, 1.8 Hz, ArH-7′), 7.39 (d, 1H, J = 1.2 Hz, ArH-5′); ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 33.2 (N-*C*H$_3$), 56.9 (O-*C*H$_3$), 62.6 (O-*C*H$_3$), 75.7 (*C*-2), 110.0 (*C*-1′), 111.3 (Ar-*C*H), 113.5 (Ar-*C*), 117.6 (Ar-*C*H), 118.8 (Ar-*C*), 119.4 (Ar-*C*H), 121.7 (Ar-*C*H), 125.4 (Ar-*C*H), 128.0 (Ar-*C*Br), 130.0 (*C*-2′), 136.1 (Ar-*C*), 141.7 (Ar-*C*), 148.2 (Ar-*C*OMe), 152.8 (Ar-*C*OMe), 167.9 (*C*═O); HRESITOFMS: *m/z* calcd for $C_{19}H_{16}NO_4NaBr$ $[M+Na]^+$ 424.0160, found 424.0151.

2.6.6. 2-(Bis(5-bromo-1-methylindol-3-yl)methyl)benzoic acid (*5b*)

Compound **5b** was synthesized from starting materials 2-carboxybenzaldehyde (**1a**) and 5-bromo-1-methylindole (**2d**). This product was purified by recrystallization from acetone-EtOAc to obtain off-white solid; IR (ATR, υ_{max}/cm^{-1}): 3061, 2935 (broad, O—H), 1676 (C═O), 1477 (C═C), 1265 (C—N), 1047 (C—O), 774 (C—Br), 722; ^{1}H NMR (400 MHz; DMSO d_6) δ (ppm): 3.69 (s, 6H, 2 × N-C*H*$_3$), 6.81 (s, 2H, 2 × H-2′), 6.90 (s, 1H, H-7), 7.23 (dd, 2H, J = 8.8, 2.0 Hz, 2 × ArH-7′), 7.29–7.33 (m, 2H, ArH-3 and ArH-5), 7.37–7.45 (m, 5H, ArH-4, 2 × ArH-5′ and 2 × ArH-8′), 7.79 (dd, 1H, J = 7.8, 1.4 Hz, ArH-6); ^{13}C NMR (101 MHz, DMSO d_6) δ (ppm): 33.0 (2 × N-*C*H$_3$), 34.1 (*C*-7), 111.7 (2 × Ar-*C*), 112.5 (2 × Ar-*C*H), 117.2 (2 × *C*-1′), 121.4 (2 × Ar-*C*H), 124.1 (2 × Ar-*C*H), 126.7 (Ar-*C*H), 129.0 (2 × *C*-2′), 129.8 (Ar-*C*H), 130.2 (Ar-*C*H), 130.3 (2 × Ar-*C*Br), 131.1 (Ar-*C*), 131.8 (Ar-*C*H), 136.1 (2 × Ar-*C*), 144.5 (Ar-*C*), 170.0 (*C*═O); HRESITOFMS: *m/z* calcd for $C_{26}H_{20}N_2O_2NaBr_2$ $[M+Na]^+$ 572.9789, found 572.9778.

3. Results and discussion

3.1. Reaction and mechanism

We have previously synthesized **3a–c** by conventional method and microwave irradiation using a domestic microwave oven (Sheryn et al., 2018). Scheme 1 and Table 1 show the eco friendly synthesis of 3-substituted phthalide derivatives by microwave irradiation (160 °C, 5 min) or reflux in water (100–120 °C, 24 h). Compounds **4a–c** were successfully synthesized using the conventional reflux method. The same reaction scheme was followed in an attempt to produce **4a–c** using the Monowave 50 reactor. However, only **4a** could be obtained in 54% yield. In a similar reaction, **5b** was obtained as the main product in 28% reaction yield instead, with a trace of **4b** (7% yield). **4b** was produced with good yield (81%) when refluxed in water. Generally, **4a–c** gave better yields by the conventional method, whereas microwave irradiation further activated the alkylation process to give bisindole **5b**.

A plausible mechanism for the synthesis of phthalide-fused indole is proposed in Scheme 2. The reaction occurred via condensation of a Fischer base with aldehyde (Chunaev et al., 1982). The first step is initiated via nucleophilic attack of the Fischer base on the aldehyde group of a 2-formylbenzoic acid derivative to form an iminium ion intermediate. Next, a carbinol intermediate is formed by intramolecular proton transfer, followed by the release of the lone pair on amine nitrogen.

Table 1 The yield (%) of synthesized compounds via different reaction methods.

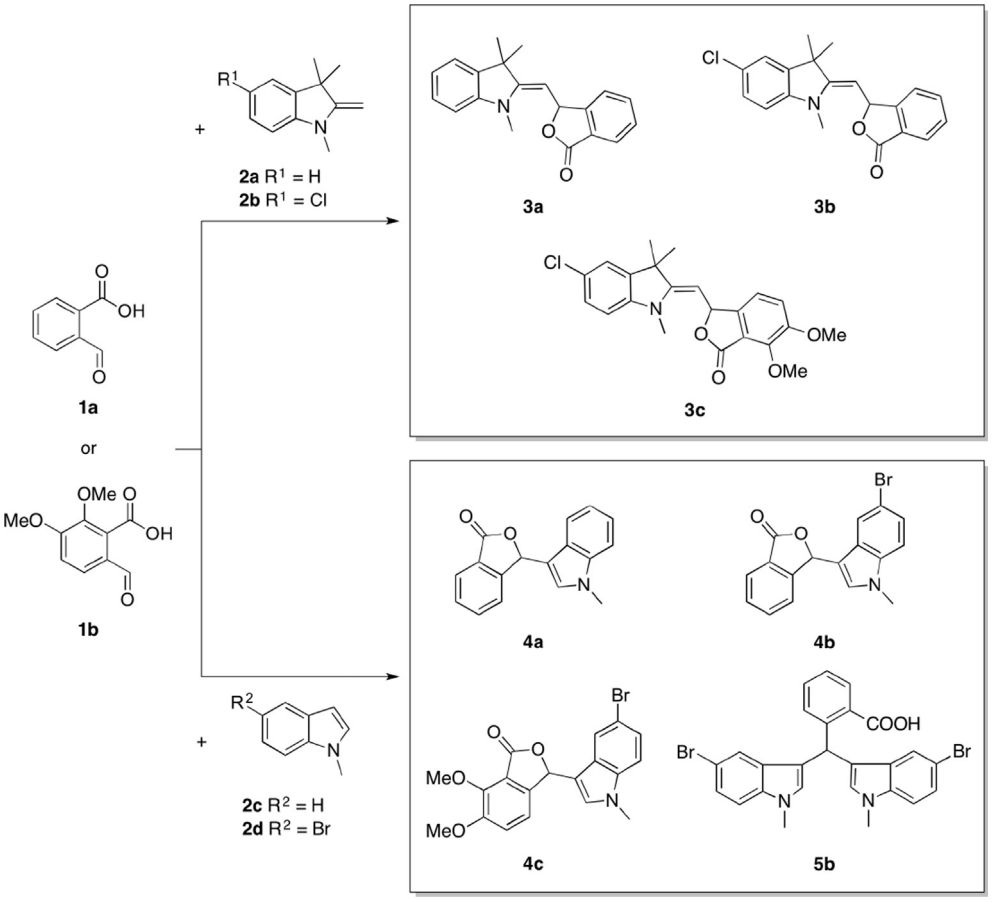
Product	Microwave irradiation		Conventional Reflux	
	Time (min)	Yield (%)	Time (h)	Yield (%)
3a[a]	5	72	24	79
3b[a]	1	80	24	77
3c[a]	1	80	24	68
4a	5	54	24	38
4b	5	7	24	81
4c	5	NO[b]	24	36
5b	5	28	24	NO[b]

[a] Previously synthesized compounds (Sheryn et al., 2018).
[b] Product not obtained.

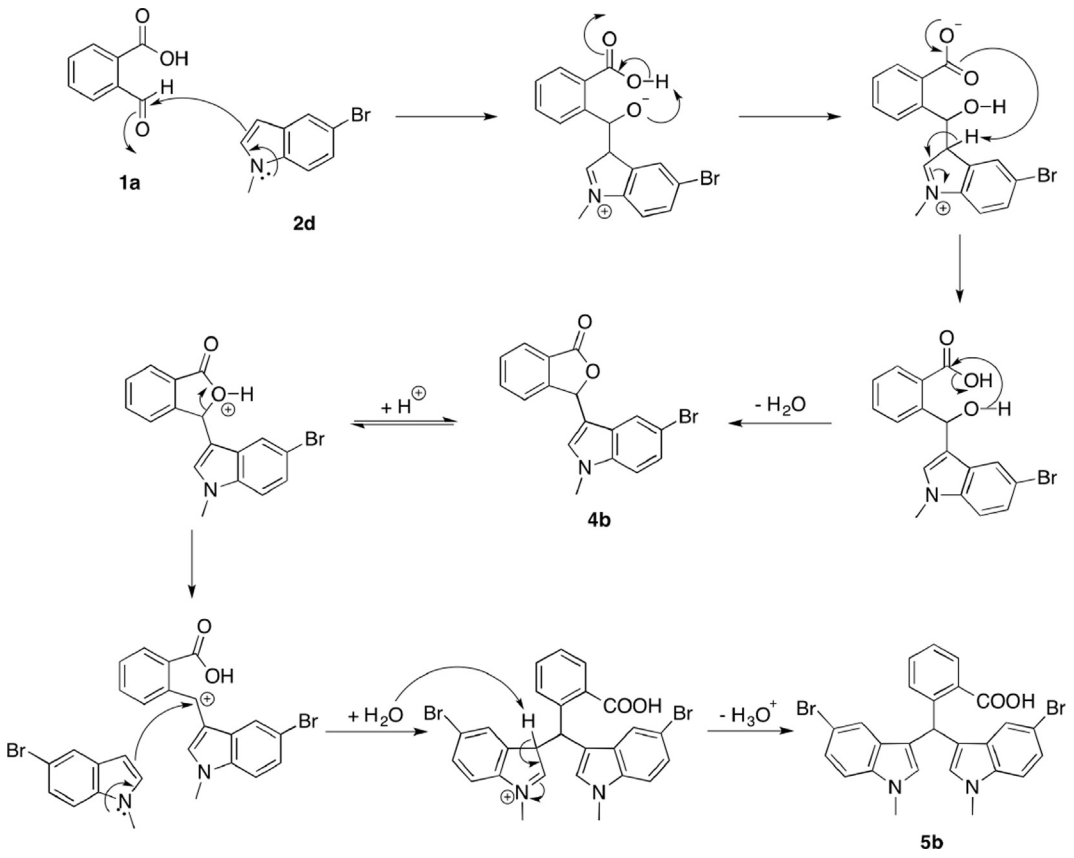
Subsequent cyclization by condensation reaction gave a five-membered ring lactone. Another unit of indole could be fused when the furanone ring was fragmented upon protonation catalyzed by 2-formylbenzoic acid (Chen et al., 2017; Lin and Sun, 2008).

3.2. Spectroscopic studies

Spectral data (FT-IR, NMR and MS) are analyzed to elucidate the structure of synthesized compounds (Supplementary Material Figs. S1–S24). The FT-IR spectra of **4a–c** exhibited



Scheme 1 Synthesis of phthalide-fused indolines (**3a–c**), phthalide-fused indoles (**4a–c**) and a bisindole (**5b**) by microwave irradiation (160 °C, 5 min) or reflux in water (100–120 °C, 24 h).



Scheme 2 Plausible mechanism for the synthesis of **4b** and **5b.**

a strong band at 1736–1750 cm^{-1}, signifying stretching frequencies of the phthalide carbonyl esters. Whereas the FT-IR spectrum of **5b** showed a strong band at 1676 cm^{-1} which is a typical stretching for carbonyl of benzoic acid and was observed together with a broad OH band. The C—O—C stretching frequencies were observed at 1046–1065 cm^{-1}, indicating the formation of lactone ring (Mahmoodi and Salehpour, 2003).

Generally, the ^{1}H NMR spectra of **4a–c** showed the presence of methylamine protons (N-C*H*$_3$) at 3.73–3.78 ppm, a chiral proton (C*H*-O) at 6.57–7.02 ppm, an olefinic proton of the enamine (C═C*H*-N) detected as a singlet at 7.01–7.50 ppm and aromatic protons resonated between 7.30 and 8.04 ppm. Two new singlets were detected in the spectrum of **4c** at 3.95 and 4.18 ppm, with an integral of 3 protons each belong to the methoxy substituents. The positions of aromatic protons on both the phthalide and indole moieties were determined through COSY analysis. The aromatic protons on phthalide moiety (7.46–8.04 ppm) were found to be slightly more deshielded as compared to aromatic protons on the indolic system (7.05–7.47 ppm) due to electron withdrawing effect caused by the carbonyl group. These values are similar with the range of the previously reported individual entities (Chitra et al., 2017; Zhang et al., 2010).

The ^{13}C NMR spectra showed methylamine carbon (N—*C*H$_3$) at 33.0–33.3 ppm, chiral carbon (*C*H—O or C-2) at 75.7–77.7 ppm, olefinic carbons overlapped with aromatic carbons between 109.1 and 152.8 ppm and a most downfield quaternary peak of the carbonyl carbon at 167.9–170.7 ppm. Two new methoxy carbon (O—*C*H$_3$) peaks were observed for **4c** at 56.9 ppm and 62.6 ppm. By comparing the spectra of **4b** and **4c**, presence of the dimethoxy substituents in **4c** exhibited a slight upfield shifts on the chiral carbon (C-2) and carbonyl carbon (C═O) by 1.7 ppm and 2.5 ppm, respectively. Analysis of 1D and 2D NMR data led us to the assignment of aromatic carbons of compounds **4a–c**. Comparison of the ^{13}C NMR spectra indicated chemical shift differences to the substituted patterns on aromatic rings (Fig. 2). By comparing the spectra of **4a** and **4b**, small downfield and upfield shifts were observed for *ortho* carbons (C-5′ and C-7′) and carbons on *meta* position (C-4′ and C-8′), respectively, due to the electron withdrawing effect of bromine atom. In addition to that, C-6′ was shifted to downfield by 5.5 ppm when bromine was directly attached to it. On the other hand, the aromatic carbons (C-3, C-4, C-7 and C-8) on the dimethoxy phthalide substituent (**4c**) showed upfield shifts due to the shielding effect of the electron donating methoxy groups, whereas C-5 and C-6 were shifted to downfield at 148.2 ppm and 152.8 ppm as an electronegative oxygen atom was attached to them.

The ^{1}H and ^{13}C NMR spectra of **5b** showed that the two indole molecules have chemically equivalent protons and carbons of the same multiplicity. A total of 17 carbon peaks was observed in ^{13}C NMR of **5b**, nine (9) of which are of higher intensity were assigned as carbon signals of the bisindole molecules.

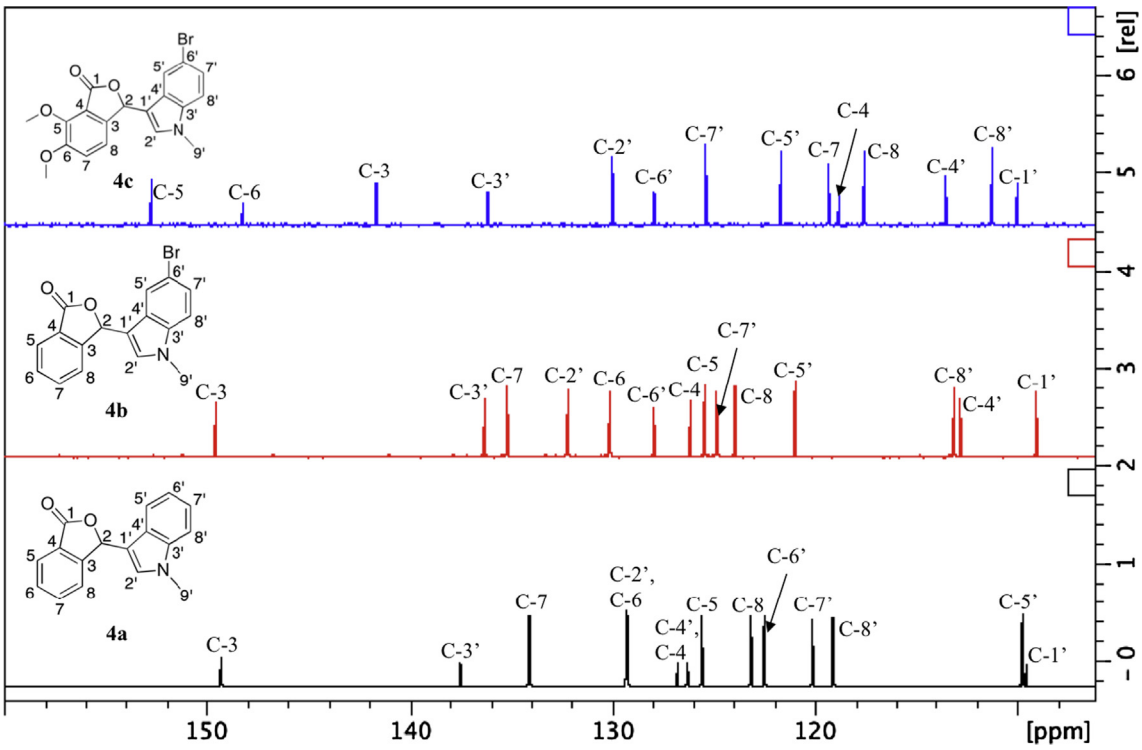


Fig. 2 The ^{13}C NMR spectra of **4a–c** at aromatic region.

Results for mass analysis were found to be in good agreement with the calculated values.

3.3. In vitro cytotoxicity

The antiproliferative activities of all the synthesized compounds were assessed against HL-60 and HepG2 cell lines using the CCK-8 assay (Kakumu et al., 2014). The cell viability results are summarized in Fig. 3 and Table 2, with noscapine hydrochloride (Nos) as the positive control. Dose-dependant cytotoxic effect was observed for all compounds on both cells except for **3a** and **3c** in HL-60 and HepG2 cells, respectively. At a final concentration of 100 μM, all compounds possessed cytotoxic potency and inhibited cell proliferation of the two cell lines. Moreover, phthalide bearing 5-chloro substituted indoline, **3b**, was the most potent compound at 100 μM with cell viability of 11.7 and 24.0% for HL-60 and HepG2 cells, respectively. However, compound **3c** was inactive and failed to inhibit the proliferation of HepG2 cells even at 100 μM.

The drug concentration (μM) giving a 50% reduction in cellular viability (IC_{50}) was calculated for each compound. The non-substituted indoline, **3a** did not show inhibition more than 50% compared to **3b**. This signifies the importance of halide substitution for inhibition activity to take place and may be associated with the hydrophobic effect of the Cl group, which improved cellular uptake of compound. Results for compounds **3a** and **4a** show that the replacement of conjugated indoline to indole moiety resulted in better activity on the two cell lines tested. On the other hand, the non-substituted indole on compound **4a** possessed greater activity than those with 5-bromo substituents (**4b**–**c**). Overall, the dimethoxy substitution on the phthalide moiety did not show any significant effect on the antiproliferative activity. Bisindole **5b** showed better cytotoxicity against HL-60 than its monomeric counterpart, **4b**. This finding was supported by a literature which revealed that bis- and tris-indole substituted compounds displayed stronger cytotoxicity than mono-indole substituted compounds (Pingaew et al., 2013).

Nuclear morphology studies were carried out to validate apoptosis induction of three selected compounds with the best cytotoxic activity. The microscopic images of HL-60 cells stained with Hoechst 33342 are shown in Fig. 4. The compounds **3b**, **4a** or **5b**-treated cancer cells (B, C, and D) showed different cell shapes from the untreated control group (A). The observed alterations caused by chromatin condensation, membrane blebbing and nuclear fragmentation are morphological features typical of apoptosis (Rello et al., 2005). These results suggested that the synthesized compounds were able to induce apoptosis in HL-60 cells.

3.4. Docking and modeling studies

Many human tumors are formed due to an overexpression of the Bcl-2 family of anti-apoptotic proteins, which also promotes the resistance of tumor cells to conventional chemotherapeutic drugs (Ashkenazi et al., 2017; Chaabane et al., 2013). The molecular docking simulation methods were carried out according to a previous report to explore their possible binding modes and interactions with this receptor and their potential in suppressing the anti-apoptotic proteins, which inhibit apoptosis in cancer cells (Gyebi et al., 2017). The top three synthesized compounds that showed the best potency were docked into the navitoclax (an inhibitor) binding hot spots of Bcl-2. The co-crystallized ligand navitoclax was re-docked as validation of

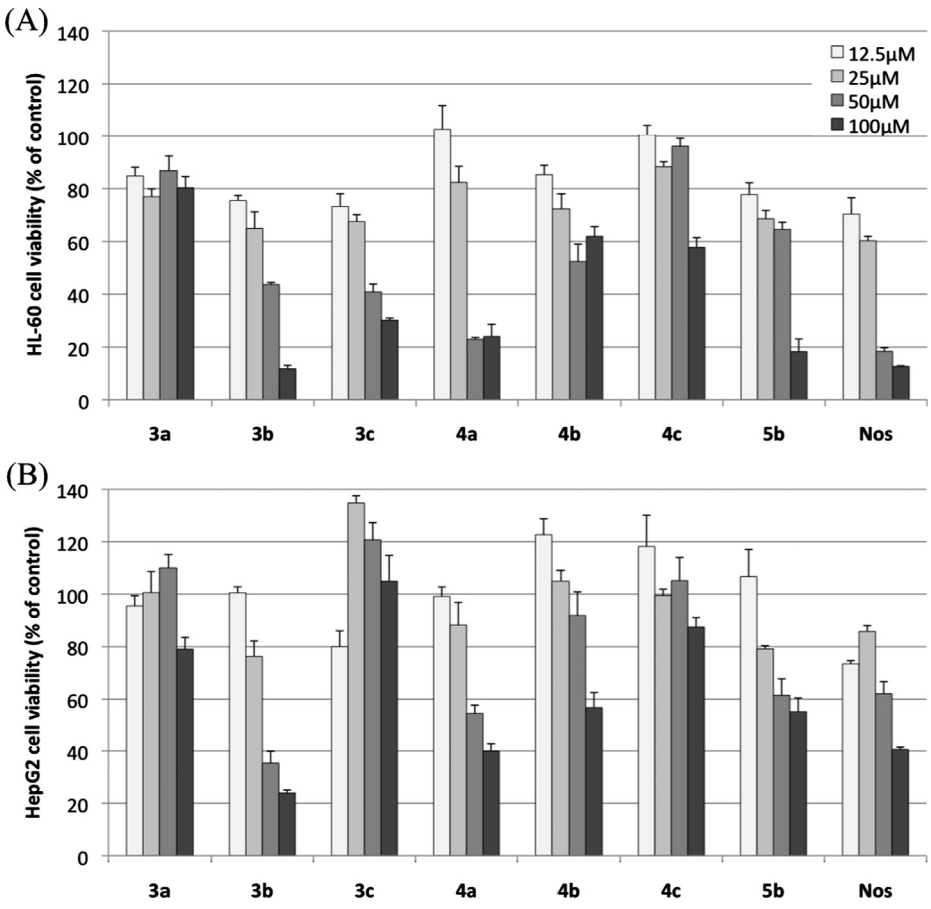


Fig. 3 Antiproliferative effects of phthalide-fused indoles and indolines on (A) HL-60 cells and (B) HepG2 cells (means ± SEMs, $n = 3$). Results are presented as percent viability of treated cells compared to that of untreated control.

Table 2 Cell viability of phthalide-fused indoles and indolines against HL-60 and HepG2 cells at a final concentration of 100 μM and their IC_{50} values.

Compound	HL-60		HepG2	
	Cell viability (% of control)	IC_{50} (μM)	Cell viability (% of control)	IC_{50} (μM)
3a	80.3 ± 4.3	> 100	79.0 ± 4.5	> 100
3b	11.7 ± 1.3	45.4	24.0 ± 1.1	57.7
3c	30.2 ± 0.8	52.8	105.0 ± 9.8	> 100
4a	24.0 ± 4.6	55.9	39.9 ± 2.7	76.9
4b	61.9 ± 3.7	> 100	56.7 ± 5.8	> 100
4c	57.7 ± 3.7	> 100	87.5 ± 3.6	> 100
5b	18.2 ± 4.8	57.8	54.9 ± 5.5	97.0
Noscapine	12.6 ± 0.3	32.5	40.5 ± 0.9	80.6

the docking protocol and yielded RMSD of 1.126 Å (Supplementary Material Fig. S25). All the docked compounds showed good fitting with the receptor and achieved best configuration with binding free energies (ΔG) of −16.61, −16.68, −16.36 and −15.10 kcal/mol for compounds **3b**, **4a**, **5b** and noscapine, respectively. Compound **4a** gave the best docking score although **3b** and noscapine was the most potent drugs, experimentally.

Generally, the docked compounds were incorporated at the P2 hot spot of the protein formed by Asp108, Phe109, Met112, Leu134, Arg143, Ala146 and Val153 (Fig. 5). Among these amino acid residues, Ala146 and Leu134 formed π-alkyl interaction with compounds **3b** and **4a** via the aromatic indole scaffold (Supplementary Material Fig. S26). On the contrary, the bisindole moieties on compound **5b** formed π-anion interactions with Asp108. A conventional H-bond was observed between Arg143 and carbonyl (C═O) on the phthalide scaffold of **4a**. Both the indole and phthalide scaffolds could attribute to the binding affinity of Bcl-2, hence, blocking the signal pathway of Bcl-2 for triggering apoptosis in tumor cells.

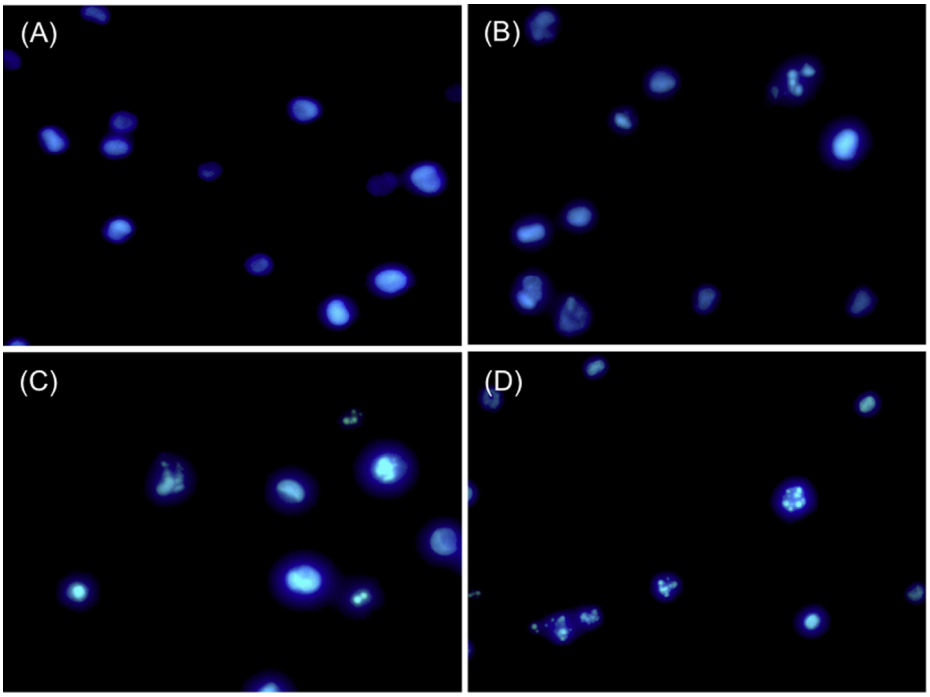


Fig. 4 Morphological changes of HL-60 cells induced by synthesized compounds at a final concentration of 100 μM for 48 h. The cells were stained in Hoechst 33342 and viewed under a fluorescence microscope. (A) Controlled HL-60 cells (DMSO alone); (B) **3b** in HL-60; (C) **4a** in HL-60; (D) **5b** in HL-60.

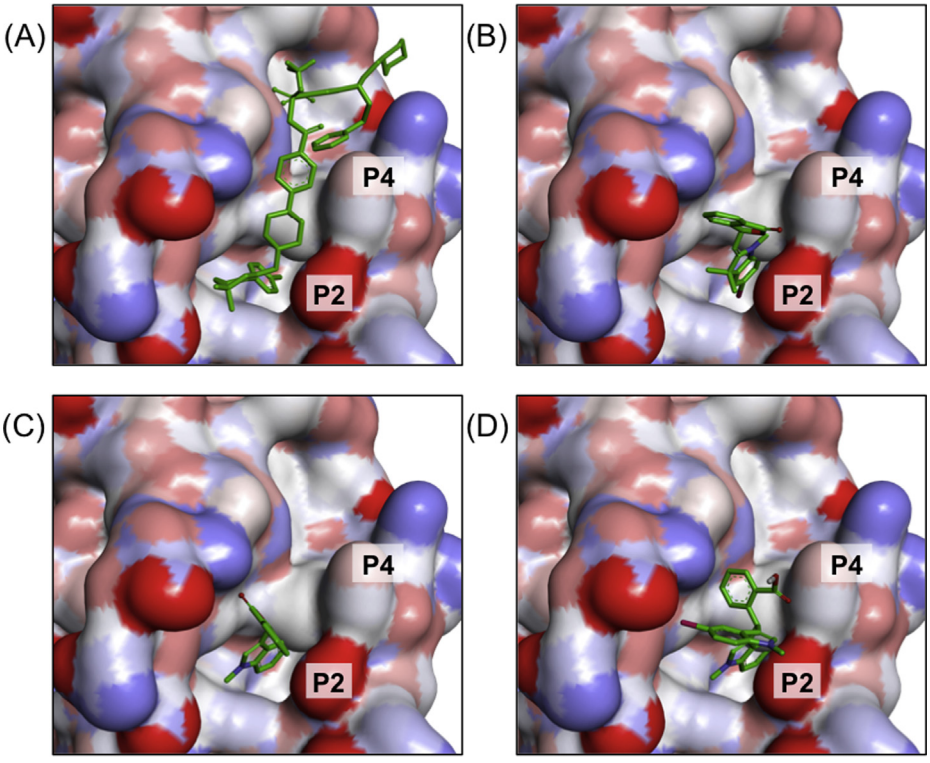


Fig. 5 3D molecular surface map of Bcl-2 showing the docked poses of (A) navitoclax, (B) **3b**, (C) **4a** and (D) **5b** into P2 and P4 binding hot spots.

4. Conclusions

The 3-substituted phthalide derivatives were successfully synthesized via the eco-friendly methods using the microwave irradiation or by reflux with water as a solvent. The synthesized phthalide-fused indoles and indolines have moderate cytotoxicity against the tested HL-60 and HepG2 cell lines. Further alteration of the structure or incorporation of varying substituents could improve the antitumor potential of these compounds and aid in the discovery of new cytotoxic agents.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2019.02.002.

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