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Full Paper

Synthesis and Biological Evaluation of Novel Indomethacin Derivatives as Potential Anti-Colon Cancer Agents

Amel Mostafa Farrag

Pharmaceutical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo, Egypt

The molecular structure of indomethacin was used as a starting scaffold for the synthesis of 20 novel analogs and to study their effects on the proliferation of three human colon cancer cell lines, HCT-116, HT-29, and Caco-2, by MTT assay. The synthesized indomethacin analogs were characterized on the basis of IR, ^{1}H NMR, ^{13}C NMR, mass spectral data, and elemental analysis results. Cytotoxicity assay results showed that the indomethacin amide analog **2** was the most potent anticancer agent ($IC_{50} = 0.78$, 0.09, and 0.0127 μg/mL) against the three colon cancer cell lines, respectively, being more potent than the standard 5-fluorouracil ($IC_{50} = 1.8$, 0.75, and 5.45 μg/mL). Interestingly, the indomethacin oxazin analog **3** and the indomethacin amide analog **8** displayed very potent anticancer activity against the HCT-116 cell line with $IC_{50} = 0.421$ and 0.27 μg/mL, respectively, much better than the reference ($IC_{50} = 1.8$ μg/mL). Additionally, analogs **3**, **4b**, **11**, **12c**, and **13a** exhibited excellent antitumor activity against Caco-2 cells, with IC_{50} ranging from 1.5 to 4.5 μg/mL. Furthermore, analogs **2** and **8** were additionally examined for their effect on the cell cycle of HCT-116 and HT-29 cells, respectively, using flow cytometric analysis. Analog **2** arrested the cell cycle of HT-29 cells at the S phase, while **8** was found to arrest the cell cycle of HCT-116 cells at the G0/G1 phase.

Keywords: G1 phase / Human colon cancer cell line / Indomethacin / S phase

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Introduction

Colon cancer is one of the most common digestive malignant tumors with increasing morbidity and mortality, which severely threatens the health of human beings. Prevention and control of colon cancer has been a research focus in the medical field for a long time [1]. Chemoprevention represents an important and feasible option for this cancer, especially in view of the limited efficacy of currently available treatments for its advanced stages [2].

Correspondence: Dr. Amel Mostafa Farrag, El Arabi Pharma Group, 2nd Floor, 4 El Marwa Buildings, Ahmed Tayseer St., Heliopolis, Cairo 11371, Egypt.
E-mail: amelfarrag@ymail.com
Fax: +202-24182105

One important theme in the body of evidence is the fact that non-steroidal anti-inflammatory drugs (NSAIDs) are able to alter intestinal tumor growth rates and modulate carcinogenesis by a variety of reported methods including inhibition of COX activity and disruption of prostaglandin homeostasis, interruption of nuclear factor kappa B (NF-kB) signaling [3–6], and of extracellular signal-regulated kinases (ERK/MAPK) [7], induction of various apoptotic pathways [8, 9], as well as effects on cell cycling [9]. All of these mechanisms either contribute and enhance, or antagonize and counterbalance, the proliferative behavior that is observed in tumor cells.

Indomethacin, an NSAID, is related to the decrease in the incidence rate and mortality rate of colon cancer when administered on a long-term basis [10]. It also exhibits anticancer activity as suggested by a report demonstrating that indomethacin significantly increased the lifespan of a group of terminally ill patients suffering from a range of cancers, mainly gastrointestinal [11]. In 1980, Waddell et al. [12–14] reported that treatment with indomethacin led to the

regression of colorectal polyps in patients with familial adenomatous polyposis. Since then, preclinical studies have established that indomethacin exhibits substantial efficacy against colon.

In the recent past, two separate studies using indomethacin in HT-29 and HCT-116 colon cancer cell lines demonstrated anti-proliferative effects with concentrations of 100 and 600 μM of the drug, respectively, after 72 h [15, 16].

It was found that indomethacin inhibits human colorectal cancer cell growth by inducing G1 arrest and apoptosis [17]. It also suppresses angiogenesis through inhibition of mitogen-activated protein kinase (MAPK) activity [18].

Phospho-tyrosol-indomethacin (PTI) [19], a novel derivative of indomethacin, possesses potent anticancer efficacy in preclinical models of human colon cancer (Fig. 1).

Development of anti-colorectal active analogs based on known inhibitor is an important strategy for the discovery of new anti-cancer drugs with high efficiency and less toxicity. It is feasible to completely improve the anti-cancer potency through chemical modification of the NSAIDs with promising anti-cancer activities [20–22]. In our effort to develop novel anti-cancer agents, we aim at the indomethacin as lead compound (Fig. 2).

In this study, indomethacin was used as lead compound and a series of analogs were synthesized through systematic modification of the carboxylic acid moiety (Fig. 2). A series of new analogs were synthesized and evaluated as antiproliferative agents against three human colon cancer cell lines, HCT-116, HT-29, CACO-2, in order to further systematically interpret the structure–activity relationship, and to explore the pathways by which cell death was induced.

Results and discussion

Chemistry

The preparation of the target compounds **2–13c** is described in Schemes 1 and 2. The reaction of indomethacin acid chloride **1** and 2-aminobenzoic acid furnished the amide analog **2** which was then refluxed in acetic anhydride [23] to obtain 2-((1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)methyl)-4*H*-benzo[d][1,3]oxazin-4-one **3**.

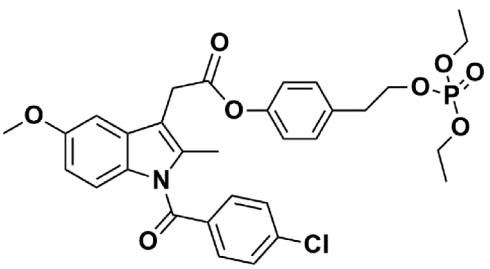

Figure 1. Chemical structure of PTI.

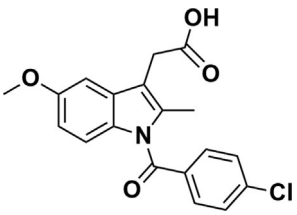

Figure 2. Chemical structure of indomethacin.

The latter compound reacted with hydrazine hydrates and/or phenyl hydrazine in an attempt to obtain 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2-(hydrazinecarbonyl derivatives)phenyl)acetamide **4a**,**b**. Condensation of **4a** with different aromatic aldehydes [24] to afford respective 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2-(2-(4-benzylidene derivatives)hydrazinecarbonyl)phenyl)acetamide **5a–d**. Accordingly, acid chloride **1** was allowed to react with methyl anthranilate via nuclophilic addition [25] to afford methyl 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol- 3-yl)acetamido)benzoate **6**, which was refluxed with hydrazine hydrate in *n*-butanol [26] to obtain 3-amino-2-((1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)methyl)quinazolin-4(3*H*)-one **7**. The key intermediate *N*-(4-acetylphenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl) acetamide **8** was obtained by the reaction of indomethacin acid chloride **1** with 4-aminoacetophenone [25]. Refluxing of the key intermediate **8** with thiosemicarbazide in acetic acid [27] yielded the corresponding thiosemicarbazone **9**. The intermediate **8** was also refluxed in ethanol with hydrazine hydrate [27] to afford the corresponding hydrazone derivative 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(4-(1-hydrazonoethyl)phenyl)acetamide **10** which upon refluxing in dioxan with cyclohexyl isothiocyanate [25] furnished 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(4-(1-(2-(cyclohexylcarbamothioyl)hydrazono)ethyl)phenyl)acetamide **11**. The Claisen–Schmidt condensation of **8** with different aromatic aldehydes in ethanolic potassium hydroxide [24] afforded the chalcone derivatives **12a–c**.

Condensation of chalcone derivatives **12a–c** with malononitrile in ethanol containing few drops of piperidine [28] yielded *N*-(4-(6-amino-5-cyano-4-phenyl-4*H*-pyran-2-yl)phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetamide dervatives **13a–c**. All products were characterized by IR, 1H, ^{13}C NMR spectroscopy as well as by MS and elemental analysis.

X-ray diffraction analysis of 6

The single colorless crystals for compound **6** was developed by slow evaporation of alcohol at room temperature, the structure was reconfirmed by crystallographic methods. ORTEP diagram of the molecule is shown in Fig. 3 (Supporting Information Table S1 lists the main crystallographic parameters).

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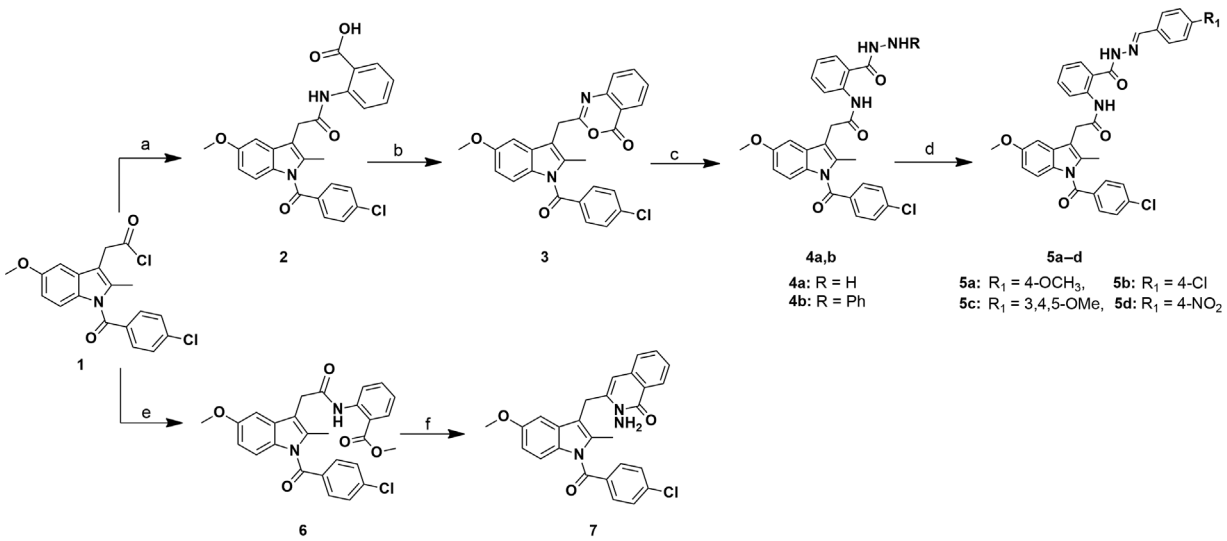


Scheme 1. Reagents and conditions: a) 2-aminobenzoic acid, DMF, pyridine, reflux, 2 h; b) AC_2O, reflux, 4 h; c) NH_2NHR, EtOH; d) **4a**, RC_6H_5CHO, EtOH, reflux, 8 h; e) methyl anthranilate, DMF, pyridine, reflux, 2 h; f) N_2H_4, *n*-butanol, reflux, 4 h.

Biological activity

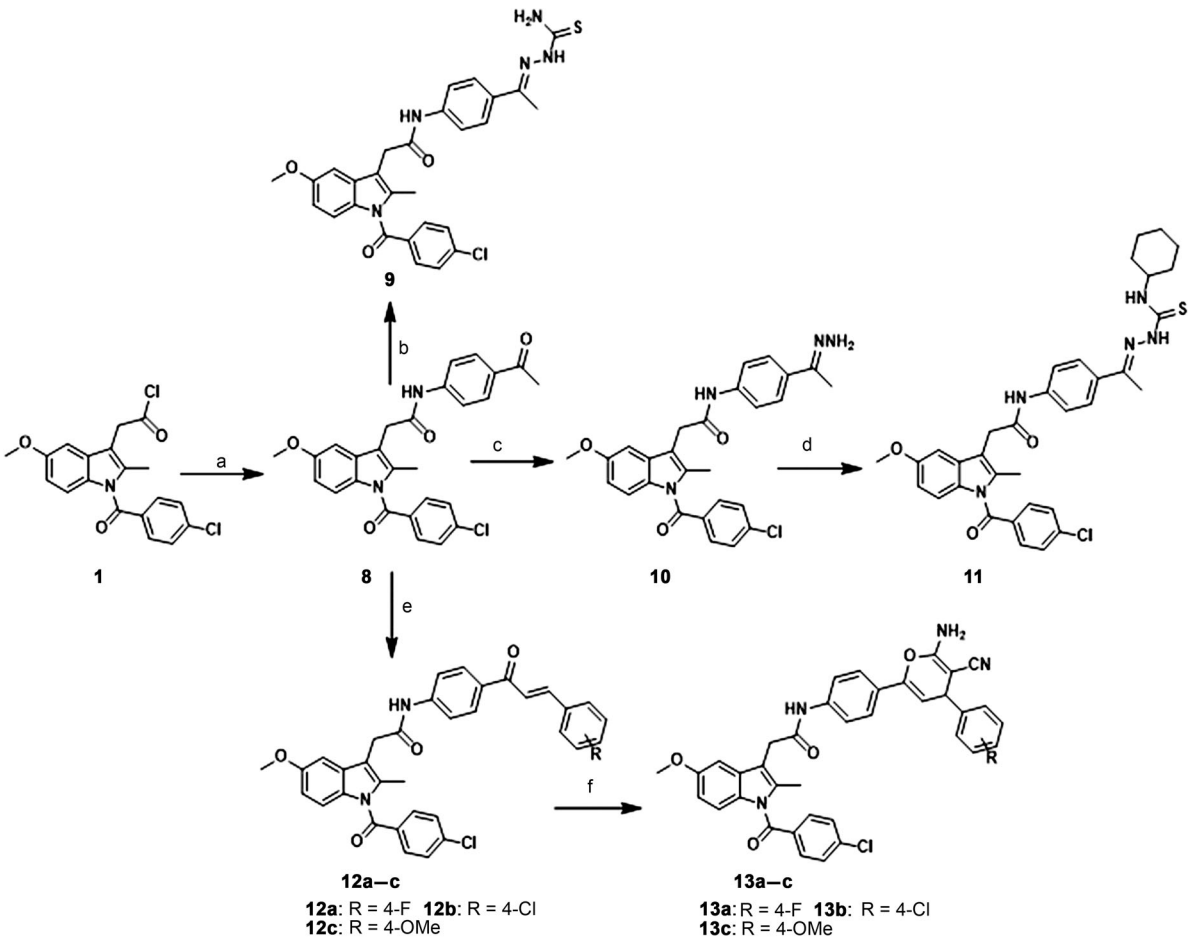
In vitro cytotoxic activity and structure–activity relationships

The synthesized compounds **2**, **3**, **4a**,**b**, **5a–d**, **6–11**, **12a–c**, and **13a–c** in addition to indomethacin were screened for their antitumor activity against HCT116, HT-29, CACO-2 colon cancer cell lines, in comparison with the activity of the known anticancer agent 5FU as a reference drug, utilizing the *in vitro* 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) standard method [29, 30]. The results were expressed as IC_{50} values which represent the compound concentrations required to produce a 50% inhibition of cell growth after 72 h of incubation. From the results showed in Table 1 and Fig. 4, it is apparent that seven of the synthesized analogs exhibit potent antitumor properties against the tested human tumor cell lines.

Considering the observed antitumor screening data against HCT116 cell line in the range of 0.27–1000 μg/mL, it was found that compounds **2**, **3**, and **8** which included 2-aminobenzoic acid, benzo-oxazone, and 4-aminoacetophenone substituents had potent cytotoxic activity with IC_{50} values 0.78, 0.421, and 0.27 μg/mL, respectively, whereas 5FU and indomethacin IC_{50} values were 1.8 and 50.11 μg/mL against HCT116, respectively. Compound **2** exhibited a very potent cytotoxic activity against HT29 cell line with an IC_{50} value of 0.09 μg/mL whereas 5FU and indomethacin had values of 0.75 and 53.7 μg/mL, respectively. CACO-2 cells were the most susceptible cells to the compounds **2**, **3**, **4b**, **11**, **12c**, and **13a**, as they exhibited the most superior anti-proliferative activity against CACO-2, colon cancer cell line with IC_{50} values of 0.0127, 0.12, 4.5, 4.3, 1.5, 1.5 μg/mL, respectively, compared with 5FU and indomethacin IC_{50} values which were 5.45 and 30.2 μg/mL, respectively.

With regard to antitumor activity, indomethacin derivatives that substituted by 2-aminobenzoic acid **2** and 4-aminoacetophenone **8** were superior to this substituted with anthranilate moiety **6**. This is obvious upon comparing compound **2** (HCT-116, IC_{50} 0.78 μg/mL), compound **8** (HCT-116, IC_{50} 0.27 μg/mL), while that of **6** (HCT-116, IC_{50} 57.5 μg/mL). Additionally, indomethacin benzo-oxazone derivative **3** was found to be strongly more active than amino-isoquinoline indomethacin derivative **7**, as their antitumor activity displayed that compound **3** possesses (HCT-116, IC_{50} 0.421 μg/mL; HT-29, IC_{50} 1.33 μg/mL; CACO-2, IC_{50} 0.12 μg/mL),while compound **7** possesses (HCT-116, IC_{50} 32.4 μg/mL; HT-29, IC_{50} 288.5 μg/mL; CACO-2, IC_{50} 135 μg/mL). On the other hand, compound **4b** bearing amino-*n*-phenylbenzohydrazide (CACO-2, IC_{50} 4.5 μg/mL) offers antitumor activity more than that of derivative bearing 2-aminobenzohydrazide **4a** (CACO-2, IC_{50} 229 μg/mL), and 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2-(2-(4-benzylidene derivatives)-hydrazinecarbonyl)phenyl)acetamide **5a–d** (CACO-2, IC_{50} 21.3–985 μg/mL).

Cyclohexyl isothiocyanate derivative **11** exhibited more potent antitumor activity (CACO-2, IC_{50} 4.3 μg/mL) than that of thiosemicarbazide derivative **9** (CACO-2, IC_{50} 338 μg/mL) and the hydrazide derivative **10** (CACO-2, IC_{50} 138 μg/mL). Furthermore, it was found that introduction of 4-methoxy benzaldehyde on the key intermediate **8** yielded much more potent derivative **12c** (CACO-2, IC_{50} 1.5 μg/mL) than introducing 4-fluorobenzaldehyde **12a** (CACO-2, IC_{50} 234 μg/mL) or 4-chlorobenzaldehyde **12b** (CACO-2, IC_{50} 900 μg/mL), while reaction of **12a** with malononitrile yielded a more potent derivative **13a** (CACO-2, IC_{50} 1.5 μg/mL) than that resulted from its reaction with **12b** or **12c** that gave **13b** (CACO-2, IC_{50} 14.5 μg/mL) and **13c** (CACO-2, IC_{50} 1020 μg/mL).



Scheme 2. Reagents and conditions: a) 4-aminoacetophenone, DMF, pyridine, reflux, 2 h; b) thiosemicarbazide, acetic acid, reflux, 2 h; c) NH_2NH_2, EtOH, reflux, 2 h; d) cyclohexyl isothiocyanate, dioxane, reflux, 8 h; e) RC_6H_5CHO, EtOH, 10% aq. KOH, stirr, 3 h; f) malononitrile, EtOh, piperidine, reflux, 8 h.

Cell cycle analysis

Compounds with the highest activity in cytotoxicity assays, namely **8** and **2** were selected for cell cycle studies in human colon cancer cells HCT-116 and HT-29, respectively, through assessing the DNA content of cells as measured by flow cytometry. As shown in Table 2 and Fig. 4, treatment of HCT-116 cells with compound **8** for 48 h enhanced cell-cycle arrest at the G0/G1 phase, resulting in significant accumulation of cells population at the G0/G1 phase compared with the control cells, concomitant with decrease of cells population at the S phase and the G2/M phase compared with that of the control cells. While treatment of HT-29 cells with compound **2** for 48 h enhanced cell-cycle arrest at the S phase and caused a significant decrease of cells population at G2/M phase of the cell cycle relative to control. These results suggested that the G1 phase arrest could be the main reason for the population decease of S and G2 phases in HCT-116 cell cycle induced by **8**. While the S phase arrest could be the main reason for the population decrease of G2 phase in HT-29 cell cycle induced by **2**.

Conclusion

In summary, the present work demonstrated the synthesis and quantitative evaluation of a series of novel indomethacin derivatives as efficient antitumor agents. Most compounds were found to be very potent compared to indomethacin and their cytotoxicities were determined by MTT method against HCT-116 cells, HT-29 cells, and CACO-2 cells. Among all of the 20 compounds, compounds **2**, **3**, **4b**, **8**, **11**, **12c**, and **13a** displayed a very potent antitumor activity more than that of 5FU that was used as the standard drug. By studying the effect of **8** on the cell cycle of HCT-116, it indicated that **8** may arrest the HCT-116 cells at G0/G1 phase, while compound **2** may arrest the HT-29 cells at S phase and further study is being developed.

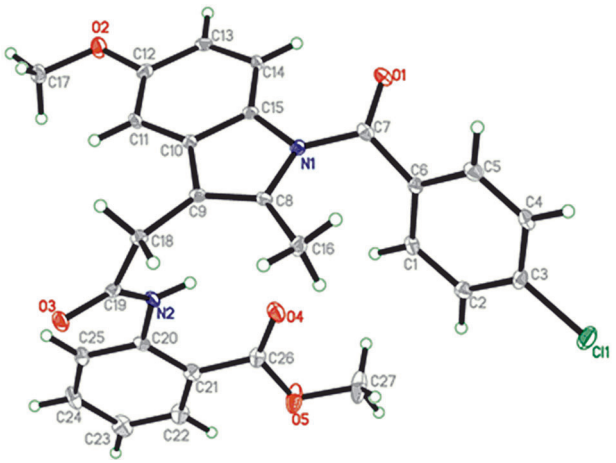


Figure 3. ORTEP diagram of the title compound. Displacement ellipsoids are plotted at the 40% probability level for non-H-atoms.

Experimental

Chemistry

General

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 NMR and ^{13}C NMR spectra were performed on Varian Gemini 300, 75 MHz spectrophotometer, respectively, using tetramethylsilane (TMS) 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 a 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 (TLC) 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-Azhar University, and they were within ±0.4% of the theoretical values.

The InChI codes of the investigated compounds are provided as Supporting Information.

*2-(2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)benzoic acid (**2**)*

A mixture of equimolar amounts (0.01 mol, 3.7 g) of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetyl chloride (**1**) and 2-aminobenzoic acid (0.01 mol, 1.4 g) 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. Yield 82%; m.p. 110–112°C. IR: cm^{-1}: 3380 (br, carboxylic OH), 3214 (NH), 3080 (CH-Ar), 2943 (CH-aliphatic), 1677 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.27 (s, 3H, CH_3); 3.71 (s, 3H, OCH_3);

Table 1. **IC_{50} (μg/mL) of the synthesized compounds against human colon cell lines (HCT-116, HT-29, and CACO-2).**

Compound	HCT-116	HT-29	CACO-2
2	0.78 ± 0.025	0.09 ± 0.01	0.0127 ± 0.001
3	0.421 ± 0.041	1.33 ± 0.386	0.12 ± 0.011
4a	9.8 ± 0.2	457 ± 5.196	229 ± 0.577
4b	4 ± 0.360	2.4 ± 0.346	4.5 ± 0.288
5a	1000 ± 15.275	950 ± 0.577	985 ± 9.073
5b	47.8 ± 0.2	95.5 ± 2.598	21.3 ± 0.173
5c	173.8 ± 0.757	296 ± 5.196	51.28 ± 0.993
5d	269.5 ± 5.634	457 ± 6.928	229 ± 2.886
6	57.5 ± 2.5	182 ± 4.618	43.7 ± 0.173
7	32.4 ± 0.2	288.5 ± 3.752	135 ± 2.886
8	0.27 ± 0.191	912 ± 10.392	645 ± 2.309
9	38.59 ± 0.980	30.9 ± 0.635	338 ± 6.429
10	120.2 ± 1.01	151.3 ± 0.981	138 ± 2.309
11	25.7 ± 0.7	12.5 ± 0.866	4.3 ± 0.173
12a	10 ± 2.51	1,000 ± 0.577	234 ± 6.351
12b	986 ± 30.315	980 ± 5.773	900 ± 6.082
12c	21.5 ± 0.763	34 ± 0.577	1.5 ± 0.173
13a	7.5 ± 0.360	6 ± 0.577	1.5 ± 0.173
13b	38 ± 3.5118	27 ± 1.732	14.5 ± 0.288
13c	983 ± 6.082	950 ± 5.773	1020 ± 12.124
Indomethacin	50.11 ± 0.548	53.7 ± 0.173	30 ± 0.461
5FU	1.8 ± 0.2	0.75 ± 0.045	5.45 ± 0.25

The values represent mean ± standard deviation of three separate experiments.

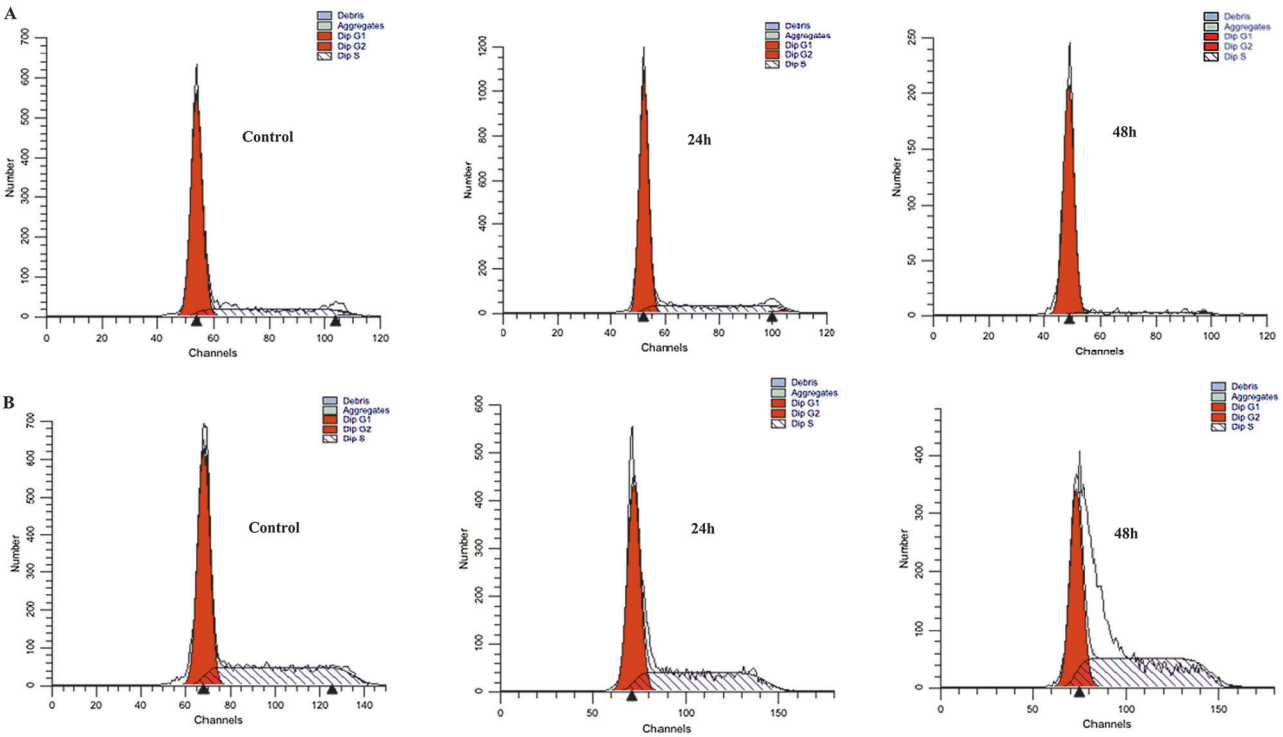


Figure 4. (A) Exposure of HCT-116 cells to **8** (0.27 μg/mL) for 48 h induced significant cell cycle arrest at the G0/G1 phase with concurrent reduction in the percentage of cells in the S and G2/M phases compared to the control. (B) Exposure of HT-29 cells to **2** (0.09 μg/mL) for 48 h resulted in a significant increase in the percentage of cells in the S phase with concurrent reduction in the percentage of cells in the G2/M phase compared to the control. The data also indicated that compound **8** arrested HCT-116 cells in the G1 phase while compound **2** arrested HT-29 cells in the S phase.

3.85 (s, 2H, CH_2); 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 (75 MHz, DMSO-d_6) δ 13.67, 33.81, 55.82, 101.71, 112.08, 112.89, 115.30, 116.46, 119.92, 123.15, 129.54, 130.93, 130.94, 131.63, 131.77, 134.54, 134.65, 136.72, 138.10, 141.13, 156.09, 168.45, 169.40, 169.58. MS, *m/z*, (%): 476, $[M]^+$, (9.02%), 477, $[M+2]^+$, (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)methyl)-4H-benzo[d][1,3]oxazin-4-one (**3**)*

A mixture of 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-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.

Table 2. **HCT-116 and HT-29 cell cycle distribution of compounds 8 and 2.**

Compound	Cell line	Conc. (μg/mL)	h	Cell cycle distribution (%)			
				Pre G	G0/G1	S	G2/M
	Control	0		0.23	75.61	23.85	0.54
8	HCT-116	0.27	24	0.14	74.68	24.14	1.18
			48	0.83	91.86	8.14	0
	Control	0		0.31	56.36	43.54	0.11
2	HT-29	0.09	24	0.08	57.46	42.54	0
			48	0.26	44.70	55.3	0

Yield 75%; m.p.168–170°C. IR: cm^{-1}: 3057 (CH-Ar), 2983, 2830 (CH-aliphatic), 1772 (CO-lactone), 1670 (CO). ^1H NMR (300 MHz, DMSO-d_6): 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 (75 MHz, DMSO-d_6) δ 13.80, 29.95, 55.82, 102.32, 111.89, 115.08, 117.03, 126.91, 128.37, 129.02, 129.11, 129.51, 130.73, 130.96, 131.69, 132.05, 134.51, 136.39, 137.35, 138.14, 146.39, 156.03, 159.63, 160.94. 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-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(hydrazinecarbonyl derivatives)phenyl)acetamide (**4a,b**)*
A mixture of **3** (4.5 g, 0.01 mol) and the appropriate hydrazine derivatives (0.012 mol) in ethanol was heated under reflux for 3 h. The reaction mixture was concentrated and the separated solid was crystallized from ethanol.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(hydrazinecarbonyl)phenyl)acetamide (**4a**)*
Yield 72%; m.p. 150–153°C. IR: cm^{-1}: 3278, 3209 (br, NH, NH_2), 3075 (CH-aromatic), 2909 (CH-aliphatic), 1653 (br, CO). ^1H NMR (300 MHz, DMSO-d_6): δ 2.29 (s, 3H, CH_3); 3.69 (s, 3H, OCH_3); 4.12 (s, 2H, CH_2); 6.56–7.07 (m, 11H, ArH); 9.02 (s, 1H, NH exchangeable with D_2O); 10.54 (s, 2H, 2NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.03, 30.12, 55.81, 100.61, 101.03, 105.06, 109.77, 111.14, 112.23, 113.45, 123.2, 124.44, 127.74, 128.72, 129.23, 130.50, 131.42, 134.15, 135.14, 137.01, 153.35, 164.86, 167.74, 170.66. MS, *m/z*, (%): 491, $[M+1]^+$, (7.3%), 493, $[M+3]^+$ (2.5%), 43, (100%). Anal. calcd. for $C_{26}H_{23}ClN_4O_4$: C, 63.61; H, 4.72; N, 11.41; found: C, 63. 89; H, 4.81; N, 11.54.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-phenylhydrazinecarbonyl)phenyl)acetamide (**4b**)*
Yield 69%; m.p. 144–146°C. IR: cm^{-1}: 3246 (br, NH), 3090 (CH-aromatic), 2938 (CH-aliphatic), 1675 (br, CO). ^1H NMR (300 MHz, DMSO-d_6): δ 2.18 (s, 3H, CH_3); 3.65 (s, 3H, OCH_3); 4.13 (s, 2H, CH_2); 6.47–8.02 (m, 16H, ArH); 9.05 (s, 2H, NH exchangeable with D_2O), 10.58 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.06, 34.59, 55.39, 100.88, 104.05, 108.85, 109.93, 111.28, 112.75, 120.52, 121.50, 126.67, 127.09, 127.37, 129.20, 129.28, 130.07, 130.55, 131.58, 134.79, 135.39, 138.23, 146.88, 147.40, 153.20, 159.65, 160.76, 166.88. MS, *m/z*, (%): 566, $[M]^+$, (2.15%), 568, $[M+2]^{+\cdot}$ (0.7%), 43.1 (100%). Anal. calcd. for $C_{32}H_{27}ClN_4O_4$: C, 67.78; H, 4.80; N, 9.88; found: C, 67.93; H, 4.78; N, 10.01.

*General procedure for synthesis of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-(4-benzylidene derivatives)hydrazinecarbonyl)phenyl)-acetamide (**5a–d**)*
A mixture of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)-*N*-(2-(hydrazinecarbonyl)phenyl)acetamide (**4a**) (4.9 g, 0.01 mol) and (0.01 mol) of appropriate aromatic aldehyde was dissolved in 30 mL of ethanol. Then refluxed for 8 h and kept aside. The solid obtained was filtered, washed with water, dried, and recrystallized from ethanol.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-(4-methoxybenzylidene)hydrazinecarbonyl)-phenyl)acetamide (**5a**)*
Yield 63%; m.p. 180–182°C. IR: cm^{-1}: 3216 (br, NH), 3065 (CH-aromatic), 2938, 2837 (CH-aliphatic), 1677 (br, CO). ^1H NMR (300 MHz, DMSO-d_6): δ 2.45 (s, 3H, CH_3); 3.67 (s, 6H, $2OCH_3$); 3.90 (s, 2H, CH_2); 6.81–8.07 (m, 16H, ArH + CH=N); 9.87 (s, 2H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.53, 31.32, 55.43, 105.36, 107.85, 113.73, 114.25, 114.96, 120.87, 121.33, 126.36, 126.68, 126.89, 130.00, 130.09, 131.77, 132.26, 132.60, 134.76, 134.95, 146.8, 151.21, 154.02, 156.02, 158.73, 161.20, 161.29, 164.66, 191.77. MS, *m/z*, (%): 609, $[M+1]^+$, (16.4%), 611 $[M+3]^+$, (5.2%), 174, (100%). Anal. calcd. for $C_{34}H_{29}ClN_4O_5$: C, 67.05; H, 4.80; N, 9.20; found: C, 67.18; H, 4.89; N, 9.36.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-(4-chlorobenzylidene)hydrazinecarbonyl)-phenyl)acetamide (**5b**)*
Yield 62%; m.p. 149–151°C. IR: cm^{-1}: 3237 (br, NH), 3042 (CH-aromatic), 2933 (CH-aliphatic), 1653 (br, CO). ^1H NMR (300 MHz, DMSO-d_6): δ 2.26 (s, 3H, CH_3); 3.62 (s, 3H, OCH_3); 3.72 (s, 2H, CH_2); 6.96–8.62 (m, 16H, ArH + CH=N); 9.88, 10.34 (2s, 2H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 11.64, 21.69, 55.71, 100.03, 102.34, 103.35, 111.65, 119.84, 128.76, 128.91, 129.08, 129.15, 129.30, 129.74, 130.48, 131.32, 131.53, 133.36, 133.39, 134.65, 134.73, 135.24, 141.02, 142.45, 145.45, 151.59, 153.55, 165.24, 171.41. MS, *m/z*, (%): 612, $[M]^+$, (15.6%), 614, $[M+2]^+$, (10.4%), 616, $[M+4]^+$, (1.56%), 174, (100%). Anal. calcd. for $C_{33}H_{26}Cl_2N_4O_4$: C, 64.61; H, 4.27; N, 9.13; found: C, 64.72; H, 4.35; N, 9.29.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-(3,4,5-trimethoxybenzylidene)-hydrazinecarbonyl)phenyl)acetamide (**5c**)*
Yield 60%; m.p. 210–212°C. IR: cm^{-1}: 3262 (br, NH), 3085 (CH-aromatic), 2936, 2833 (CH-aliphatic), 1674 (br, CO). ^1H NMR (300 MHz, DMSO-d_6): δ 1.92 (s, 3H, CH_3); 3.62 (s, 12H, $4OCH_3$); 3.79 (s, 2H, CH_2); 6.39–8.10 (m, 14H, ArH + CH=N); 9.88, 10.89 (2s, 2H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.84, 33.23, 55.90, 56.12, 60.08, 100.06, 104.78, 106.03, 112.24, 113.46, 120.92, 123.28, 124.45, 125.82, 127.74, 128.48, 129.32, 131.35, 132.16, 134.22, 135.15, 137.73, 138.82, 140.02, 141.54, 147.26, 150.90, 152.31, 160.49, 160.94, 165.23. MS, *m/z*, (%): 669, $[M+1]^+$, (8.6%), 671, $[M+3]^+$, (2.8%), 347, (100%). Anal. calcd. for $C_{36}H_{33}ClN_4O_7$: C, 64.62; H, 4.97; N, 8.37; found: C, 64.89; H, 5.05; N, 8.51.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(2-(2-(4-nitrobenzylidene)hydrazinecarbonyl)-phenyl)acetamide (**5d**)*
Yield 68%; m.p. 130–132°C. IR: cm^{-1}: 3261 (br, NH), 3105 (CH-aromatic), 2814 (CH-aliphatic), 1675 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.91 (s, 3H, CH_3); 3.84 (s, 3H, OCH_3); 3.88 (s, 2H, CH_2); 6.52–8.43 (m, 16H, ArH + CH=N); 10.16, 10.99 (2s, 2H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.47, 31.3, 56.66, 105.15, 107.25, 110.69, 121.32, 123.73, 124.39, 124.55, 124.73, 125.59, 126.34, 126.72, 127.26, 130.29, 131.09, 132.49, 134.99, 138.84, 140.51, 145.20, 147.00, 147.67, 150.76, 151.08, 160.78, 161.39, 192.80. MS, *m/z*, (%): 624, $[M+1]^+$, (23.8%), 626, $[M+3]^+$, (7.8%), 347, (100%). Anal. calcd. for $C_{33}H_{26}ClN_5O_6$: C, 63.51; H, 4.20; N, 11.22; found: C, 63.66; H, 4.24; N, 11.48.

*Methyl 2-(2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamido)benzoate (**6**)*
A mixture of equimolar amounts of compound **1** (0.01 mol, 3.7 g) and methyl anthranilate (1.5 g, 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. Yield 80%; m.p. 94°C. IR: cm^{-1}: 3290 (br, NH), 3097 (CH-aromatic), 2830 (CH-aliphatic), 1698 (CO ester), 1671 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.08 (s, 3H, CH_3); 3.43 (s, 2H, CH_2); 3.78 (s, 3H, OCH_3); 3.88 (s, 3H, $COOCH_3$); 6.51–8.50 (m, 11H, ArH); 10.74 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.58, 31.34, 51.85, 56.26, 107.49, 109.59, 112.07, 113.85, 115.62, 117.26, 120.62, 121.67, 123.59, 129.53, 131.04, 131.81, 134.49, 138.24, 140.47, 145.13, 149.52, 151.15, 156.01, 168.17, 171.37. MS, *m/z*, (%): 490 $[M]^+$, (6.9%), 492 $[M+2]^+$, (2.4%), 138, (100%). Anal. calcd. for $C_{27}H_{23}ClN_2O_5$: C, 66.06; H, 4.72; N, 5.71; found: C, 66.34; H, 4.79; N, 5.76.

*3-Amino-2-((1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)methyl)quinazolin-4(3H)-one (**7**)*
A mixture of compound **6** (4.9 g, 0.01 mol) and hydrazine hydrate (0.012 mol) in *n*-butanol (30 mL) was refluxed for 4 h then allowed to cool. The solid product was collected and recrystallized from ethanol. Yield 60%; m.p. 200°C. IR: cm^{-1}: 3312 (br, NH), 3025 (CH-aromatic), 2934 (CH-aliphatic), 1670 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.91 (s, 3H, CH_3); 3.34 (s, 2H, CH_2); 3.78 (s, 3H, OCH_3); 6.49–8.15 (m, 11H, ArH); 10.84 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.36, 30.95, 55.46, 101.05, 104.40, 105.52, 107.29, 109.87, 110.00, 111.25, 120.21, 126.34, 126.53, 127.27, 129.39, 130.60, 134.46, 134.52, 134.70, 146.97, 152.96, 153.22, 157.59, 161.03. MS, *m/z*, (%): 472, $[M+1]^+$, (8.7%), 474, $[M+3]^+$, (2.6%), 334, (100%). Anal. calcd. for $C_{26}H_{21}ClN_4O_3$: C, 66.03; H, 4.48; N, 11.85; found: C, 66.17; H, 4.52; N, 11.98.

*N-(4-Acetylphenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**8**)*
A mixture of equimolar amounts (2.4 g, 0.01 mol) of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetyl chloride (**1**) and 4-aminoacetophenone (0.01 mol, 1.3 g) 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. Yield 85%; m.p. 140°C. IR: cm^{-1}: 3282 (br, NH), 3070 (CH-aromatic), 2946 (CH-aliphatic), 1678 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3); 2.48 (s, 3H, $COCH_3$); 3.69 (s, 2H, CH_2); 3.80 (s, 3H, OCH_3); 6.69–7.94 (m, 11H, ArH); 10.58 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.86, 26.63, 31.35, 55.86, 100.65, 106.34, 112.25, 113.49, 121.57, 128.70, 129.00, 129.31, 131.14, 131.33, 135.27, 136.69, 137.60, 140.16, 142.92, 154.00, 167.77, 168.96, 197.22. MS, *m/z*, (%): 474, $[M]^+$, (2.46%), 476, $[M+2]^+$, (0.9%), 139, (100%). Anal. calcd. for $C_{27}H_{23}ClN_2O_4$: C, 68.28; H, 4.88; N, 5.90; found C, 68.51; H, 4.96; N, 5.97.

*N-(4-(1-(2-carbamothioylhydrazono)ethyl)phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-acetamide (**9**)*
A mixture of compound **8** (4.7 g, 0.01 mol) and thiosemicarbazide (0.9 g, 0.01 mol) in acetic acid (30 mL) was refluxed for 2 h then allowed to cool, the solid product was filtered off and recrystallized from acetic acid. Yield 65%; m.p. 180°C. IR: cm^{-1}: 3246 (br, NH), 3082 (CH-aromatic), 2929 (CH-aliphatic), 1664 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.29 (s, 3H,CH_3); 2.36 (s, 3H, $NH_2CSNHN{=}CCH_3$); 3.75 (s, 2H, CH_2); 3.82 (s, 3H, OCH_3); 6.63–8.13 (m, 11H, ArH); 8.45, 10.65 (2s, 4H, NH, NH_2 exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 13.86, 26.87, 31.21, 55.90, 109.70, 110.00, 110.25, 114.25, 115.04, 115.96, 118.87, 129.52, 129.94, 130.70, 131.33, 131.62, 134.65, 135.95, 138.05, 156.02, 162.77, 166.86, 168.33, 172.47. MS, *m/z*, (%): 547, $[M]^+$, (3.49%), 549, $[M+2]^+$, (1.3%), 111, (100%). Anal. calcd. for $C_{28}H_{26}ClN_5O_3S$: C, 61.36; H, 4.78; N, 12.78; found: C, 61.49; H, 4.81; N, 12.89.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(1-hydrazonoethyl)phenyl)acetamide (**10**)*
A mixture of compound (**8**) (4.7 g, 0.01 mol) and hydrazine hydrate (0.012 mol) in ethanol (30 mL) was refluxed for 2 h then allowed to cool, the solid product was collected and recrystallized from ethanol. Yield 65%; m.p. 170°C. IR: cm^{-1}: 3288 (br, NH), 3055 (CH-aromatic), 2935 (CH-aliphatic), 1653 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.98 (s, 3H, CH_3); 2.51 (s, 3H, NH_2NCCH_3); 3.62 (s, 2H, CH_2); 3.71 (s, 3H, OCH_3); 6.60–7.85 (m, 11H, ArH); 9.38, 10.62 (2s, 3H, NH, NH_2 exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 11.69, 12.07, 33.10, 55.80, 100.91, 105.04, 109.82, 111.27, 118.84, 119.15, 125.50, 129.27, 129.85, 130.56, 131.59, 132.19, 134.36, 135.10, 138.81, 142.54, 145.63, 153.42, 170.20. MS, *m/z*, (%): 488, $[M]^+$, (13.3%), 490, $[M+2]^+$, (4.2%), 78, (100%). Anal. calcd. for $C_{27}H_{25}ClN_4O_3$: C, 66.32; H, 5.15; N, 11.46; found: C, 66.74; H, 5.27; N, 11.61.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(1-(2-(cyclohexylcarbamothioyl)hydrazono)ethyl)-phenyl)acetamide (**11**)*

A mixture of equimolar amount of compound (**10**) (4.4 g, 0.01 mol) and cyclohexyl isothiocyanate (1.4 g, 0.01 mol) in dioxane (20 mL) was refluxed for 8 h on water bath. The reaction mixture was then concentrated, cooled, and kept overnight in the refrigerator. The solid separated out was filtered, dried, and crystallized from ethanol. Yield 60%; m.p. 220°C. IR: cm^{-1}: 3242 (br, NH), 3137 (CH-aromatic), 2930 (CH-aliphatic), 1665 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.24 (m, 4H, $2CH_2$); 1.64 (m, 2H, CH_2); 1.76 (m, 4H, $2CH_2$); 2.23 (s, 3H, CH_3); 2.34 (s, 3H, $C_6H_{11}NHCSNHNCCH_3$); 2.53 (m, 1H, CH); 3.54 (s, 2H, CH_2); 3.69 (s, 3H, OCH_3); 6.58–7.91 (m, 11H, ArH); 9.14, 10.21, 10.63 (3s, 3H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.08, 14.89, 25.01, 25.49, 32.03, 32.28, 55.81, 58.00, 100.89, 103.86, 104.91, 106.56, 109.84, 110.98, 111.30, 119.08, 122.41, 127.02, 127.56, 128.79, 130.19, 130.57, 134.25, 134.42, 141.23, 149.77, 153.46, 157.91, 170.54. MS, *m/z*, (%): 630, $[M+1]^+$, (2.11%), 632, $[M+3]^+$, (0.7%), 139, (100%). Anal. calcd. for $C_{34}H_{36}ClN_5O_3S$: C, 64.80; H, 5.76; N, 11.11; found: C, 64.98; H, 5.82; N, 11.18.

*General procedure for synthesis of 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-cinnamoylphenyl)-acetamide derivatives (**12a–c**)*

A mixture of *N*-(4-acetylphenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl)acetamide (**8**) (4.7 g, 0.01 mol), appropriate aromatic aldehyde (0.01 mol), and 10% aqueous potassium hydroxide (10 mL) in ethanol (25 mL) was stirred at room temperature for about 3 h. The resulting solid was filtered off, rinsed with water, dried, and crystallized from ethanol.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(3-(4-fluorophenyl)acryloyl)phenyl)acetamide (**12a**)*

Yield 67%; m.p. 145°C. IR: cm^{-1}: 3263 (br, NH), 3098 (CH-aromatic), 2928 (CH-aliphatic), 1667 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.27 (s, 3H, CH_3); 3.72 (s, 2H, CH_2); 3.78 (s, 3H, OCH_3); 6.68–7.91 (m, 13H, ArH + CHCH); 10.51 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 13.84, 32.52, 55.89, 102.38, 111.64, 113.88, 114.22, 115.04, 115.88, 116.67, 118.87, 129.51, 129.94, 130.71, 131.29, 131.61, 132.20, 132.99, 134.63, 135.69, 135.96, 138.06, 143.88, 156.02, 168.31, 169.53, 172.50, 196.92. MS, *m/z*, (%): 580, $[M]^+$, (11.9%), 582, $[M+2]^+$, (3.5%), 44, (100%). Anal. calcd. for $C_{34}H_{26}ClFN_2O_4$: C, 70.28; H, 4.51; N, 4.82; found: C, 70.43; H, 4.58; N, 4.91.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(3-(4-chlorophenyl)acryloyl)phenyl)acetamide (**12b**)*

Yield 70%; m.p. 147°C. IR: cm^{-1}: 3260 (br, NH), 3098 (CH-aromatic), 2929 (CH-aliphatic), 1669 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.26 (s, 3H, CH_3); 3.72 (s, 2H, CH_2); 3.78 (s, 3H, OCH_3); 6.68–7.91 (m, 13H, ArH + CHCH); 10.52 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 13.85, 32.51, 55.88, 102.36, 111.64, 114.21, 115.04, 118.86, 128.75, 129.52, 129.95, 130.70, 131.29, 131.62, 132.19, 134.62, 135.96, 138.06, 143.88, 144.46, 147.21, 147.12, 149.05, 150.25, 156.02, 168.32, 169.54, 196.95. MS, *m/z*, (%): 596, $[M]^+$, (2.5%), 598, $[M+2]^+$, (1.6%), 600, $[M+4]^+$, (0.3%), 127, (100%). Anal. calcd. for $C_{34}H_{26}Cl_2N_2O_4$: C, 68.35; H, 4.39; N, 4.69; found: C, 68.49; H, 4.47; N, 4.78.

*2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)-N-(4-(3-(4-methoxyphenyl)acryloyl)phenyl)acetamide (**12c**)*

Yield 72%; m.p. 280°C. IR: cm^{-1}: 3270 (br, NH), 3063 (CH-aromatic), 2933 (CH-aliphatic), 1657 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.47 (s, 3H, CH_3); 3.69 (s, 2H, CH_2); 3.79 (s, 6H, $2OCH_3$); 6.58–8.11 (m, 13H, ArH + CH=CH); 10.42 (s, H, NH exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.07, 33.18, 55.81, 100.84, 104.70, 109.87, 111.32, 114.83, 118.75, 118.86, 119.88, 127.87, 129.25, 129.90, 130.19, 130.58, 131.13, 131.59, 132.84, 134.50, 143.74, 144.07, 144.93, 153.47, 154.93, 161.69, 170.94, 187.79. MS, *m/z*, (%): 592, $[M]^+$, (22.52%), 594, $[M+2]^+$, (7.5%), 43, (100%). Anal. calcd. for $C_{35}H_{29}ClN_2O_5$: C, 70.88; H, 4.93; N, 4.72; found: C, 71.04; H, 4.98; N, 4.87.

*General procedure for synthesis N-(4-(6-amino-5-cyano-4-phenyl-4H-pyran-2-yl)phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide dervatives (**13a–c**)*

A mixture of chalcone compound (**12a–c**) (0.001 mol) and malononitrile (0.0011 mol) in absolute ethanol (20 mL) containing few drops of piperidine as a catalyst was refluxed for 8 h. The resulting crude product was filtered off, dried, and recrystallized from ethanol.

*N-(4-(6-Amino-5-cyano-4-(4-fluorophenyl)-4H-pyran-2-yl)-phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**13a**)*

Yield 75%; m.p. 130°C. IR: cm^{-1}: 3250 (br, NH), 3033 (CH-aromatic), 2916 (CH-aliphatic), 2209 (CN), 1677 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H,CH_3); 3.61 (s,2H, CH_2); 3.70 (s, 3H, OCH_3); 4.06 (d, $J = 6$ Hz, H, CH), 5.21 (d, $J = 6$ Hz, H, CH), 6.59–7.95 (m, 15H, ArH); 10.41, 10.65 (2s, 3H, NH, NH_2 exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.06, 29.71, 33.17, 55.81, 58.12, 100.83, 104.63, 109.87, 111.32, 113.85, 115.76, 115.97, 118.75, 118.78, 129.20, 129.23, 129.90, 130.07, 130.56, 130.75, 130.83, 131.07, 131.58, 134.49, 134.53, 138.23, 144.56, 153.47, 166.89, 170.99, 195.20. MS, *m/z*, (%): 646, $[M]^+$, (6.08%), 648, $[M+2]^+$, (2%), 76, (100%). Anal. calcd. for $C_{37}H_{28}ClFN_4O_4$: C, 68.68; H, 4.36; N, 8.66; found: C, 68.91; H, 4.40; N, 8.79.

*N-(4-(6-Amino-4-(4-chlorophenyl)-5-cyano-4H-pyran-2-yl)-phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**13b**)*

Yield 80%; m.p. 150°C. IR: cm^{-1}: 3209 (br, NH), 3033 (CH-aromatic), 2938 (CH-aliphatic), 2209 (CN), 1640 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.29 (s, 3H,CH_3); 3.67 (s, 2H, CH_2); 3.83 (s, 3H, OCH_3); 4.10 (d, $J = 6$ Hz, H, CH), 5.15 (d, $J = 6$ Hz, H, CH), 6.60–7.92 (m, 15H, ArH); 10.45, 10.64 (2s, 3H, NH, NH_2 exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.06, 20.16, 33.15, 55.81, 58.12, 100.87, 104.89, 109.87, 111.32, 118.76, 119.02, 119.70, 128.14, 129.03, 129.15, 129.23, 129.27, 129.62, 129.90, 130.57, 130.84, 130.92, 130.95, 134.38, 134.49, 134.99, 143.60, 153.47, 159.30, 171.05, 196.95. MS, *m/z*, (%): 662, $[M]^+$, (8.2%), 664, $[M+2]^+$, (5.4%), 666, $[M+4]^+$, (0.9%), 143, (100%). Anal. calcd. for $C_{37}H_{28}Cl_2N_4O_4$: C, 66.97; H, 4.25; N, 8.44; found: C, 67.12; H, 4.31; N, 8.52.

*N-(4-(6-Amino-5-cyano-4-(4-methoxyphenyl)-4H-pyran-2-yl)phenyl)-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl)acetamide (**13c**)*

Yield 80%; m.p. 180°C. IR: cm^{-1}: 3199 (br, NH), 3028 (CH-aromatic), 2931 (CH-aliphatic), 2199 (CN), 1645 (br, CO). ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.28 (s, 3H, CH_3); 3.70 (s, 2H, CH_2); 3.81, 3.84 (2s, 6H, $2OCH_3$); 4.10 (d, $J = 6$ Hz, H, CH), 4.44 (d, $J = 6$ Hz, H, CH), 6.62–7.94 (m, 15H, ArH); 10.49, 10.64 (2s, 3H, NH, NH2 exchangeable with D_2O). ^{13}C NMR (75 MHz, DMSO-d_6) δ 12.94, 20.23, 32.15, 55.81, 58.22, 100.65, 106.39, 110.37, 111.42, 118.81, 119.10, 119.75, 121.52, 126.52, 128.14, 129.30, 129.90, 130.03, 130.57, 130.84, 131.13, 131.37, 134.52, 135.19, 140.01, 143.60, 153. 47, 154.00, 159.30, 171.05, 196.95. MS, *m/z*, (%): 658, $[M]^+$, (2.4%), 660, $[M+2]^+$, (0.78%), 43, (100%). Anal. calcd. for $C_{38}H_{31}ClN_4O_5$: C, 69.24; H, 4.74; N, 8.50; found: C, 69.52; H, 4.81; N, 8.64.

*X-ray structure determinations of **6***

A colorless plate-like crystal (0.41 × 0.14 × 0.14 mm) of compound **6** was selected for X-ray diffraction. Intensity data were collected at room temperature ($T = 100$ K) using a Bruker APEX-II D8 venture diffractometer with Mo Kα monochromatic radiation (λ = 0.71073 Å) and cell refinement and data reduction were carried out by Bruker SAINT [31]. For compound **6**, a total of 5254 reflections to a maximum θ of 26.8° were measured. The crystal structure for compound **6** was solved by direct methods and refined anisotropically with full matrix least square on F2 using SHELXL-97 program [32]. The main crystallographic parameters were inserted in the supporting information. Crystallographic data for compound **6** have been deposited with the Cambridge Crystallographic Data Center as Supplementary Publication No. CCDC 1473378. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CH21EZ, UK (fax: 033 336 1223 44 or e-mail: deposit@ccdc.cam.ac.uk).

In **6** crystal structure, the centeral indol moiety (N1/C8–C15) makes dihedral angles 69.14 and 81.76° with the chlorobenzoyl group (C1–C6) and the benzoate group (C20–C25), respectively. Regarding the methyl group and methoxy group present in the same plane of the indol ring, the crystal structure was stabilized with two intra-molecular hydrogen bonds between N2–H1N2···O4 and C1–H1A···O4 with H···*A* 2.07 (2) Å and 2.57 Å and bond angles 134 (2)° and 156°, respectively. On other hand, the molecules are packed together with one non-classical intermolecular hydrogen bond between C11–H11A···O3^{i} with bong length 2.54 Å and bond angle 152° and symmetry code: (i) $-x+1, -y+1, -z+2$.

Biological assays

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

Cancer cells from different cancer cell lines (HCT116, colon cancer cell line, HT-29, colon cancer cell line, CACO-2, colon cancer cell line) were purchased from American Type Cell Culture Collection (ATCC, Manassas, USA) and grown on the appropriate growth medium Dulbecco's modified Eagle's medium (DMEM) or Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in a humidified, 5% (v/v) CO_2 atmosphere at 37°C.

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 μM) for 72 h. Then the viability of treated cells were determined using MTT technique as follows: 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 was 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 was measured at 570 nm using a Stat Fax^R 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 (IC_{50}) was determined using GraphPad Prism version 5 software (GraphPad software Inc., CA) (1,2).

Cell cycle analysis

HT-29 cells and HCT-116 cells at a density of 4×10^6 cell/T 75 flask were exposed to compounds **2** and **8**, respectively, at its GI_{50} concentration for 24 and 48 h. The cells then were collected by trypsinization, washed with phosphate-buffered saline (PBS), and fixed in ice-cold absolute alcohol. Thereafter,

cells were stained, using Cycletest™ Plus DNA Reagent Kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Cell cycle distribution was determined using a FACS Calibur flow cytometer (BD Biosciences).

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