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ORIGINAL RESEARCH

Synthesis, characterization and biological evaluation of some new indomethacin analogs with a colon tumor cell growth inhibitory activity

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Abstract Focusing particularly on colorectal cancer, and suggesting research strategies that may help to accelerate the future clinical application of indomethacin for the treatment of cancer, the molecular structures of indomethacin was used as starting scaffold to design novel amide analogs, and the effects of those analogs on the proliferation of human cancer cells were evaluated against three colon cancer cell lines, namely, HCT-116, CACO-2, and HT-29. Compared to indomethacin, the new derivatives displayed significantly increased activities. Interestingly two of the indomethacin analogs **7a** and **8c** displayed high growth inhibitory activity in nano-molar to micro-molar range against all three human colon cancer cell lines with IC50 values ranging from 0.055 to 4.0 µg/ml compared to 0.7–5.45 µg/ml for 5-fluorouracil (5-FU). Moreover, the potential mechanisms of the cytotoxic activity of the promising compounds **7a** and **8c** on the HT-29 and HCT-116 cell lines respectively were studied. The results indicated that compounds **7a** and **8c** arrested the cell cycle at G1/S and G0/G1 phase in HT-29 and HCT-116 cells respectively and might induced apoptosis via caspase-3 dependent pathway.

Keywords Synthesis · Indomethacin · Anticancer activity · Colon cancer · Apoptosis · Cell cycle arrest

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Introduction

Cancer colon is one of the most common malignant tumors affecting humans, and represents the third leading cause of cancer death among adults (Sinha and Richa 2009). The disease sometimes begins as a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer (Connell et al. 2014). Apoptosis is the programmed cell death which maintains the healthy survival/death balance in normal cells. Disruption of apoptotic pathway has been established as a prominent hallmark of several cancers (Cotter 2009). Evidences are increasingly available to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer and its poor response to chemotherapy and radiation (Watson 2004). Deeper understanding of the molecular mechanisms of apoptosis and its defective status opens the gate for a new class of targeted therapeutics (Hassan et al. 2014). There is a growing body of evidence suggests that the non-steroidal anti-inflammatory drug indomethacin has a potent anticolorectal cancer activity as well as chemopreventive effect from in vitro and in vivo models of colorectal cancer by a variety of reported methods including cyclooxygenase inhibition and peroxisome proliferator-activated receptor c activation (Pollard and Luckert 1983; Blitzer and Huang 1983; Heneghan 1985; Michael et al. 2002; Kumar et al. 2013; Chan 2002; Hawcraft et al. 2002; Zhu et al. 1999a; Blidner et al. 2015). In recent years, it is widely reported that, inhibiting cell proliferation and inducing cell apoptosis is another mechanism by which indomethacin and other non steroidal anti-inflammatory drugs inhibit tumor. The fact that the anti-proliferative effects of indomethacin in a variety of transformed cells that do not express cyclooxygenase (COX)-enzyme would argue against the

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relevance of COX inhibition as a target in colorectal epithelial cells (Groot et al. 2007, 2005; Rigas and Shiff 1999; Rigas and Kashfi 2005; Hanif et al.1996; Zhu et al. 1999a, b; Schror 2011; Elder et al. 1997; Kralj et al. 2001; Piazza et al. 1997, 1995; Wu and Xu 2001; Jones et al. 1999; Tinsley et al. 2010). This study is an attempt to improve the indomethacin anticancer potency through chemical modification to attain an active antitumor agent with potentiated activity and selectivity toward colon cancerous cells, hence indomethacin was used as a lead compound for the synthesis of a series of new derivatives through systematic modification of carboxylic acid moiety. The newly synthesized derivatives were evaluated as antiproliferative agents against colon cancer cells,namely, HCT-116, CACO-2, and HT-29. For the most potent compounds, the effect on normal cell cycle profile and apoptosis were performed to analyze the possible mechanism underlying their antiproliferative effect.

Experimental

Chemistry

All chemicals and reagents used in the current study were of analytical grade. Melting points (uncorrected) were determined on open capillary tubes using Griffin melting point apparatus. All the ^{1}H (nuclear magnetic resonance) NMR and ^{13}C NMR spectra were performed on Varian Gemini 300 MHz, 75 MHz spectrophotometer, respectively, using tetramethylsilane as internal standard. Chemical shift values (δ) are given using parts per million scale (ppm) at the Armed Forces Laboratories. The infrared (IR) spectra were recorded using Bruker ATR/FTIR Spectrophotometer at the Armed Forces Laboratories. Mass spectra were made on a DI-150 Unit of Shimadzu GC/MS-QP 5050A at the Regional Centre for Mycology and Biotechnology, AL-Azhar University. Analytical thin-layer chromatography was performed on precoated silica gel plates 60-F-254 (Merck; 0.25 mm). Elemental analyses (C, H, N) were performed by Micro Analytical Center, The Regional Center for Mycology and Biotechnology, Al-Azher University, and they were within ±0.4% of the theoretical values.

*General procedure for synthesis of 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) N-substituted acetamide derivatives) (**2a–e**)*

A mixture of equimolar amounts (0.01 mol, 3.7 g) of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetyl chloride (**1**) and appropriate amine (0.01 mol) in dimethylformamide solvent (DMF) (10 mL) containing few drops of pyridine was refluxed for 2 h. The reaction mixture was poured over crushed ice, few drops of HCl was added and the separated solid product was filtered, dried and recrystallized from ethanol to give:

Ethyl 4-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)benzoate (**2a**) Beige needles (EtOH): Yield 67%; m.p. 120–122 °C. IR (KBr) νmax:3292 (NH), 3060 (CH–Ar), 2908 (CH-aliphatic), 1713 (CO, ESTER),1668 (br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz,) δ: 1.24 (t, 3H, $CH_2\underline{CH_3}$), 2.25 (s, 3H, CH_3); 3.74 (s, 2H, CH_2); 3.80 (s, 3H, OCH_3); 4.28 (m, 2H, $\underline{CH_2}CH_3$), 6.58–7.92(m, 11H, ArH); 10.61 (s, 1H, NH exchangeable with D_2O).^{13}C NMR (DMSO, 75 MHz,);δ 13.89 ($\underline{CH_3}$ pyrole), 14.65 ($COOCH_2$–CH_3), 31.62($NHCOCH_2$), 55.95 ($O\underline{CH_3}$), 60.85(COO-CH_2-CH_3),102.49(CH, C_5 indole), 111.70 (C, C_3 indole), 114.37 (CH, C_7 indole), 115.00 (CH, C_8 indole), 116.94 (C, C_4 indole), 118.92 (C, C_9 indole), 124.00 (CH, C_{2b} Ph-COO), 124.63 (CH, C_{6b} Ph-COO), 129.50 (C, C_{4b} Ph-COO), 130.63 (CH, C_{3b} Ph-COO), 130.77 (CH, C_{5b} Ph-COO), 131.33 (CH, C_{2a} Cl-benzoyl), 131.61 (CH, C_{6a} Cl-benzoyl), 134.64 (C, C_{1a} Cl-benzoyl), 135.92 (C, C_2 indole), 138.03 (CH, C_{3a} Cl-benzoyl), 138.07 (CH, C_{5a} Cl-benzoyl) 144.03 (C, C_{1b} Ph-COO), 156.02 (C,C_{4a} Cl-benzoyl),165.02 (C, carboxyl), 156.97 (C,C_6 indole), 168.15 (C, carbonyl), 169.63 (C, amide). EI MS *m/z*: (%): 504 ([M+2]$^+$, 3.04), 504 ([M+]$^+$, 13.31), 41.01 (100). Anal. calcd. for $C_{28}H_{25}ClN_2O_5$: C, 66.60; H, 4.99; N, 5.55; found: C, 66.82; H, 5.06; N, 5.64.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido) benzoic acid (**2b**) Dark yellow(EtOH): Yield 78%; m.p. 110–112 °C. IR (KBr) νmax:3380 (br, carboxylic OH), 3214 (NH), 3080 (CH- Ar), 2943 (CH-aliphatic), 1677 (Br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz,) δ: 2.27 (s, 3H, CH_3); 3.71 (s, 2H, CH_2); 3.85 (s, 3H, OCH_3); 6.68–8.60 (m, 11H, ArH); 11.21(s, 1H, NH exchangeable with D_2O); 13.42(s, 1H, COOH exchangeable with D_2O). ^{13}C NMR (d_6-DMSO, 75 MHz,) δ 13.67 ($\underline{CH_3}$ pyrole), 33.81 ($NHCOCH_2$), 55.82 ($O\underline{CH_3}$), 101.71 (CH, C_5 indole), 112.08 (C, C $_3$ indole), 112.89 (CH, C_7 indole), 115.30 (CH, C_8 indole), 119.92 (C, C_4 indole), 123.15 (C, C_9 indole), 129.54 (CH, C_{2b} Ph-COOH), 130.93 (CH, C_{6b} Ph-COOH), 130.94 (C, C_{4b} Ph-COOH), 131.63 (CH, C_{3b} Ph-COOH), 131.77 (CH, C_{5b} Ph-COOH), 134.54 (CH, C_{2a} Cl-benzoyl), 134.56 (CH, C_{6a} Cl-benzoyl), 136.62 (C, C_{1a} Cl-benzoyl), 138.10 (C, C_2 indole), 141.13 (CH, C_{3a} Cl-benzoyl), 138.07 (CH, C_{5a} Cl-benzoyl) 144.03 (C, C_{1b} Ph-COOH), 154.02 (C, C_{4a}Cl-benzoyl),165.09 (C, carboxyl), 168.45 (C,C_6 indole),169.40 (C, carbonyl), 169.58 (C, amide).

EI MS *m/z*: (%): 476 ([M], 9.02), 477 ([M+]$^+$, 2.9), 139 (100). Anal. calcd. for $C_{26}H_{21}ClN_2O_5$: C, 65.48;H, 4.44; N, 5.87; found: C, 65.69; H, 4.49; N, 5.96.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-1-(piperazin-1-yl)ethanone (**2c**) Red needles (EtOH): Yield 69%; m.p. >300 °C. IR (KBr) νmax: 3382 (NH),3063 (CH–Ar), 2926 (CH-aliphatic), 1677 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz,) δ: 2.19 (s, 3H, CH_3); 2.96 (m, 4H, 2(CH_2), piperazine); 3.05 (m, 4H, 2(CH_2, piperazine);; 3.73 (s, 2H, CH_2); 3.85 (s, 3H, OCH_3) 6.69–7.76 (m, 7H, ArH); 12.24(s, 1H, NH exchangeable with D_2O) ^{13}C NMR (DMSO, 75 MHz) δ 13.82 (CH_3 pyrole), 29.81 ($NHCOCH_2$), 42.00 (CH, piprazine), 43.70 (CH, piprazine), 55.86 (OCH_3), 101.71 (CH, C_5 indole), 111.48 (C, C_3 indole), 114.40 (CH, C_7 indole), 115.30 (CH, C_8 indole), 119.51 (C, C_4 indole), 123.15 (C, C_9 indole), 130.71 (CH, C_{2a} Cl-benzoyl), 134.67 (CH, C_{6a} Cl-benzoyl), 138.01 (C, C_{1a} Cl-benzoyl), 138.01 (C, C_2 indole), 141.17 (CH, C_{3a} Cl-benzoyl), 138.22 (CH, C_{5a} Cl-benzoyl) 154.02 (C,C_{4a}Cl-benzoyl), 155.45 (C,C_6 indole), 169.40 (C, carbonyl), 168.25 (C, amide). EI MS *m/z*: (%): 426 ($[M+1]^+$, 1.93), 42.2, (100) Anal. calcd. for $C_{23}H_{24}ClN_3O_3$: C, 64.86;H, 5.68; N, 9.87; found: C, 65.03; H, 5.65; N, 10.04.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(naphthalen-2-yl)acetamide (**2d**) Dark yellow needles (EtOH): Yield 69%; m.p. 280–282 °C. IR (KBr) νmax:3208 (NH), 3004 (CH–Ar), 2793 (CH-aliphatic), 1675 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz,) δ: 2.35 (s, 3H, CH_3); 3.76 (s, 2H, CH_2); 3.91 (s, 3H, OCH_3); 6.79–8.08 (m, 14H, ArH); 10.11(s, 1H, NH exchangeable with D_2O). ^{13}C NMR (DMSO, 75 MHz) δ 13.93 (CH_3 pyrole), 32.05 ($NHCOCH_2$), 55.90 (OCH_3), 102.24 (CH, C_5 indole), 111.86 (C, C_3 indole), 114.72 (CH, C_7 indole), 115.11 (CH, C_8 indole), 122.46 (C, C_4 indole), 123.07 (C, C_9 indole), 125.88 (CH, C_{2b} naphthalene), 126.01 (CH, C_{11b} naphthalene), 126.24 (C, C_{3b} naphthalene), 126.49 (CH, C_{4b} naphthalene), 128.34 (CH, C_{5b} naphthalene), 129.49 (CH, C_{6b} naphthalene), 130.78 (CH, C_{7b} naphthalene), 131.77 (CH, C_{8b} naphthalene), 131.62 (C, C_{9b} naphthalene), 133.39 (CH, C_{10b} naphthalene), 131.62 (CH, C_{2a} Cl-benzoyl), 133.39 (CH, C_{6a} Cl-benzoyl), 134.14 (C, C_{1a} Cl-benzoyl), 134.70 (C, C_2 indole), 135.92 (CH, C_{3a} Cl-benzoyl), 138.03 (CH, C_{5a} Cl-benzoyl) 156.02 (C,C_{4a} Cl-benzoyl), 156.09 (C, C_6 indole),168.34 (C, carbonyl), 169.68 (C, amide). EI MS *m/z*: (%): 482 ($[M]^+$, 0.97), 104, (100). Anal. calcd. for $C_{29}H_{23}ClN_2O_3$; C, 72.12; H, 4.80; N, 5.80; found: C, 72.31; H, 4.87; N, 5.89.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)acetamide (**2e**) Yellowish brown needles (EtOH): Yield 73%; m.p. 110–112 °C. IR (KBr) νmax: 3267 (NH), 3018 (CH–Ar), 2926 (CH-aliphatic), 1660 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz): δ 2.05 (s, 3H, $CH_{3\text{-pyrazole}}$); 2.26 (s, 3H, CH_3); 3.02 (s, 3H, N-CH_3); 3.70 (s, 2H,CH_2) 3.77 (s, 3H, OCH_3); 6.68–7.70 (m, 12H, ArH); 9.28(s, 1H, NH exchangeable with D_2O). ^{13}C NMR (DMSO, 75 MHz,) δ 11.64 (CH_3 pyrazole), 13.93 (CH_3 pyrole), 32.05 ($NHCOCH_2$), 36.48 (NCH_3 pyrazole), 55.88 (OCH_3), 102.22 (CH, C_5 indole), 108.04 (C=CCH_3 pyrazole),111.87 (C, C_3 indole), 114.67 (CH, C_7 indole), 115.00 (CH, C_8 indole), 123.86 (C, C_4 indole), 129.49 (CH, C_{2b} benzene), 129.53 (CH, C_{6b} benzene), 130.26 (CH, C_{4b} benzene), 131.2 (CH, C_{3b} benzene), 131.63 (CH, C_{5b} benzene), 131.62 (C, C_{1a} Cl-benzoyl), 134.69 (CH, C_{5a} Cl-benzoyl), 134.14 (CH, C_{3a} Cl-benzoyl), 135.49 (C, C_{1b} benzene), 135.66 (CH, C_{2a} indole), 135.49 (CH, C_{6a} Cl-benzoyl), 138.01 (C=CCH_3 pyrazole), 152.81 (C, C_{4a} Cl-benzoyl), 156.02 (C, C_6 indole), 162.23 (C, CO pyrazole), 168.34 (C, carbonyl), 169.68 (C, amide). EI MS *m/z* (%): 542 ($[M]^+$, 0.77), 105, (100%). Anal. calcd. for $C_{30}H_{27}ClN_4O_4$; C, 66.36; H, 5.01; N, 10.32; found: C, 66.52; H, 5.08; N, 10.47.

*N-(2-aminoethyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**3**)*

A mixture of equimolar amounts of compound (**1**) (0.01 mol, 3.7 g) and ethylenediamine (0.01 mol) in DMF (10 mL) containing few drops of pyridine was refluxed for 2 h. The reaction mixture was poured over crushed ice, few drops of HCl was added and the separated solid product was filtered, dried, and recrystallized from ethanol. Dark yellow needles (EtOH), 84%; m.p. 260–262 °C. IR (KBr) νmax:3281 (Br, NH), 3069 (CH–Ar), 2926 (CH-aliphatic), 1677(Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz)δ: 2.08 (s, 3H, CH_3); 2.91 (m, 2H, CH_2-CH_2-NH_2); 3.31 (s, 2H, CH_2); 3.41 (m, 2H, CH_2-CH_2-NH_2); 3.84 (s, 3H, OCH_3); 6.67–7.96 (m, 7H, ArH); 8.07, 8.96 (2s, 2NH exchangeable with D_2O). ^{13}C NMR (DMSO, 75 MHz) δ 13.77 (CH_3 pyrole), 31.59 ($NHCOCH_2$), 37.62 (CH_2CH_2), 39.08 (CH_2CH_2)), 55.87 (OCH_3), 102.26 (CH, C_5 indole), 111.69 (C, C 3 indole), 114.56 (CH, C_7 indole), 114.96 (CH, C_8 indole), 129.45 (C, C_4 indole), 130.72 (C, C_9 indole), 131.28 (CH, C_{2a} Cl-benzoyl), 131.59 (CH, C_{6a} Cl-benzoyl), 134.69 (C, C_{1a} Cl-benzoyl), 135.64 (C, C_2 indole), 137.96 (CH, C_{3a} Cl-benzoyl), 138.22 (CH, C_{5a} Cl-benzoyl) 154.02 (C, C_{4a}Cl-benzoyl), 155.99 (C, C_6 indole), 168.28 (C, carbonyl), 170.10 (C, amide). EI MS *m/z* (%): 399 ($[M]^+$, 3.43), 105, (100). Anal. calcd. for $C_{21}H_{22}ClN_3O_3$; C, 63.08; H, 5.55; N, 10.51; found: C, 63.21; H, 5.62; N, 10.76.

*General procedure for synthesis of (E)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-((4-substituted-benzylidene)amino)ethyl)acetamide (**4a–c**)*

A mixture of equimolar amounts (0.01 mol, 3.7 g) of compound (**3**) and appropriate aldehyde (0.01 mol) in glacial

acetic acid (10 mL) was refluxed for 2 h. The reaction mixture was poured over crushed ice, and the separated solid product was filtered, dried and recrystallized from ethanol to give:

(E)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-((4-methoxybenzylidene)amino)ethyl)acetamide (**4a**) Beige needles (EtOH): Yield 65%; m.p. 230–232 °C. IR (KBr) νmax: 3281 (NH), 3075 (CH–Ar), 2928 (CH-aliphatic), 1660 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz) δ: 2.18 (s, 3H, CH_3); 3.07 (m, 2H, $\underline{CH_2}$-CH_2-N=Ph); 3.32 (m, 2H, CH_2-$C\underline{H_2}$-N=Ph); 3.76 (s, 2H,$C\underline{H_2}CO$); 3.82, 3.86 (2s, 6H, $2OCH_3$); 6.90–8.69 (m, 11H, ArH); 9.84 (s, 1H, NH exchangeable with D_2O); ^{13}C NMR (DMSO, 75 MHz) δ 13.78 ($\underline{CH_3}$ pyrole), 31.56 (NHCO$\underline{CH_2}$), 43.83 ($\underline{C}H_2CH_2$), 45.74 ($CH_2\underline{C}H_2$)), 55.87 (O$\underline{CH_3}$), 55.91 (O$\underline{CH_3}$), 102.30 (CH, C_5 indole), 111.72 (C, C 3 indole), 114.64 (CH, C_7 indole), 114.6 (CH, C_8 indole), 114.6 (CH, C_{3b} benzylidene), 114.6 (CH, C_{5b} benzylidene), 126.99 (C, C_4 indole), 129.45 (C, C_9 indole), 130.42 (CH, C_{2b} benzylidene), 130.70 (CH, C_{6b} benzylidene), 131.29 (C, C_{1b} benzylidene), 131.58 (CH, C_{2a} Cl-benzoyl), 132.59 (CH, C_{6a} Cl-benzoyl), 134.69 (C, C_{1a} Cl-benzoyl), 135.62 (C, C_2 indole), 137.96 (CH, C_{3a} Cl-benzoyl), 138.22 (CH, C_{5a} Cl-benzoyl) 154.02 (C, C_{4a}Cl-benzoyl), 155.99 (C, C_6 indole), 162.11 (CH, N=$\underline{C}$H),168.28 (C, carbonyl), 170.04 (C, C_{4b} benzylidene), 170.13 (C,amide). EI MS *m/z* (%): 518 ($[M]^+$, 0.86%), 287, (100%). Anal. calcd. for $C_{29}H_{28}ClN_3O_4$: C, 67.24; H, 5.45; N, 8.11; found: C,67.39; H, 5.52; N, 8.24.

(E)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-((4-chlorobenzylidene)amino)ethyl)acetamide (**4b**) Light brown needles (EtOH): Yield 71%; m.p. 235–237 °C. IR (KBr) νmax: 3280 (NH),3077 (CH–Ar), 2927 (CH-aliphatic), 1666 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz) δ: 2.16 (s, 3H, CH_3); 2.72 (m, 2H, $\underline{CH_2}$-CH_2-N=Ph); 2.88 (m, 2H, CH_2-$C\underline{H_2}$-N=Ph); 3.06 (s, 2H, CH_2); 3.74 (s, 3H,OCH_3); 6.67–8.20 (m, 12H, ArH+NH). ^{13}C NMR (DMSO, 75 MHz) δ 13.74 ($\underline{CH_3}$ pyrole), 31.59 (NHCO$\underline{CH_2}$), 36.22 ($\underline{C}H_2CH_2$), 39.07 ($CH_2\underline{C}H_2$)), 55.84 (O$\underline{CH_3}$), 102.23 (CH, C_5 indole), 111.67 (C, C 3 indole), 114.50 (CH, C_7 indole), 114.6 (CH, C_{3b} benzylidene), 114.6 (CH, C_{5b} benzylidene), 115.01 (CH, C_8 indole), 129.37(C, C_4 indole), 129.45 (C, C_9 indole), 129.54 (CH, C_{2b} benzylidene), 129.47 (CH, C_{6b} benzylidene), 131.29 (C, C_{1b} benzylidene),131.27 (CH, C_{2a} Cl-benzoyl), 131.59 (CH, C_{6a} Cl-benzoyl), 133.05 (C, C_{1a} Cl-benzoyl), 134.67 (C, C_2 indole), 135.66 (C, C_{4b} benzylidene), 137.96 (CH, C_{3a} Cl-benzoyl), 138.22 (CH, C_{5a} Cl-benzoyl) 139.02 (C, C_{4a}Cl-benzoyl), 155.98 (C, C_6 indole), 160.11 (CH, N=$\underline{C}$H), 168.28 (C, carbonyl), 170.13 (C, amide). EI MS *m/z* (%): 521 ($[M]^+$, 0.52), 54, (100). Anal. calcd. for $C_{28}H_{25}Cl_2N_3O_3$: C, 64.37; H, 4.82; N, 8.04; found: C, 64.52; H, 4.89; N, 8.13.

(E)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-((4-fluorobenzylidene)amino)ethyl)acetamide (**4c**) A dark yellow needle, Yield: 66%; m.p. 229–231 °C. IR (KBr) νmax: 3281 (NH), 3080 (CH–Ar), 2967 (CH-aliphatic), 1668 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz) δ: 2.18 (s, 3H, CH_3); 2.70 (m, 2H, $\underline{CH_2}$-CH_2-N=Ph); 3.12 (m, 2H, CH_2-$C\underline{H_2}$-N=Ph); 3.30 (s, 2H, CH_2); 3.75 (s, 3H,OCH_3); 6.99–8.01 (m, 12H, ArH+NH). ^{13}C NMR (DMSO, 75 MHz) δ 12.05 ($\underline{CH_3}$ pyrole), 25.33 (NHCO$\underline{CH_2}$), 28.79 ($\underline{C}H_2CH_2$), 30.22 ($CH_2\underline{C}H_2$)), 55.81 (O$\underline{CH_3}$), 100.84 (CH, C_5 indole), 104.67 (C, C 3 indole), 109.87 (CH, C_7 indole), 110.00 (CH, C_{3b} benzylidene), 111.35 (CH, C_{5b} benzylidene), 113.25 (CH, C_8 indole), 129.20 (C, C_4 indole), 129.23 (C, C_9 indole), 130.04 (CH, C_{2b} benzylidene), 130.04 (CH, C_{6b} benzylidene), 130.34 (C, C_{1b} benzylidene),130.57 (CH, C_{2a} Cl-benzoyl), 131.59 (CH, C_{6a} Cl-benzoyl), 132.55 (C, C_{1a} Cl-benzoyl), 132.89 (C, C_2 indole), 134.52 (CH, C_{3a} Cl-benzoyl), 138.25 (CH, C_{5a}Cl-benzoyl), 144.26 (C, C_{4a} Cl-benzoyl), 153.47 (C, C_{4b} benzylidene) 155.98 (C,C_6 indole), 166.92 (CH, N=$\underline{C}$H), 171.03 (C, carbonyl), 186.89 (C, amide). EI MS *m/z* (%): 507 ($[M+2]^+$, 1.61%), 505 $[M]^+$, (2.08), 40.18, (100). Anal. calcd. for $C_{28}H_{25}ClFN_3O_3$: C, 66.47; H, 4.98; N, 8.30; found: C, 66.63; H, 5.06; N, 8.45.

*General procedure for synthesis of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(N-(5-substituted-3-yl)sulfamoyl)phenyl)acetamide derivatives (**5a–c**)*

A mixture of equimolar amounts (0.01 mol, 3.7 g) of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetyl chloride (**1**) and appropriate sulpha drug (0.01 mol) in DMF (10 mL) containing few drops of pyridine was refluxed for 4 h. The reaction mixture was poured over crushed ice, few drops of HCl was added and the separated solid product was filtered, dried and recrystallized from ethanol to give:

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-sulfamoylphenyl)acetamide (**5a**) Dark yellow needles (EtOH), Yield 83%; m.p. 80–82 °C. IR (KBr) νmax: 3286 (Br NH), 3050(CH–Ar), 2988 (CH-aliphatic), 1671 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz) δ: 2.16 (s, 3H, CH_3); 3.70 (s, 2H, CH_2); 3.782 (s, 3H, OCH_3); 6.69–8.73 (m, 14H, Ar H+NH & NH_2). ^{13}C NMR (DMSO, 75 MHz) δ 13.67 ($\underline{CH_3}$ pyrole), 34.63 (NHCO$\underline{CH_2}$), 55.84 (O$\underline{CH_3}$), 102.02 (CH, C_5 indole), 111.74 (C, C_3 indole), 111.89 (CH, C_7 indole), 113.27 (CH, C_8 indole), 113.88 (C, C_4 indole), 115.05 (C, C_9 indole), 129.19 (CH, C_{2b} Ph), 129.51 (CH, C_{6b} Ph), 130.18 (C, C_{4b} Ph), 130.64 (CH, C_{3b} Ph), 130.65 (CH, C_{5b} Ph), 130.97 (CH, C_{2a} Cl-benzoyl), 131.61 (CH, C_{6a} Cl-benzoyl), 134.55 (C, C_{1a} Cl-benzoyl), 134.60 (C, C_2 indole), 135.60 (CH, C_{3a} Cl-benzoyl), 135.80 (CH,

C_{5a} Cl-benzoyl) 138.10 (C, C_{1b} Ph),156.01 (C, C_{4a}Cl-benzoyl), 168.31 (C, $C_{6\ indole}$), 170.96 (C, carbonyl), 172.5 (C, amide). EI MS *m/z* (%): 511 ($[M]^+$, 0.78), 137, (100). Anal. calcd. for $C_{25}H_{22}ClN_3O_5S$: C, 58.65; H, 4.33; N, 8.21; found: C, 58.79; H, 4.39; N, 8.34.

N-(4-(N-acetylsulfamoyl)phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**5b**) Brown needle (EtOH): Yield 74%; m.p. 100–102 °C. IR (KBr) νmax: 3280 (Br NH), 3046 (CH–Ar), 2988 (CH-aliphatic), 1671 (Br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): 2.17 (s, 3H, CH_3); δ 2.43 (s, 3H, $COCH_3$); 3.71; (s, 2H, CH_2); 3.85 (s, 3H, OCH_3); 6.76–8.70 (m, 13H, Ar H+2NH). ^{13}C NMR (DMSO, 75 MHz) *δ*; 13.67 ($CH_{3\ pyrole}$), 29.82 ($COCH_3$), 34.66 ($NHCOCH_2$), 55.84 (OCH_3), 102.02 (CH, $C_{5\ indole}$), 111.75 (C, $C_{3\ indole}$), 111.89 (CH, $C_{7\ indole}$), 113.27 (CH, $C_{8\ indole}$), 113.88 (C, $C_{4\ indole}$), 115.05 (C, $C_{9\ indole}$), 129.19 (CH, C_{2b} Ph), 130.56 (CH, C_{6b} Ph), 130.18 (C, C_{4b} Ph), 130.65 (CH, C_{3b} Ph), 130.87 (CH, C_{5b} Ph), 130.97 (CH, C_{2a} Cl-benzoyl), 131.65 (CH, C_{6a} Cl-benzoyl), 134.53 (C, C_{1a} Cl-benzoyl), 134.61 (C, $C_{2\ indole}$), 135.90 (CH, C_{3a} Cl-benzoyl), 135.81 (CH, C_{5a} Cl-benzoyl) 138.09 (C, C_{1b} Ph), 155.99 (C, C_{4a}Cl-benzoyl), 168.31 (C, $C_{6\ indole}$), 170.96 (C, carbonyl), 172.5 (C, amide). EI MS *m/z* (%): 554 ($[M]^+$, 4.07), 280, (100). Anal.calcd. for $C_{27}H_{24}ClN_3O_6S$: C, 58.53; H, 4.37; N, 7.85; found: C, 58.70; H, 4.41; N, 7.67.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(N-(thiazol-2-yl)sulfamoyl)phenyl)acetamide (**5c**) Dark yellow needles (EtOH), Yield 79%; m.p. 150–152 °C. IR (KBr) νmax: 3283 (Br NH),3102 (CH–Ar), 2988 (CH-aliphatic), 1699 (Br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): *δ* 2.26 (s, 3H, CH_3); 3.65 (s, 2H, CH_2); 3.75 (s, 3H, OCH_3); 6.69–8.76 (m, 15H, Ar H+2NH). ^{13}C NMR (DMSO, 75 MHz) *δ* 13.67 ($CH_{3\ pyrole}$), 34.70 ($NHCOCH_2$), 55.85 (OCH_3), 102.01 (CH, $C_{5\ indole}$), 102.15 (CH, $C_{4\text{-}thiazole}$), 111.75 (C, $C_{3\ indole}$), 111.89 (CH, $C_{7\ indole}$), 113.27 (CH, $C_{8\ indole}$), 113.88 (C, $C_{4\ indole}$), 115.02 (C, $C_{9\ indole}$), 129.52 (CH, C_{2b} Ph), 130.63 (CH, C_{6b} Ph), 130.97 (C, C_{4b} Ph), 131.18 (CH, C_{3b} Ph), 131.60 (CH, C_{5b} Ph), 134.56 (CH, C_{2a} Cl-benzoyl), 134.61 (CH, C_{6a} Cl-benzoyl), 135.60 (C, C_{1a} Cl-benzoyl), 135.81 (C, $C_{2\ indole}$), 138.04 (CH, C_{3a} Cl-benzoyl), 138.09 (CH, C_{5a} Cl-benzoyl) 138.90 (C, C_{1b} Ph), 155.99 (CH, $C_{5\text{-}thiazole}$) 156.99 (C, C_{4a}Cl-benzoyl), 168.31 (C, $C_{6\ indole}$), 170.02 (C, $C_{2\text{-}thiazole}$), 170.96 (C, carbonyl), 172.50 (C, amide). EI MS *m/z* (%): 594 ($[M1]^+$, 8.47), 69, (100). Anal. calcd. for $C_{28}H_{23}ClN_4O_5S_2$: C, 56.51; H, 3.90; N, 9.41; found: C, 56.59; H, 3.87; N, 9.52.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acetamide (**5d**) Dark brown needles (EtOH), Yield 61%; m.p. 70–72 °C. IR (KBr) νmax: 3280 (Br, NH), 3041 (CH–Ar), 2987 (CH-aliphatic), 1699 (Br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): *δ* 2.17 (s, 3H, CH_3); 2.48 (s, 3H, CH_3-isoxazol); 3.65 (s, 2H, CH_2); 3.75 (s, 3H, OCH_3); 6.69–7.96 (m, 13H, 12Ar H+NH); 8.43 (s, NH exchangeable with D_2O). ^{13}C NMR (DMSO, 75 MHz) *δ* 13.67 ($CH_{3\ pyrole}$), 14.58 ($CH_{3\ methylisoxazole}$), 34.64 ($NHCOCH_2$), 55.83 (OCH_3), 102.00 (CH, $C_{5\ indole}$), 111.75 (CH, $C_{2\text{-}\ isoxazole}$), 111.89 (C, $C_{3\ indole}$), 113.88 (CH, $C_{7\ indole}$), 115.05 (CH, $C_{8\ indole}$), 129.51 (C, $C_{4\ indole}$), 130.62 (C, $C_{9\ indole}$), 130.64 (CH, C_{2b} Ph), 130.97 (CH, C_{6b} Ph), 131.18 (C, C_{4b} Ph), 131.56 (CH, C_{3b} Ph), 131.60 (CH, C_{5b} Ph), 131.62 (CH, C_{2a} Cl-benzoyl), 134.55 (CH, C_{6a} Cl-benzoyl), 134.61 (C, C_{1a} Cl-benzoyl), 135.60 (C, $C_{2\ indole}$), 135.81 (CH, C_{3a} Cl-benzoyl), 138.04 (CH, C_{5a} Cl-benzoyl) 138.09 (C, C_{1b} Ph), 150.99 (C, $C_{1\text{-}\ isoxazole}$) 156.00 (C, C_{4a}Cl-benzoyl), 168.31 (C, $C_{6\ indole}$), 168.31 (C, $C_{3\text{-}isooxazole}$), 170.96 (C, carbonyl), 172.50 (C, amide). EI MS *m/z* (%): 592 ($[M]^+$, 5.88), 137, (100). Anal.calcd. for $C_{29}H_{25}ClN_4O_6$: C, 58.73; H, 4.25; N, 9.45; found: C, 58.91; H, 4.32; N, 9.62.

2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(N-(pyrimidin-2-yl)sulfamoyl)phenyl)acetamide (**5e**) Dark yellow needles (EtOH), Yield 59%; m.p. 80–82 °C. IR (KBr) νmax: 3287 (Br, NH), 3080 (CH–Ar), 2927 (CH-aliphatic), 1687 (Br, CO) cm^{-1}. ^{1}H NMR (DMSO, 300 MHz): *δ* 2.14 (s, 3H, CH_3); 3.65 (s, 2H, CH_2); 3.75 (s, 3H,OCH_3); 6.69–7.96 (m, 15H, Ar H+NH); 12.39 (s, 1H, NH exchangeable with D_2O). ^{13}C NMR (DMSO, 75 MHz) *δ* 13.64 ($CH_{3\ pyrole}$), 34.55 ($NHCOCH_2$), 55.85 (OCH_3), 102.14 (CH, $C_{5\ indole}$), 111.75 (CH, $C_{5\text{-}\ diazine}$), 111.89 (C, $C_{3\ indole}$), 113.89 (CH, $C_{7\ indole}$), 115.01 (CH, $C_{8\ indole}$), 127.26 (C, $C_{4\ indole}$), 129.52 (C, $C_{9\ indole}$), 130.62 (CH, C_{2b} Ph), 131.59 (CH, C_{6b} Ph), 131.61 (C, C_{4b} Ph), 134.60 (CH, C_{3b} Ph), 135.60 (CH, C_{5b} Ph), 135.80 (CH, C_{2a} Cl-benzoyl), 138.09 (CH, C_{6a} Cl-benzoyl), 134.61 (C, C_{1a} Cl-benzoyl), 135.60 (C, $C_{2\ indole}$), 135.81 (CH, C_{3a} Cl-benzoyl), 138.04 (CH, C_{5a} Cl-benzoyl) 138.09 (C, C_{1b} Ph), 143.38 (CH, $C_{4\text{-}\ diazine}$), 145.33 (CH, $C_{6\text{-}\ diazine}$), 156.00 (C,C_{4a}Cl-benzoyl), 168.31 (C, $C_{6\ indole}$), 168.31 (CH, $C_{2\text{-}diazine}$), 170.96 (C, carbonyl), 172.50 (C, amide). EI MS *m/z* (%): 589 ($[M]^+$, 0.89), 264 (100). Anal. calcd. for $C_{29}H_{24}ClN_5O_5S$: C, 59.03; H, 4.10; N, 11.87; found: C, 59.34; H, 4.19; N, 11.98.

*2-((1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)methyl)-4H-benzo[d][1,3]oxazin-4-one (**6**)*

A mixture of 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)benzoic acid **(2)** (14 g, 0.03 mol) and acetic anhydride (30 g, 0.3 mol) was heated under reflux for 4 h. The solvent was removed under

reduced pressure. The residue was triturated with petroleum ether 40–60. The separated solid was collected by filtration, washed with petroleum ether 40–60, dried and crystallized from ethanol to give a yellowish needle (EtOH), Yield 75 %; m.p.168–170 °C. IR (KBr) νmax: 3057 (CH–Ar), 2983, 2830 (CH-aliphatic), 1772 (CO-lactone),1670 (CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz): δ 2.29 (s, 3H, CH_3); 3.71 (s, 3H, OCH_3); 4.13 (s, 2H, CH_2); 6.69–8.08 (m, 11H, ArH). ^{13}C NMR (DMSO, 75 MHz) δ 13.80 ($\underline{CH_3}$ pyrole), 29.95 (NHCO$\underline{CH_2}$), 55.82 (O$\underline{CH_3}$), 102.32 (CH, C_5 indole), 111.89 (C, C_3 indole), 115.08 (CH, C_7 indole), 117.03 (CH, C_8 indole), 126.91 (C, C_4 indole), 128.36 (C, C_9 indole), 129.02 (CH, C_{2a} Cl-benzoyl), 129.11 (CH, C_{6a} Cl-benzoyl), 129.51 (C, C_{1a} Cl-benzoyl), 129.54 (CH, benoxazine), 130.93 (CH, benoxazine), 130.94 (CH, benoxazine), 130.73 (CH, C_{3a} Cl-benzoyl), 130.96 (CH, C_{5a} Cl-benzoyl), 131.96 (C, C_2 indole), 132.05 (CH, benoxazine), 134.51 (C, benoxazine), 136.39 (C, benoxazine), 146.39 (C, C_{4a}Cl-benzoyl),156.03 (C, carboxyl), 159.63 (C, C_6 indole), 160.40 (C, carbonyl), 164,20 (C, N=$\underline{C}$-OCO) 169.58 (C, amide). EI MS *m/z* (%): 458, [M], (35.62), 460, [M+2], (11.6), 139, (100). Anal. calcd. for $C_{26}H_{19}ClN_2O_4$: C, 68.05; H, 4.17; N, 6.10; found: C, 68.23; H, 4.21; N, 6.19.

*General procedure for synthesis of 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetamido)-N-(4-(N-(4-substituted)sulfamoyl)phenyl) benzamide (**7a–c**)*

A mixture of equimolar amounts (0.01 mol, 4.5 g) of of benzoxazinone derivative **(6)** and appropriate sulpha drug (0.01 mol) in DMF (10 mL) containing few drops of pyridine was refluxed for 4 h. The reaction mixture was poured over crushed ice, few drops of HCl was added and the separated solid product was filtered, dried and recrystallized from ethanol to give:

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-sulfamoylphenyl)benzamide (**7a**) Beige needles (EtOH); Yield 68%; m.p. 100–102 °C; IR (KBr) νmax: 3349 (Br, NH), 3075 (CH–Ar), 2931, 2833 (CH-aliphatic.),1679 (Br, CO) cm^{-1}: ^1H NMR (DMSO, 300 MHz) δ:2.30 (s, 3H, CH_3); 3.74 (s, 2H, CH_2); 3.87 (s, 3H,OCH_3); 6.70–8.63 (m, 17H, ArH+NH_2); 11.16 (s, 1H, NH cancelled with D_2O). ^{13}C NMR (DMSO, 75 MHz) δ: 11.64 ($\underline{CH_3}$ pyrole), 34.55 (NHCO$\underline{CH_2}$), 55.85 (O$\underline{CH_3}$), 100.14 (CH, C_5 indole), 111.54 (C, C_3 indole), 116.03 (CH, C_7 indole), 119.01 (CH, C_8 indole), 129.26 (C, C_4 indole), 129.49 (C, C_9 indole), 129.61 (CH, C_{2c}- Ph), 129.61 (CH, C_{6c}- Ph), 130.07 (CH, C_{2b} Ph), 130.74 (CH, C_{6b}- Ph), 131.50 (C, C_{4b} Ph), 131.59 (CH, C_{3b} Ph), 131.72 (CH, C_{5b} Ph), 131.75 (CH, C_{3c}- Ph), 131.75 (CH, C_{3c}- Ph), 134.59 (CH, C_{2a} Cl-benzoyl), 138.09 (CH, C_{6a} Cl-benzoyl), 134.61 (C, C_{1a} Cl-benzoyl), 135.60 (C, C_2 indole), 135.29(CH, C_{3a} Cl-benzoyl), 135.58 (CH, C_{5a} Cl-benzoyl), 135.90 (CH, C_{4c}- Ph), 138.90 (CH, C_{1c} Ph) 139.23 (C, C_{1b} Ph), 156.00 (C, C_{4a}Cl-benzoyl), 168.31 (C, C_6 indole), 166.90 (C, carbonyl), 169.77 (C, amide), 172.50 (C, amide). EI MS *m/z* (%): 631 $[M+1]^+$, 630 $[M]^{+\cdot}$, 155, (100). Anal. calcd. for $C_{32}H_{27}ClN_4O_6S$: C, 60.90; H, 4.31; N, 8.88; found: C, 61.03; H, 4.35; N, 9.02.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-(N-pyrimidin-2-ylsulfamoyl)phenyl) benzamide (**7b**) Brown needles (EtOH); Yield 65%; m.p. 151–153 °C; IR (KBr) νmax: 3216 (Br, NH), 3085 (CH–Ar), 2932 (CH-aliphatic), 1679 (Br, CO) cm^{-1}: ^1H NMR (DMSO, 300 MHz) δ: 2.38 (s, 3H, CH_3), 3.72 (s, 2H, CH_2); 4.08 (s, 3H, OCH_3); 6.61–8.62 (m, 18H, ArH); 10.5, 11.14 (2s, 2H, NH cancelled with D_2O). ^{13}C NMR (DMSO, 75 MHz) δ: 11.60 ($\underline{CH_3}$ pyrole), 34.51(NHCO$\underline{CH_2}$), 55.83 (O$\underline{CH_3}$), 100.14 (CH, C_5 indole), 111.53 (C, C_3 indole), 111.45 (CH, C_5- diazine), 117.03 (CH, C_7 indole), 119.01 (CH, C_8 indole), 129.26 (C, C_4 indole), 129.49 (C, C_9 indole), 129.61 (CH, C_{2c}- Ph), 129.61 (CH, C_{6c}- Ph), 130.09 (CH, C_{2b} Ph), 130.94 (CH, C_{6b}- Ph), 131.50 (C, C_{4b} Ph), 131.59 (CH, C_{3b} Ph), 131.72 (CH, C_{5b} Ph), 131.75 (CH, C_{3c}- Ph), 131.75 (CH, C_{3c}- Ph), 134.59 (CH, C_{2a} Cl-benzoyl), 138.09 (CH, C_{6a} Cl-benzoyl), 134.61 (C, C_{1a} Cl-benzoyl), 135.60 (C, C_2 indole), 135.29 (CH, C_{3a} Cl-benzoyl), 135.58 (CH, C_{5a} Cl-benzoyl), 135.90 (CH, C_{4c}- Ph), 138.90(CH, C_{1c}- Ph) 139.23 (C,C_{1b} Ph), 149.38 (CH, C_4- diazine), 149.33 (CH, C_6- diazine), 156.00 (C, C_{4a}Cl-benzoyl), 168.31 (CH, C_2- diazine), 168.31 (C,C_6 indole), 166.81 (C, carbonyl), 169.70 (C, amide), 171.50 (C, amide). EI MS *m/z* (%): 708 $[M]^+$, 174, (100). Anal. calcd. for $C_{36}H_{29}ClN_6O_6S$: C, 60.97; H, 4.12; N, 11.85; found: C, 61.42; H, 4.17; N, 12.02.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-(N-(4-methylisoxazol-3-yl)sulfamoyl)phenyl)benzamide (**7c**) Brown needles (EtOH); Yield 64%; m.p. 140–142 °C; IR (KBr) νmax: 3230 (Br, NH), 3085 (CH–Ar.), 2932(CH-aliphatic), 1678 (Br, CO) cm^{-1}: ^1H NMR (DMSO, 300 MHz): δ 2.29, 2.38 (2s, 6H, $2CH_3$), 3.70 (s, 2H, CH_2); 3.77 (s, 3H, OCH_3), 6.62–8.64 (m, 16H, ArH); 10.74, 11.14 (2s, 2H, NH cancelled with D_2O); ^{13}C NMR (DMSO, 75 MHz) δ: 11.64 ($\underline{CH_3}$ pyrole), 13.76 ($\underline{CH_3}$ methyl-isoxazole), 34.55 (NHCO$\underline{CH_2}$), 55.71 (O$\underline{CH_3}$), 100.12 (CH, C_5 indole), 103.47 (CH, C_2- isoxazole), 110.21 (C, C_3 indole), 111.53 (CH, C_7 indole), 116.07 (CH, C_8 indole), 119.78 (C, C_4 indole), 122.67 (C, C_9 indole), 129.11 (CH, C_{2c}- Ph), 129.20 (CH, C_{6c}- Ph), 130.40 (CH, C_{2b} Ph), 130.50 (CH, C_{6b}- Ph), 130.75 (C, C_{4b} Ph), 131.57 (CH, C_{3b} Ph), 131.72 (CH, C_{5b} Ph), 131.75 (CH, C_{3c}- Ph), 131.75 (CH, C_{3c}- Ph), 134.59 (CH, C_{2a} Cl-benzoyl), 138.09 (CH, C_{6a} Cl-benzoyl), 134.61 (C, C_{1a} Cl-benzoyl), 135.60

(C, $C_{2\ indole}$), 135.29 (CH, C_{3a} Cl-benzoyl), 135.58 (CH, C_{5a} Cl-benzoyl), 135.90 (CH, C_{4c-} Ph), 138.90 (CH, C_{1c-} Ph) 139.23 (C, C_{1b} Ph), 141.60 (C, $C_{1-\ isoxazole}$) 153.61 (C, C_{4a}Cl-benzoyl), 164.31 (C,$C_{6\ indole}$), 165.35 (C, $C_{3-isooxazole}$), 166.89 (C, carbonyl), 169.76 (C, amide), 171.02 (C, amide). EI MS *m/z* (%): 711 $[M]^+$, 137, (100). Anal. calcd. $C_{36}H_{30}ClN_5O_7S$: C, 60.71; H, 4.25; N, 9.83; found: C, 60.89; H, 4.34; N, 9.79.

*General procedure for synthesis of 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) acetamido)-N-(4-substituted phenyl)benzamides (**8a–c**)*

A mixture of equimolar amounts (0.01 mol, 4.5 g) of benzoxazinone derivative **(6)** and appropriate aromatic amines (0.01 mol) in DMF (10 mL) containing few drops of pyridine was refluxed for 2 h. The reaction mixture was poured over crushed ice, few drops of HCl was added and the separated solid product was filtered, dried and recrystallized from ethanol to give.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-fluorophenyl)benzamide (**8a**) Yellow needles (EtOH); Yield 74%; m.p. 60–62 °C; IR (KBr) νmax: 3377 (Br, NH), 3080 (CH-arom.), 2936 (CH-aliph.), 1679 (Br, CO) cm^{-1}. ^1H NMR (DMSO, 300 MHz) *δ*: 2.34 (s, 3H, CH_3), 3.76 (s, 3H, OCH_3), 3.94 (s, 2H,CH_2), 6.64–8.63 (m, 15H, Ar–H), 9.44, 11.18 (2s, 2H, NH cancelled with D_2O): ^{13}C NMR (DMSO, 75 MHz) *δ*:11.40 ($\underline{CH_3}$ $_{pyrole}$), 34.51 (NHCO$\underline{CH_2}$), 55.75 (O$\underline{CH_3}$), 101.14 (CH, $C_{5\ indole}$), 111.64 (C, $C_{3\ indole}$), 115.03 (CH, $C_{7\ indole}$), 118.05 (CH, $C_{8\ indole}$), 129.36 (C, $C_{4\ indole}$), 129.60 (C, $C_{9\ indole}$), 129.61 (CH, C_{2c-} Ph), 129.63 (CH, C_{6c-} Ph), 130.07 (CH, C_{2b} Ph), 130.84 (CH, C_{6b-} Ph), 131.44 (C, C_{4b} Ph), 131.59 (CH, C_{3b} Ph), 131.62 (CH, C_{5b} Ph), 131.75 (CH, C_{3c-} Ph),131.79 (CH, C_{3c-} Ph), 134.59 (CH, C_{2a} Cl-benzoyl), 138.09 (CH, C_{6a} Cl-benzoyl), 134.51 (C, C_{1a} Cl-benzoyl), 135.60 (C, $C_{2\ indole}$), 135.29 (CH, C_{3a} Cl-benzoyl), 135.55 (CH, C_{5a} Cl-benzoyl), 135.93 (CH, C_{4c-} Ph), 138.67 (CH, C_{1c-} Ph) 139.23 (C,C_{1b} Ph), 156.03 (C,C_{4a}Cl-benzoyl), 166.60 (C, carbonyl), 168.43 (C, $C_{6\ indole}$), 169.00 (C, amide), 171.40 (C, amide). EI MS *m/z* (%): 570 $[M]^{+\cdot}$, 339 (100). Anal. calcd. for $C_{32}H_{25}ClFN_3O_4$: C, 67.43; H, 4.42; N, 7.37; found: C, 67.75; H, 4.51; N, 7.52.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-chlorophenyl)benzamide (**8b**) Yellowish brown needles (EtOH); Yield 75%; m.p. 120–122 °C; IR (KBr) νmax: 3238 (Br, NH), 3065 (CH-arom.), 2887 (CH-aliph.), 1669 (Br, CO) cm^{-1}, ^1H NMR (DMSO, 300 MHz): *δ* 2.27 (s, 3H, CH_3), 3.66 (s, 3H, OCH_3), 3.76 (s, 2H, CH_2), 6.61–8.28 (m, 15H, ArH); 10.33, 11.14 (2s, 2H, NH cancelled with D_2O); ^{13}C NMR (DMSO, 75 MHz)*δ*: 11.85 ($\underline{CH_3}$ $_{pyrole}$), 34.55 (NHCO$\underline{CH_2}$), 55.70 (O$\underline{CH_3}$), 103.43 (CH, $C_{5\ indole}$), 116.04 (C, C $_{3\ indole}$), 119.41 (CH, $C_{7\ indole}$), 121.41 (CH, $C_{8\ indole}$), 122.35 (C, $C_{4\ indole}$), 127.56 (C, $C_{9\ indole}$), 128.94 (CH, C_{2c-} Ph), 129.09 (CH, C_{6c-} Ph), 129.20 (CH, C_{2b} Ph), 129.22 (CH, C_{6b-} Ph), 129.50 (C, C_{4b} Ph), 129.69 (CH, C_{3b} Ph), 129.75 (CH, C_{5b} Ph), 130.07 (CH, C_{3c-} Ph),130.12 (CH, C_{3c-} Ph), 130.75 (CH, C_{2a} Cl-benzoyl), 131.50 (CH, C_{6a} Cl-benzoyl), 131.56 (C, C_{1a} Cl-benzoyl), 131.58 (C, $C_{2\ indole}$), 133.79 (CH, C_{3a} Cl-benzoyl), 137.58 (CH, C_{5a} Cl-benzoyl), 138.30 (CH, C_{4c-} Ph), 138.41 (CH, C_{1c-} Ph) 141.61 (C, C_{1b} Ph), 160.18 (C, C_{4a}Cl-benzoyl), 164.96 (C, $C_{6\ indole}$), 166.89 (C, carbonyl), 169.77 (C, amide), 171.05 (C, amide). EI MS *m/z* (%): 588 [M+2], 586 $[M]^+$, 139, (100). Anal. calcd. for $C_{32}H_{25}Cl_2N_3O_4$: C, 65.54; H, 4.30; N, 7.16; found: C, 60.56; H, 4.34; N, 7.33.

2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(2,6-dimethylphenyl)benzamide (**8c**) Brown needles (EtOH); Yield 70%; m.p. 114–116 °C; IR (KBr) νmax: 3372 (Br, NH), 3071 (CH-arom.), 2830 (CH-aliph.), 1679 (Br, CO) cm^{-1}; ^1H NMR (DMSO, 300 MHz) *δ*: 2.17, 2.27, 2.38 (3s, 9H, $3CH_3$), 3.77 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2), 6.61–8.14 (m, 14H, Ar–H), 8.68, 12.50 (2s, 2H, NH cancelled with D_2O). ^{13}C NMR (DMSO, 75 MHz) *δ*: 11.54 ($\underline{CH_3}$ $_{-Ph}$), 19.23 ($\underline{CH_3}$ $_{-Ph}$), 19.50 ($\underline{CH_3}$ $_{pyrole}$), 34.45 (NHCOCH_2), 55.75 (O$\underline{CH_3}$), 102.13 (CH, $C_{5\ indole}$), 112.53 (C, C $_{3\ indole}$), 116.73 (CH, $C_{7\ indole}$), 119.00 (CH, $C_{8\ indole}$), 129.23 (C, $C_{4\ indole}$), 129.48 (C, $C_{9\ indole}$), 129.67 (CH, C_{2c-} Ph), 129.69 (CH, C_{6c-} Ph), 130.00 (CH, C_{2b} Ph), 131.14 (CH, C_{6b-} Ph), 131.50 (C, C_{4b} Ph), 131.60 (CH, C_{3b} Ph), 131.72 (CH, C_{5b} Ph), 131.75 (CH, C_{3c-} Ph), 131.79 (CH, C_{3c-} Ph), 133.19 (CH, C_{2a} Cl-benzoyl), 133.89 (CH, C_{6a} Cl-benzoyl), 134.51 (C, C_{1a} Cl-benzoyl), 135.60 (C, $C_{2\ indole}$), 135.20 (CH, C_{3a} Cl-benzoyl), 135.50 (CH, C_{5a} Cl-benzoyl), 135.90 (CH, C_{4c-} Ph), 137.80 (CH, C_{1c-} Ph) 139.23 (C,C_{1b} Ph), 156.23 (C,C_{4a}Cl-benzoyl), 168.01 (C,$C_{6\ indole}$), 165.90 (C, carbonyl), 169.07 (C, amide), 172.00 (C, amide). EI MS *m/z* (%): 580 $[M]^+$, (0.66), 139, (100). Anal. calcd. for $C_{34}H_{30}ClN_3O_4$: C, 70.40; H, 5.21; N, 7.24; found: C, 70.55; H, 5.27; N, 7.41.

In vitro anticancer screening of the synthesized compounds against human colon cell lines HCT-116, HT-29, and CACO-2

Cell culture

Cancer cells from different colon cancer cell lines HCT-116, HT-29 or Caco-2 were originally purchased from American type Cell Culture collection (ATCC, Manassas,

USA) and were maintained in the tissue culture lab of the Egyptian company for vaccines and sera (Vacsera, Giza. Egypt). Cells were then transferred to our lab and were grown on Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 1% of 100 mg/ mL streptomycin, 100 units/mL penicillin and 10% of heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37 °C (Huang et al. 2013; Sheen et al. 2003).

Cytotoxicity assay by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Exponentially growing cells from different cancer cell lines were trypsinized, counted, and seeded at the appropriate densities (2000-1000 cells/0.33 cm^2 well) into 96-well microtiter plates. Cells then were incubated in a humidified atmosphere at 37 ˚C for 24 h. Then, cells were exposed to different concentrations of compounds (0.1, 10, 100, 1000 µg/ml) for 72 h. Then the viability of treated cells were determined using MTT technique as follow. Media were removed; cells were incubated with 200 µl of 5% MTT solution/well (Sigma Aldrich, MO) and were allowed to metabolize the dye into a colored-insoluble formazan crystals for 2 h. The remaining MTT solution were discarded from the wells and the formazan crystals were dissolved in 200 µl/well acidified isopropanol for 30 min, covered with aluminum foil and with continuous shaking using a MaxQ 2000 plate shaker (Thermo Fisher Scientific Inc, MI) at room temperature. Absorbance were measured at 570 nm using a Stat FaxR 4200 plate reader (Awareness Technology, Inc., FL). The cell viability were expressed as percentage of control and the concentration that induces 50% of maximum inhibition of cell proliferation (IC50) were determined using Graph Pad Prism version 5 software (Graph Pad software Inc, CA).

Cell cycle analysis

The cell cycle profile was assayed by flow cytometry after staining with PI/RNase. HT-29 and HCT-116 cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed, and cells were treated with **7a** and **8c**, at IC50 concentrations (**7a** 0.1 µg/ml, **8c** 0.4 µg/ml). Cells were incubated for 48 h before harvesting. The cells were fixed gently with 80% ethanol before being placed in a freezer for 2 h. They were then treated with 0.25% triton X-100 for 5 min in an ice bath. The cells were resuspended in 30 µl of phosphate buffered saline (PBS) containing 40 µg/ml propidium iodide and 0.1 mg/ml RNase. Cells were incubated in a dark room for 20 min at room temperature before cell cycle analysis with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the FlowJo software (Ashland, OR). For each measurement, at least 10,000 cells were counted.

Apoptotic analysis

HT-29 and HCT-116 cells were seeded in 24-well tissue culture plates. After 24 h, the medium was changed and **7c** and **8c** at IC50 concentrations were added. After treatment for 48 h, cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then culture medium containing 10% FBS (and floating cells) was added to inactivate trypsin. When gentle pipetting was completed, the cells were centrifuged for 5 min at 1500 g. The supernatant was removed and cells were stained with annexin V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's instructions. Untreated cells were used as control for double staining. Immediately after staining the cells were analyzed by a FACScan flow cytometer. For each measurement, at least 20,000 cells were counted.

Caspase activity assay

Caspase activities were assayed using caspase enzyme Assay kits (Geno Technology). In brief, HT-29 and HCT-116 cells were treated with IC50 concentration of **7a** and **8c** respectively for 24 or 48 h, collected by trypsinization, washed once with PBS, and cell pellets resuspended in 350 ml lysis buffer. The cells were lysed by freeze and thaw five times. The lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. Supernatants were collected and used for measuring caspase-3, and -9, activities by Enzyme-linked Immunosorbent Assay (ELISA) cell-based assay according to the manufacturer's instructions. Caspase-3, and caspase-9 activities were detected using the specific caspases fluorogenic substrate, DEVD peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). Samples were read on a microplate reader at 405 nm (ThermoLabsystems, Chantily, Va.)

CDK2/A enzyme assay

ELISA was used for profiling evaluation of protein kinase targets. The microtiter plate provided in this kit has been pre-coated with an antibody specific to CDK-2A. Samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to CDK2. Next, Avidin conjugated to Horseradish peroxidase (HRP) is added to each microplate well and incubated. Then the TMB substrate solution is added, only those wells that contain CDK2, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a change in color. The

enzyme-substrate reaction is terminated by the addition of 1N sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 10 nm. The concentration of CDK2 in the samples is then determined by blotting the O.D. of the samples to the standard curve.

Results and discussion

Chemistry

The synthesis of the target compound is outlined in Schemes 1 and 2. Indomethacin was used as starting scaffolds to design our targeted new monoamide and diamide analogs containing various substituent with different electronic environment which might be beneficial to their biological activity. Indomethacin acid chloride (**1**) which was prepared from indomethacin via reported process (Eissa et al. 2013 and Khalifa et al. 2012) was used as key intermediate for successful transformation of a carboxylate moiety of the parent compound into, amide or sulphonamide functions, that were known to contribute to the enhancement of the antitumor activity, via a single chemical derivatization method (amidation) (Kalgutkar et al. 2000). The reaction between the key intermediate (**1**) and different aromatic amine containing compounds afforded the 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl) N-substituted acetamide analogues (**2a–e**). Different sulfonamide derivatives 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(N-(5-substituted-3-yl)sulfamoyl)phenyl)acetamide (**5a–e**) were prepared to investigate the effect of replacement of the amide function in (**2a–e**) derivatives with a different sulfonamide moites. On the other hand, acid chloride key intermediate (**1**) was allowed to react with the corresponding ethylenediamine to generate the N-(2-aminoethyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide intermediate (**3**), which subsequently reacted via condensation with different appropriate aromatic aldehydes in glacial acetic acid to afford the respective, arylidene derivatives, (E)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-((4-substituted-benzylidene) amino)ethyl)acetamide (**4a–c**), Scheme 1.

In order to evaluate the effect of substituents variation (linker group variation) on the cytotoxic activity of the synthesized derivatives, benzoxazin-4-one derivative, named as, 2-((1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)methyl)-4H-benzo[d][1,3]oxazin-4-one (**6**) was obtained in reasonable yield via refluxing,the amide analog, 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido) benzoic acid (**2b**) in acetic anhydride (Noolvi et al. 2011; Al-ObaidA et al. 2009). The latter compound (**6**) reacted in the similar design strategy to generate the diamide derivatives 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-(N-(4-substituted)sulfamoyl)phenyl)benzamide derivatives (**7a–c**) and 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)-N-(4-substituted phenyl)benzamides (**8a–c**) where the benzoxazinone derivative (**6**) underwent nucleophilic ring opening via the reaction with different aromatic amines or appropriate sulfa drug in dry pyridine, Scheme 2.

All the synthesized compounds were crystallized using appropriate solvents and characterized by H^1, C^{13}, NMR as well as by MS and elemental analysis.

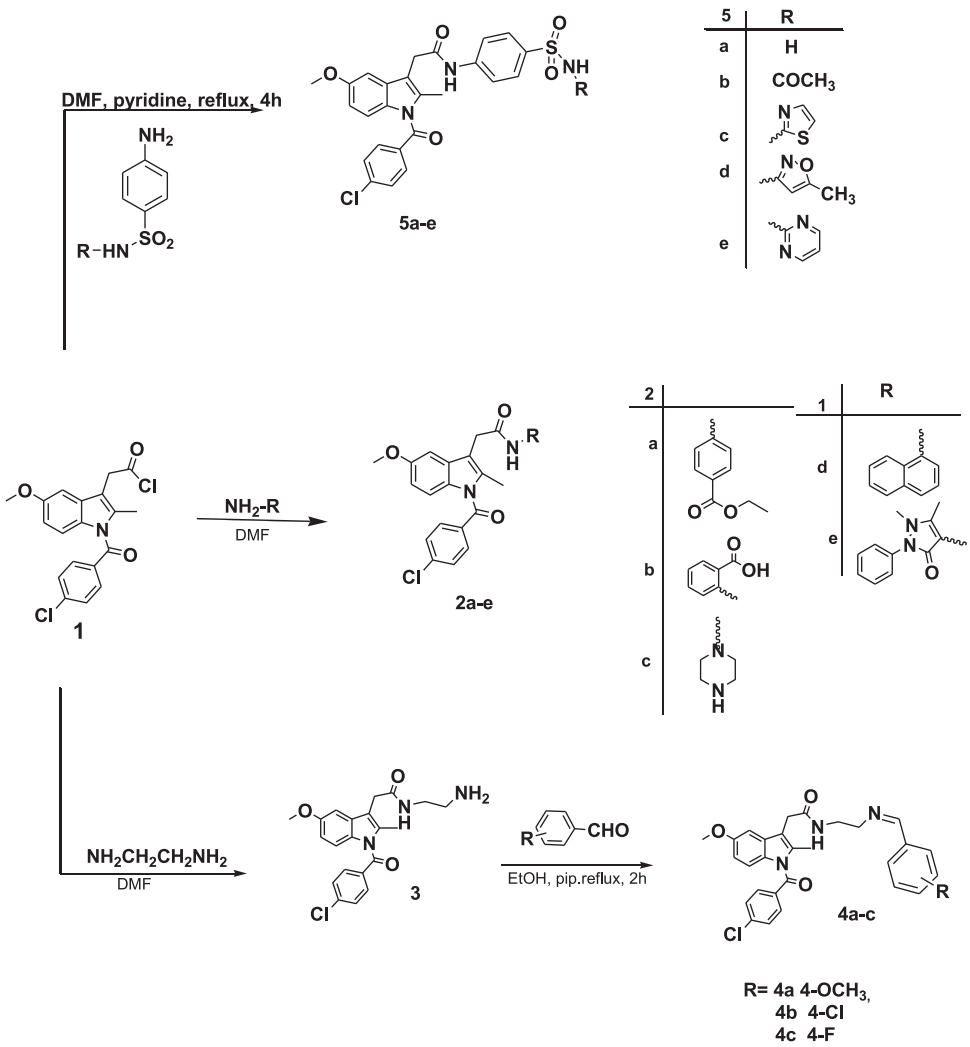
Biological activity

In vitro cytotoxic activity

Based on the widly reported colo-rectal cancer activity of indomethacin, the cytotoxic activity of the newly synthesized analoges was evaluated using (MTT) in vitro assay (Huang et al. 2013) against three human colon cancer cell lines: HCT-116, CACO-2, and HT-29, compared to the parent compound (indomethacin) and (5-FU) as a reference standard (cell lines selection was encouraged by the reported antiproliferative activity of indomethacin against colon cancer cell lines). The concentrations that cause 50% inhibition of cancer cell growth against various cell lines are expressed as IC50 values and are summarized in Table 1. Analysis of the data in Table 1 showed that, all the tested cell lines were susceptible to the influence of the tested compounds. With the exception of compounds **2a**, **4a**, **b**, **5a–e**, all the tested compounds exhibited stronger cytotoxic activities than that of parent compound for all three human cancer cell lines.

Interestingly, three of our tested compounds **7a**, **8a**, and **8c** were found to exhibit excellent anti-proliferation activities for almost all three human cell lines. Surprisingly, compound **7a** showed 99 fold higher cytotoxic activities of 5-FU against CACO-2 cell line with IC50 = 0.055 µg/ml, 7.5 fold higher cytotoxic activities of 5-FU against HT-29 with IC50 = 0.1 µg/ml, nearly half of cytotoxic activity of 5-FU with IC50 = 4.0 µg/ml. The diamide derivative **8a** had potent growth inhibitory activity for all the tested cell lines with the IC50 values smaller than 1 µg/ml (IC50 = 0.78, 0.505, 0.13 µg/ml compared to 1.8, 0.75, 5.45 µg/ml for 5-FU against HCT-116, HT-29, and CACO-2 cell lines respectively.

At the same time compound **8c** of the same series exhibited high cytotoxic activity against HCT-116 and CACO-2 with (IC50 = 0.4, 4.7 µg/ml compared to 1.8, 5.45 µg/ml for 5-FU respectively, Table 1.



Scheme 1 General procedure for preparation of target compounds **2a–e**, **3**, **4a–c** and **5a–e**

Cell cycle analysis

In response to DNA damage, cells arrest primarily at the G1/S and G2/M boundaries. G1/S arrest blocks entry of damaged DNA into the S phase, and G2/M arrest prohibits entry of damaged DNA into mitosis (Sheen et al. 2003). To analyze the possible mechanism underlying the anti-proliferative effect exerted by our new compounds, HT-29 and HCT-116 cell cycle progression was assessed following treatment with compounds **7a** and **8c** (compounds that had the marked highest antiproliferative activity (IC50 0.1 and 0.4 µg/ml) on the human colorectal adenocarcinoma HT-29 and HCT-116 cell line respectively for 24 using propidium iodide flow cytometry analysis. The apoptosis inducing activity of **7a** and **8c** was also characterized by flow cytometric analysis of the DNA profile in HT-29 and HCT-116 cells respectively (Table 2, Figs. 1, 2, 3).

The cell cycle distribution was monitored by flow cytometry analysis after propidium iodide staining of the cellular DNA (Huang et al. 2013; Sheen et al. 2003). As seen in (Figs. 1, 2, 3) in comparison with control cells, exposure of HT-29 cells to **7a** (0.1 µg/ml) for 24 h increase in the percentage of cells at the pre-G phase (pre-G1 apoptosis) and induced cell cycle arrest at G1/S phase, manifested by an increase of the cell number in the G1/S phase with a concomitant reduction in the percentage of cells at G2/M phase compared to control, Meanwhile, exposure of HCT-116 cells to **8c** for 24 h markedly increase in the percentage of cells at the pre-G phase (pre-G1 apoptosis) and induced cell cycle arrest at G0/G1 phase manifested by an increase of the cell number in the G0/G1 phase with a concomitant reduction in the number of cells in S phase compared to the control. These results indicate that, compound **7a** and **8c** arrested cells in G1/S and G0/G1 phase respectively, with subsequent induction of apoptosis.

Caspase-3 activity (key executor of apoptosis)

Study of apoptosis has considered the basis for novel targeted therapies that can bring death in malignant cells

Scheme 2 General procedure for preparation of target compounds **6**, **7a–c** and **8a–c**

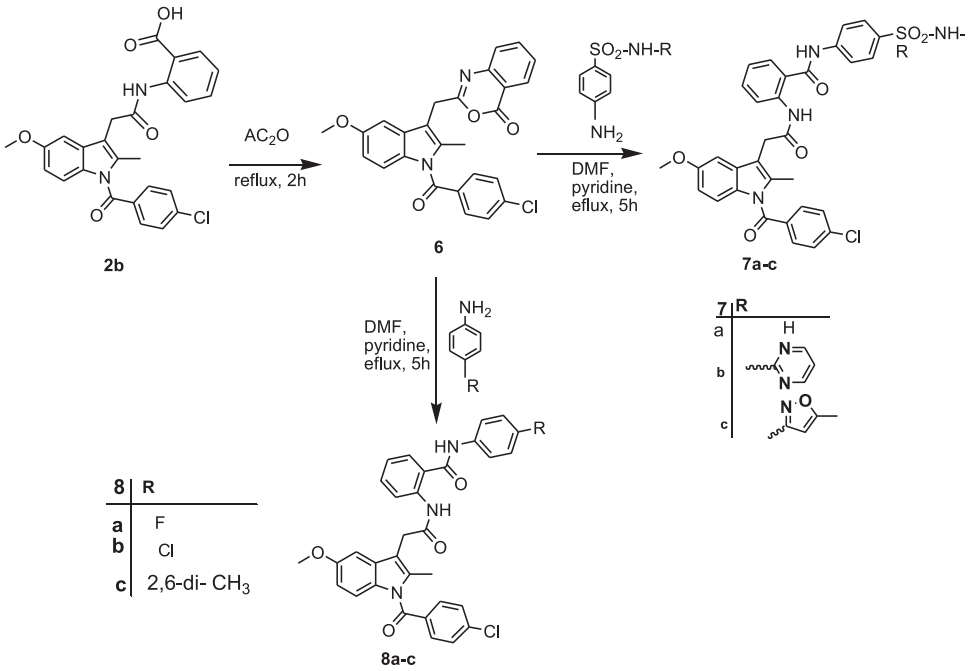


Table 1 Cytotoxic activity of the newly synthesized compounds against human colon cell lines (HCT-116, HT-29, and CACO-2). IC50 values are mean of three separate experiments ± S.D

Compound	HCT-116 IC50 µg/mL (µM)	HT-29 IC50 µg/mL (µM)	CACO-2 IC50 µg/mL (µM)
2a	43.6 ± 0.503	155 ± 3.603	77.6 ± 1.20
2c	13.5 ± 0.136	2.9 ± 0.1	15.5 ± 0.351
2d	8 ± 0.251	1.0 ± 0.386	22 ± 2.51
2e	32.5 ± 0.461	13.5 ± 0.251	18 ± 1
3	1.0 ± 0.2	4.5 ± 0.152	1.0 ± 0.163
4a	120 ± 2.081	26 ± 1.525	1.0 ± 6.082
4b	347 ± 7.505	160 ± 3.601	616 ± 2.645
4c	10.5 ± 0.208	9.5 ± 0.152	14 ± 1
5a	46 ± 3.055	21.5 ± 0.152	51 ± 2
5b	102 ± 5.866	27 ± 2.516	53.7 ± 3.156
5c	76 ± 4.509	14.5 ± 0.152	52.5 ± 1.60
5d	43.6 ± 0.416	69 ± 3.511	36 ± 3.511
5e	51 ± 3.055	91 ± 3.511	72.4 ± 1.793
7a	4 ± 0.215	0.1 ± 0.511	0.055 ± 0.285
7b	5.7 ± 0.3	5.5 ± 0.288	2.9 ± 0.152
7c	5.1 ± 0.251	5.52 ± 0.3	2.66 ± 0.02
8a	0.78 ± 0.011	0.505 ± 0.221	0.13 ± 0005
8b	446.2 ± 5.047	10.9 ± 0.152	8.7 ± 0.251
8c	0.4 ± 0.219	28.8 ± 0.757	4.7 ± 0.152
Indomethacin	50.11 ± 0.548	53.7 ± 0.173	30.2 ± 0.461
5-FU	1.8 ± 0.2	0.75 ± 0.045	5.45 ± 0.25

Table 2 HCT-116 and HT-29 cell cycle distribution of compounds **6a** and **7c**

Comp.no.		Cell cycle distribution (%)			
Sample code	Used conc. µg/ml	%G0-G1	%S	%G2-M	%Apoptosis
7a/HT-29	**0.1**	61.31	24.62	0.42	13.62
8c/HCT-116	**0.4**	78.6	14.82	0.0	6.58
Cont.HT-29	**0**	71.18	23.45	4.85	0.52
Cont. HCT116	**0**	68.7	23.49	7.33	0.48

Bold values are significantly different from control at $p<0.05$

(Hassan et al. 2014). Consequently, the activation of apoptosis is emerging as a key approach to treat colorectal tumor. The unique characteristics of apoptosis is the activation various enzymes that are cysteine protease families called caspases (Sheen et al. 2003). Caspase-3 and caspase-9 are two key proteins of the caspase family, which are highly conserved in multicellular organisms and function as central regulators of apoptosis (Alnemri et al. 1996; Kim et al. 2000; Creagh and Martin 2001; D'Amelio 2010; Janicke et al. 1998) Clarifying the role of caspases in newly synthesized indomethacin analogue-induced colon cancer cell death is important for understanding molecular mechanisms of the antineoplastic effect of those analogue. To elucidate a pathway leading to new derivatives induced cell death we determined the ability of compounds **7a** and **8c** to activate caspase-3/9 in colon cancer cell lines HT-29

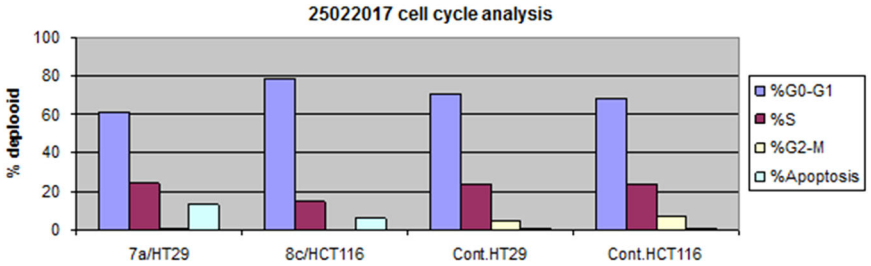


Fig. 1 Percentage of each cell cycle phase with various treatments or with control

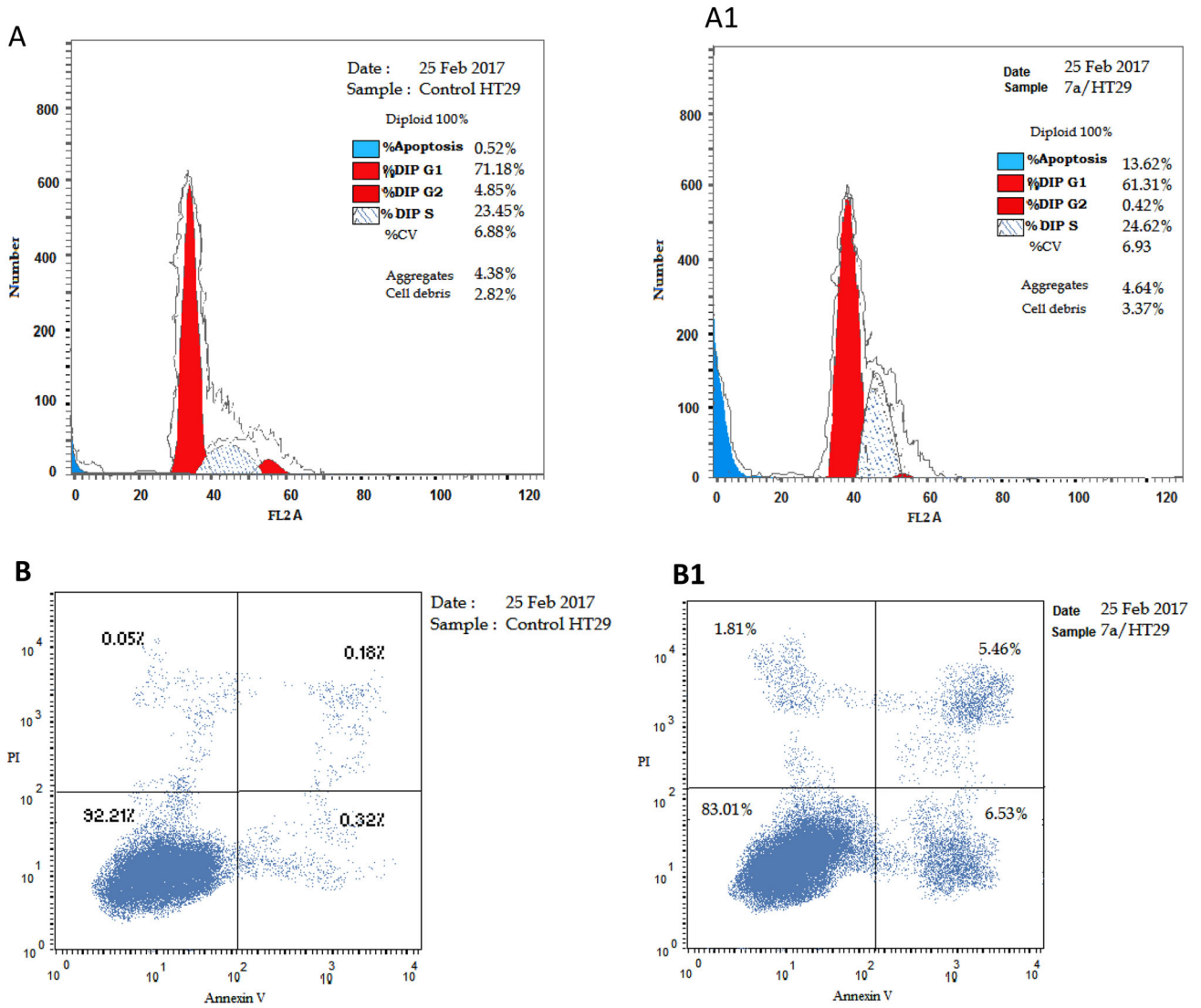


Fig. 2 **a**, **a1** Effects of **7a** on HT-29 cell cycle profile at its IC50 compared to untreated cell using flow cytometry data for 24 h. **a** Untreated HT-29 cell **a1** HT-29 cell treated with 8C. **b**, **b1** **7a** Induced apoptosis in human HT-29 cell as assayed by annexin V staining compared to control cell using flow cytometryfor 24 h., **b** control cell, **b1** Representative scatter Plots of PI (y-axis) vs. annexin V (x-axis) showing the effects of **7a** on HT-29 cell apoptosis

and HCT-116. Compared with the control, we tested the increase in caspase-3 and -9 activities in HT-29 and HCT-116 cells in response to **7a** and **8c** treatment at its IC50, the level of active caspase-3/9 was measured in ng/g protein using caspase apoptosis assay kits (ELISA-based assay) for 24 and 48 h. As shown in Table 3, Fig. 4, the activities of caspase-3 and caspase-9 were increased following the treatment of HT-29 and HCT-116 cells with **7a** and **8c**

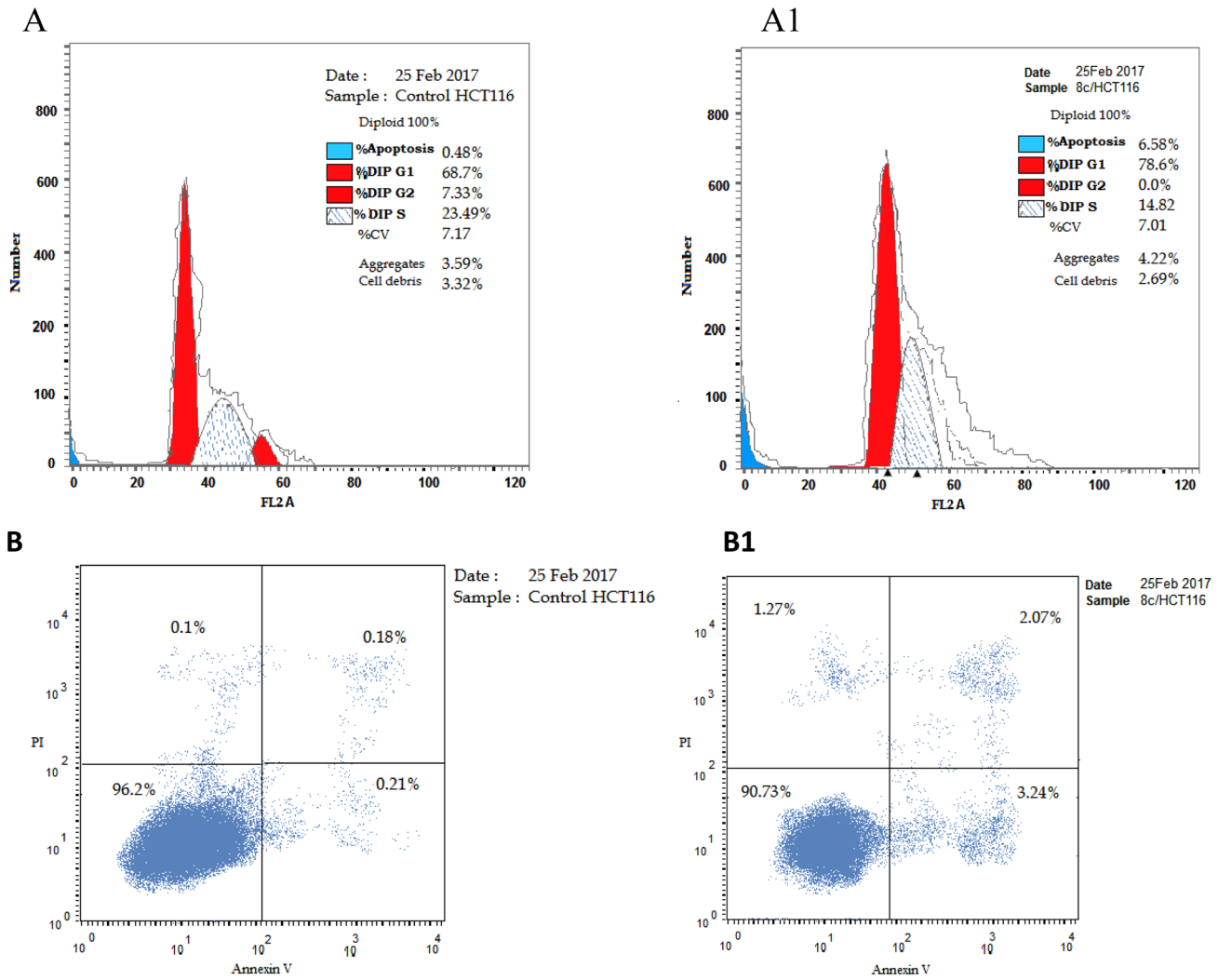


Fig. 3 **a**, **a1** Effects of **8c** on HCT-116 cell cycle profile at its IC50 compared to untreated cell using flow cytometry data. **a** Untreated HCT-116 cell **a1** HCT-116 cell treated with **8c**. **b**, **b1** **8c** Induced apoptosis in human HCT-116 cell as assayed by annexin V staining compared to control cell using flow cytometry, **b** control cell, **b1** Representative scatter Plots of PI (y-axis) vs. annexin V (x-axis) showing the effects of **8c** on HCT-116 cell apoptosis. Data are mean ± SEM. * significantly different from control at $p < 0.05$

Table 3 **7a** and **8c** induced increases in caspase-3/9 activity in human HT-29 and HCT-116 cells treated with IC50 of compound **7a** and **8c** for 24 and 48 h respectively measured by ELISA-based assay

	Cell line	Comp	24 h	Control 24 h	Ratio of activation	48 h	Control 48 h	Ratio of activation
Caspase-3 conc. ng/ml	HT-29	**7a**	0.3119	0.03849	8 fold	0.5055	0.04884	10.5 fold
	HCT-116	**8c**	0.1925	0.02293	8.5 fold	0.2952	0.03277	9 fold
Caspase-9 conc. ng/ml	HT-29	**7a**	19.74	2.707	7.3 fold	28.33	2.064	13.7 fold
	HCT-116	**8c**	13.44	4.188	3 fold	25.66	3.169	8 fold

respectively. In details, treatment of HT-29 cells with **7a** for 24 and 48 h caused a significant increase in caspase-3 level by about 8 and 10.5 folds respectively, compared to control, while treatment of HCT-116 cells with **8a** for 24 and 48 h caused a significant increase in caspase-3 level by about 8.5 and 9 folds respectively, compared to control. At the same time treatment of HT-29 cells with **7a** for 24 and 48 h caused a significant increase in caspase-9 level by about 8 and 10.5 folds respectively, compared to control, while treatment of HCT-116 cells with **8a** for 24 and 48 h caused a significant increase in caspase-9 level by about 8.5 and 9 folds respectively, compared to control. These results suggest that **7a** and **8c** may induce apoptosis in of HT-29 and HCT-116 cells via a caspase-dependent pathway.

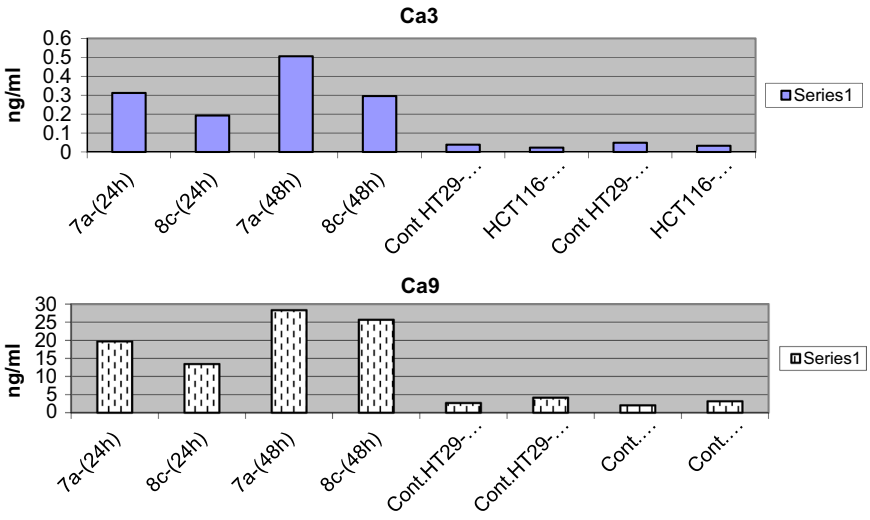


Fig. 4 Effect of **7a** and **8c** on Caspase-3/9 activity in HT-29 and HCT-116 cells for 24 and 48 h respectively measured by ELISA-based assay. The experiment was done in triplicate. Data are mean ± SEM. *Significantly different from control at $p < 0.05$

Compound	Results			
Cpd. code.	**IC50 ug/ml**	**Cell line**	CDK2**A**	
			Residual conc.ng/ml	**% inhibition**
7a-(24h)	0.1	HT29	1.076	56.15322
8c-(24h)	0.4	HCT116	1.299	54.05023
7a-(48h)	0.1	HT29	0.6059	85.91585
8c-(48h)	0.4	HCT116	0.8157	79.55127
Cont HT29-(24h)			2.454	0
Cont HCT116-(24h)			2.827	0
Cont HT29-(48h)			4.302	0
Cont HCT116-(48h)			3.989	0

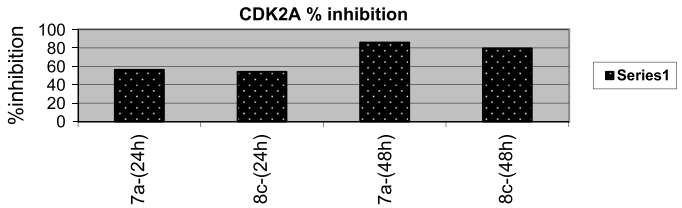


Fig. 5 The effect of **7a** and **8c** (expressed as % inhibitionon) on CDK2 expression in HT-29 7 HCT-116 cells respectivelly, compared to control cells using using radioisotope ELISA assay. The experiment was done in triplicate. Data are mean ± SEM. *Significantly different from control at $p < 0.05$

c:

Cyclin-dependent kinases (CDKs) are a family of protein kinases that are participating in the regulation of the cell cycle and promote cell proliferation. Different types of cyclins and CDKs play their roles at various stages of the cell cycle (Woo et al. 1998) and conidered the main regulatory point to start cell cycle is in G1 phase. During G1 phase, growth-dependent CDK, and specifically, CDK-2A activity promotes DNA replication and initiates G1-to-S phase transition and considered the rate-limiting step for progression of G1 phase (Pan et al. 2002). Activation of CDK2 and CDK4 can yield many cell cycle related proteins, thus prompting progression from G1 phase to S phase (Wolter et al. 2001). To verify if the G1/S phase arrest caused by compound **7a** and G0/G1 phase arrest caused by compound **8c** is mediated through CDK-2A inhibition, the kinase inhibitory effect of **7a** and **8c** was evaluated against CDK-2A at IC50 concentrations using ELISA assay (Bertoli et al. 2013 and Ma et al. 2008). The profiling data in Fig. 5 showed that CDK-2A activities of HT-29 were inhibited by 55 and 79% after 24 and 48 respectivelly compared to control cell due to the effect of **7a**. On the other hand, **8c** showed potent inhibition of enzyme at its IC50 concentration, the CDK-2A activities were inhibited by 93 and 90% respectively compared to control. In summary, experiments might have proved that **7a** and **8c** might inhibits cell proliferation, alters cell cycle and arrests G1/S and G1phase, respectively, possibly through down-regulating CDK-2A (Fig. 5).

Conclusion

In this study, the molecular structure of indomethacin was used as starting scaffolds to design novel analogs and their

effects on the proliferation of three human colon cancer cell lines were evaluated compared to indomethacin and 5FU as a reference. The results indicated that 12 compounds, out of 21, **2c**, **2d**, **2e**, **3**, **4a**, **4c**, **7a**, **7b**, **7c**, **8a**, **8b**, and **8c** exhibited cytotoxic activities stronger than that of parent compound (indomethacin) for all three human cancer lines. Interestingly, three of our tested compounds **7a**, **8a**, and **8c** were found to exhibit excellent anti-proliferation activities for almost all three human cell lines. The most potent compound **7a**, showed 99 and, 7.5, fold higher cytotoxic activities against CACO-2 and HT-29 cell lines respectively than 5-FU, while the diamide derivative **8C** had potent growth inhibitory activity for all the tested with the IC50 values smaller than 1 μg/ml. Furthermore, we observed the combined effect on cell apoptosis, cell cycle, caspase, and CDK-2A assays to elucidate the possible mechanism of **7a** and **8c** effects on colorectal cancer cells. The data indicated that **7a** and **8c** inhibited tumor proliferation and inducing cell cycle arrest in the G1/S and G0/G1 respectively might be through potent inhibition of CDK-2A and induced cancer cell apoptosis, propably, via caspase-3/9 dependent pathway. Previous findings have shown that the most active candidates may serve as promising structures in the search for powerful and selective colorectal-anticancer agents; further studies are being planned to analyze more biological molecules that are important for cell proliferation in all cancer cell lines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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