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Anti-cancer effect of Indanone-based thiazolyl hydrazone derivative on colon cancer cell lines

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ABSTRACT

Colorectal cancer is the third leading cause of cancer related deaths in the United States. Currently, Irinotecan, a topoisomerase I inhibitor, is an approved anti-cancer drug for the treatment of patients with advanced or recurrent colorectal cancer. Considering low response rate and events of high toxicity caused by irinotecan, we evaluated a series of thirteen thiazolyl hydrazone derivatives of 1-indanone for their potential antineoplastic activity and four compounds showed promising anti-cancer activity against most of the tested colon cancer cell lines with IC_{50} values ranging from 0.41 ± 0.19 to 6.85 ± 1.44 μM. It is noteworthy that the compound, *N*-Indan-1-ylidene-N'-(4-Biphenyl-4-yl-thiazol-2-yl)-hydrazine (ITH-**6**) is found to be more effective than irinotecan against colon cancer cells, HT-29, COLO 205, and KM 12. Mechanistic studies reveal that ITH-**6** arrests these cancer cell lines in G2/M phase of the cell cycle, induces apoptosis and causes an increase in ROS level with a significant reduction in the GSH level. The mechanism of inhibition relates to the inhibition of tubulin polymerization in the mitotic phase. These findings suggest that ITH-**6** is a novel drug candidate for the treatment of colorectal cancer.

1. Introduction

Cancer till date remains the most intriguing disease of human populations in terms of its types, progression and treatment (Anreddy et al., 2014; Gupta et al., 2016a, 2018; Kathawala et al., 2015). Despite of the advances in the field of cancer research and translational medicine, which has indeed result in higher cure rates for various tumor types, cancer still remains the second leading health challenge, after heart related disorders in both developing and developed countries (Gupta et al., 2017a,b; Mokhtari et al., 2012).

Among malignancies, colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer related deaths globally (Arnold et al., 2016). CRC is considered to be an environmental disease, affected by cultural, social and lifestyle practices (Cepowicz et al., 2017). Studies done in the past have shown that endocrine factors and obesity are the two major contributors to an increase in the risk of colorectal cancer (Albanes, 1990). Moreover, weight gain during the middle age and metabolic dysfunctions can predispose to abdominal obesity which positively correlates with colorectal cancer (Kono et al., 1999). It has also been found that the dietary habits influence the risk of colorectal cancer. The dietary fat especially from animal sources has earlier been demonstrated to be metabolized into a carcinogen by colonic flora (Moore and LaMont, 1984). Moreover, the genetic makeup of individuals also plays an important role in its genesis and mutations in chromosome 18q have resulted in errors in DNA replication which account for 15–20% of sporadic colon cancer (Gryfe et al., 2000; Wynder and Shigematsu, 1967).

According to the American cancer society, around 135,430 CRC cases were diagnosed in 2017 in the United States with around 50,260 deaths estimated from the disease (Siegel et al., 2017). Studies have shown that approximately 30% of colorectal cancer cases are hereditary in nature (O'Brien, 2000). The etiology remains unknown for around 75% cases of CRC and the remaining small percentage of cases are due to familial incidences or inflammatory bowel disease. Around 33% of familial cases have a genetic basis (Hisamuddin and Yang, 2004). Surgery is the primary treatment option for most cases of colorectal cancer (Compton, 2003). The current pharmacological management of primary colorectal cancer is based on the drug regimens such as FOLFOX and FOLFIRI for metastatic CRC. Though therapeutically

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efficacious, these anti-cancer agents bear a number of side effects such as nausea, vomiting, neurotoxicity, and infections which frequently reach to the level of causing a halt of the treatment (Schuell et al., 2005; Wiela-Hojeńska et al., 2015). Targeted specific drugs such as regorafenib, cetuximab, and bevacizumab have now been approved as alternatives for the treatment of CRC (Wang et al., 2017; Mirone et al., 2016). Although these drugs are effective and increase the overall survival, the existence of drug resistance mechanisms and toxicity remain serious concerns (Anreddy et al., 2014; Kathawala et al., 2015).

Since previous studies have established that the indanone ring exerts anti-cancer activity (Ganellin, 1967; Klaus, 1983; Vilums et al., 2015; Yao et al., 2003), here we investigate the anti-cancer effects of a series of indanone-based thiazolyl hydrazones on different human cancer cell lines. Moreover, in this study we explored the mechanism of action of most active derivative which caused the inhibition of colon cancer cells' proliferation, produced cell cycle arrest and induced apoptosis. The effects of this derivative on tubulin polymerization, production of reactive oxygen species (ROS), and glutathione depletion were also determined.

2. Materials and methods

2.1. Chemicals and equipment

All the thiazolyl hydrazone derivatives were synthesized at the University of Karachi, Pakistan (Supplementary Fig. 1). Regorafenib was obtained from Bayer HealthCare Pharmaceuticals Inc. (Whippany, NJ) and irinotecan hydrochloride from (Alfa Aesar, MA). A stock solution (10 mM) of all the compounds in DMSO was prepared and a series of dilutions were made. Fig. 1A shows the chemical structure of ITH-**6**. Dulbecco's modified Eagle's Medium (DMEM, IX), fetal bovine serum (FBS), Phosphate Buffer Saline (PBS), 10,000 IU/ml penicillin and 10,000 µg/ml streptomycin, and trypsin 0.25% were purchased from Hyclone (Waltham, MA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) (MTT), Dimethyl Sulfoxide (DMSO), and other chemicals were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO). Propidium Iodide (PI)/RNase staining buffer was purchased from BD biosciences (SanJose, CA) and the apoptosis kit was purchased from Biotium (Hayward, CA). 5-(and-6)-chloromethyl-20,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) was purchased from Molecular probes™ (Eugene, OR). GSH kit from Abcam (Cambridge, MA) and HTS-Tubulin Polymerization Assay Biochem Kit from Cytoskeleton (Denver, CO).

2.2. Cell lines and cell culture

HEK293 (human embryonic kidney cell line), 3T3 (mouse fibroblast) and human colon cancer cell lines (COLO 205, HCT-15, SW620, KM 12, HT-29) were used in this study. SW620 cell line was obtained from Dr. Susan E. Bates (Columbia University, New York) and HEK293 from Suresh Ambudkar (NIH, MD). All other cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The cell lines were cultured at 37 °C, 5% CO_2 with DMEM containing 10% FBS and 1% penicillin/streptomycin.

2.3. Cell proliferation assay

The anti-cancer effects of ITH-**6,** regorafenib and irinotecan were determined by a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) calorimetric assay (Śliwka et al., 2016). Cells were cultured, counted and seeded onto 96 well plates with a cell density of 6×10^3 cells per well. Following 24 h incubation, the cells were treated with different drugs (ranging from a concentration of 0–30 µM). After 68 h, 20 µl of 4 mg/ml MTT, was added to each well and the plates were further incubated 37 °C for 4 h. Subsequently, the MTT was removed from all wells and 100 µl of DMSO was added to dissolve the formazan crystals formed by the reduction of MTT in the mitochondria of the viable cells. The optical density (OD) was measured at 570 nm by an Opsys microplate reader (Dynex technologies, VA).

2.4. Cell cycle analysis

Based on the cytotoxic effects of ITH-**6**, the cell cycle analysis was carried out on colon cancer cell lines HT-29, COLO 205, and KM 12. The cells were treated with ITH-**6** at three different concentrations (0.3, 1 and 3 µM) for 24 h and the cell cycle analysis was performed as

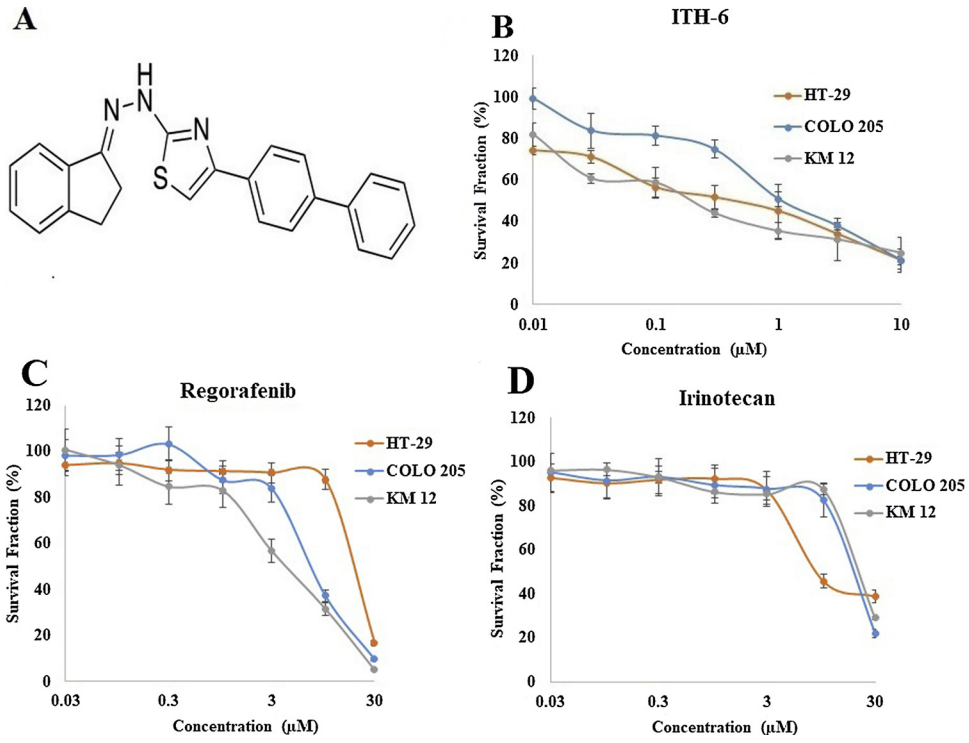


Fig. 1. Chemical structure of N-Indan-1-ylidene-N'-(4-Biphenyl-4-yl-thiazol-2-yl)-hydrazine (ITH-6) and cytotoxicity of ITH-6, Regorafenib and Irinotecan in HT-29, COLO 205, and KM 12 cell lines. The chemical structure of ITH-**6** was drawn using Chem Draw (A). Survival fraction (%) (B) was measured after treatment with ITH-**6** for 72 h in HT-29 (orange), COLO 205 (blue), and KM 12 (grey) cell lines. Points with error bars represent the mean ± SD for independent determinations in triplicate. The figures are representative of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1
The effect of ITH-6 on normal cell lines.

Compounds Code	HEK293 IC_{50} (μM)	3T3
ITH-6	> 30	> 30

μM-Micromole.
The cytotoxic effects of the test compounds on HEK293 (human embryonic kidney cells) and 3T3 (mouse fibroblasts).
Values in tables are representative of at least three independent experiments performed in triplicates.
IC_{50}: concentration that inhibits cell survival by 50% (mean ± SD).

described previously (Gupta et al., 2017a). In brief, the cells were harvested and centrifuged at 800 rpm for 5 min. The supernatant was removed and the cell pellet was washed with 1X PBS. The cells were fixed overnight in ice cold 70% ethanol at 4 °C. The fixed cells were stained with 50 μg/ml PI and 100 μg/ml of RNase at 37 °C for 30 min in the dark. The flow cytometric analysis was performed and the percentage of cells in different phases of cell cycle were determined (G0/G1, S, G2/M).To determine whether the effect of ITH-6 on the cell cycle phases are permanent or not, another flow cytometric experiment was performed, after 24 h incubation in the drug free medium following the 24 h treatment using FL-2 of BD Accuri™ C6 flow cytometer from BD Biosciences, CA (Gupta et al., 2017a).

2.5. Tubulin polymerization assay

The action of the test compound, ITH-**6** on the tubulin polymerization was assessed by tubulin polymerization kit. The preparation of samples and assay protocol was carried out as per manufacturer's instructions (Schneider et al., 2003). ITH-**6** (100 μM) was used a test compound while paclitaxel and colchicine (10 μM) were used as controls.

2.6. Apoptosis analysis

The cells were incubated with ITH-**6** for 24 h at concentrations of 0.3, 1 and 3 μM. After 24 h, the cells were washed, harvested and stained with FITC-labeled annexin-V and PI at 37 °C for 30 min. The degree of apoptosis was measured at FL-1 and FL-2 of the flow cytometer.

2.7. Intracellular ROS measurement

In order to investigate the effects of ITH-**6** on the intracellular levels of ROS, the cells were treated with ITH-**6** at different concentrations ranging from 0 to 3 μM for 24 h. After 24 h, the cells were washed and harvested. Subsequently, 10 μM of CM-H2DCFDA was added. The CM-H2DCFDA dye enters into the cells, gets converted into the fluorescent (5-chloromethyl-20-7′-dichlorofluorescein (DCF)) product by the action of intracellular peroxides. The cells were incubated in dark at 37 °C for 30 min. Intracellular ROS levels were measured using the flow cytometer.

2.8. Intracellular GSH assay

In order to better understand the inverse relation between oxidative stress and GSH, the colon cancer cell lines were treated with ITH-**6** at different concentrations 0.3, 1, and 3 μM for 24 h. The intracellular GSH was measured using GSH assay kit and the protocol was carried out as per manufacturer's instructions (Kim et al., 2016). The samples were prepared and analyzed as per manufacturer's protocol using FL-1 of flow cytometer.

2.9. Statistical analysis

All experiments were repeated at least three times and the differences were determined using a one-way analysis of variance (ANOVA). The statistical significance was determined at $p < 0.05$, $p < 0.001$ and $p < 0.0001$. The post hoc analysis was performed using Tukey's test. The data were analyzed using GraphPad Prism, version 6.

3. Results

3.1. Non-cytotoxic effect of ITH-6 on normal cell lines

To determine the cytotoxic effect of ITH-6 on normal healthy cell lines, MTT was done against human embryonic kidney cell line, HEK293 and mouse fibroblast cell, 3T3. ITH-6 did not show any cytotoxicity on these cell lines and IC_{50} was more than 30 μM (Table 1).

3.2. ITH-6 inhibits cell proliferation of colon cancer cell lines

In order to determine the cytotoxicity of synthesized compounds on colon cancer cell lines, MTT assay was performed against 7 colon cancer cell lines (as mentioned in cell lines and cell culture). Among all compounds, four compounds exhibited remarkable cytotoxic activities against most of the tested colon cell lines (Table 2). For the five types of tested human colorectal adenocarcinoma cells SW620, COLO 205, KM 12 and HT-29 cells, ITH-**1** had cytotoxic effects, with IC_{50} values of > 10 μM, 1.37 μM, 2.50 μM, and 0.86 μM, respectively. Also, ITH-**3** had cytotoxicity on the same colon cancer cell lines with IC_{50} value of > 10 μM, 2.64 μM, 2.91 μM, and 1.99 μM, respectively. The IC_{50} of ITH-6 on HT-29, COLO 205, SW620, and KM 12 cell lines (Fig. 1B). were 0.4 μM, 0.98 μM, 6.85 μM, and 0.41 μM respectively. The IC_{50} was more than 10 μM on HCT-15 cell line. For ITH-**12,** IC_{50} of 2.14 μM on COLO 205, 2.90 μM on KM 12 and 1.17 μM on HT-29 cells were exhibited. IC_{50} of Regorafenib on HT-29, COLO 205 and KM 12 were 22.7 μM, 9.43 μM and 5.02 μM respectively (Fig. 1C). Irinotecan had IC_{50} of 8.49 μM, 22.84 μM and 23.15 μM on HT-29, COLO 205 and KM 12 cells (Fig. 1D). These results indicate that ITH-**6** has a significant effect on the cell viability of HT-29, COLO 205 and KM 12 cells compared to the other cell lines, suggesting that the drug is more potent to colon cancer cells and chosen for the detailed study of its possible mechanism.

3.3. ITH-6 arrests the colon cancer cells in the G2/M phase of the cell cycle

In order to investigate the mechanism by which ITH-**6** inhibits the proliferation of colon cancer cells, its effects on the progression of cell cycle were studied. On treatment with ITH-**6** (0.3, 1, and 3 μM), a concentration dependent increase in the percentage of cells in G2/M phase of the cell cycle of all the three cell lines was observed. The concentrations were selected based on the IC_{50} values. ITH-**6** increased the percentage of cells from 37.5% to 72.1% in HT-29 (Fig. 2A), 15.1% to 33.4% in COLO 205 (Fig. 2B), and 24.1% to 77.8% in KM 12 cells (Fig. 2C). These results suggest that ITH-**6** arrests the cells in G2/M phase with negligible effect on other phases of cell cycle in all the three cell lines. To study the permanent cytotoxicity of ITH-6, the experiment was performed 24 h after incubating the cells in drug free medium following a 24 h ITH-6 treatment (0.3, 1, and 3 μM). The results showed there was no effect on G2/M phase (Fig. 3A–C).

3.4. ITH-6 inhibits tubulin polymerization in the mitotic phase

To further elucidate the mechanism by which ITH-**6** arrests the colon cancer cells in G2/M phase of the cell cycle, tubulin polymerization assay was performed according to the manufacturer's protocol. The test drug (ITH-**6**) was compared against control drugs, paclitaxel and colchicine. Our results indicated that paclitaxel (10 μM) stabilizes the microtubule by enhancing the tubulin polymerization for

Table 2
The effect of synthesized compounds on colon cancer cell lines.

Compounds Code	CELL LINES				
	COLO 205 IC_{50} (μM)	HCT-15	SW620	KM 12	HT-29
ITH-**1**	1.37 ± 0.29	> 10	> 10	2.50 ± 0.30	0.86 ± 0.17
ITH-**2**	> 10	> 10	> 10	> 10	> 10
ITH-**3**	2.64 ± 0.35	> 10	> 10	2.91 ± 0.17	1.99 ± 0.29
ITH-**4**	> 10	> 10	> 10	> 10	> 10
ITH-**5**	> 10	> 10	> 10	> 10	> 10
ITH-**6**	0.98 ± 0.06	> 10	6.85 ± 1.44	0.41 ± 0.19	0.44 ± 0.06
ITH-**7**	> 10	> 10	> 10	> 10	> 10
ITH-**8**	> 10	> 10	> 10	> 10	> 10
ITH-**9**	> 10	> 10	> 10	> 10	> 10
ITH-**10**	> 10	> 10	> 10	> 10	> 10
ITH-**11**	> 10	> 10	> 10	> 10	> 10
ITH-**12**	2.14 ± 0.36	> 10	> 10	2.90 ± 0.06	1.17 ± 0.27
ITH-**13**	> 10	> 10	> 10	> 10	> 10

μM-Micromole.
The cytotoxic effects of the test compounds on COLO 205, HCT-15, SW620, KM 12, and HT-29 (human colon cancer cell lines).
Values in tables are representative of at least three independent experiments performed in triplicates.
IC_{50}: concentration that inhibits cell survival by 50% (mean ± SD).

a period of 1 h while colchicine (10 μM) acted as a tubulin polymerization inhibitor. Interestingly, ITH-**6** at 100 μM, it inhibited the tubulin polymerization thus suggesting that ITH-**6** acted on the G2/M phase of the cell cycle by inhibiting the tubulin polymerization activity, an effect similar to colchicine however, less potent than colchicine (Fig. 4).

3.5. ITH-6 induces apoptosis in colon cancer cells

To understand the apoptotic effects of ITH-**6** on colon cancer cell lines, the cells were treated at different concentrations (0.3, 1, and 3 μM) of ITH-**6** for 24 h. In all the three cell lines, most of the cells were viable in the control group and no apoptosis was observed. ITH-**6** exhibited a concentration dependent increase in the early and late apoptosis of HT-29 (Fig. 5A), COLO 205 (Fig. 5B), and KM 12 (Fig. 5C) cells. Moreover, ITH-**6** did not induce any significant necrosis in all the three cell lines (Fig. 5 and Supplementary Fig. 2).

3.6. ITH-6 elevates ROS production in colon cancer cells

Since an increase in intracellular ROS is a measure of induction in apoptosis, we investigated the effects of ITH-**6** on the intracellular ROS production. The cells were treated at the indicated concentrations for 24 h and the intracellular ROS levels were measured using the flow cytometer. As shown in the Fig. 6, ROS percentage increased from 5.98% (control) to 66.3% (ITH-**6** at 3 μM) in HT-29, 1.88% (control) to 71.7% (ITH-**6** at 3 μM) in COLO 205, and 4.26% (control) to 69.57% (ITH-**6** at 3 μM) in KM 12 cells. These results suggested that ITH-**6** elevates intracellular ROS levels and cause apoptosis in colon cancer cells.

3.7. ITH-6 inhibits GSH levels in colon cancer cells

Since a decrease in GSH levels is known to induce ROS and in turn induce apoptosis, the effects of ITH- **6** on the intracellular GSH levels

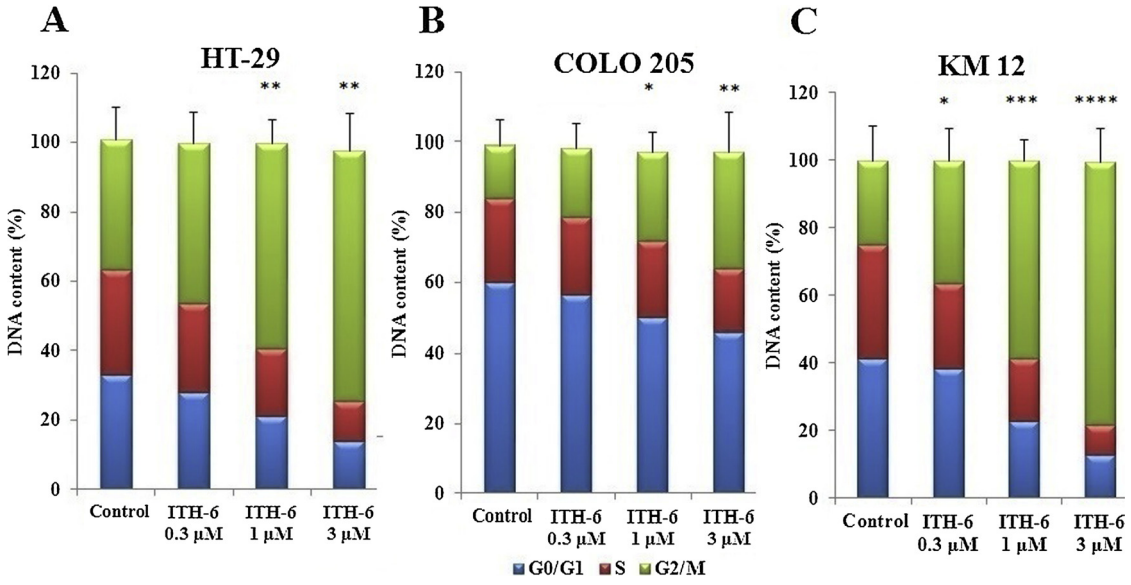


Fig. 2. Effect of ITH-6 on the cell cycle of HT-29, COLO 205, and KM 12 cell lines. HT-29 (A), COLO 205 (B), and KM 12 (C) cells were treated with ITH-**6** (24 h) in a concentration-dependent manner, stained with propidium iodide (PI), and analyzed by flow cytometer. Quantification of the PI staining data is presented as the percentage of distribution through stages of the cell cycle: blue-G0/G1; red- S; green- G2/M. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

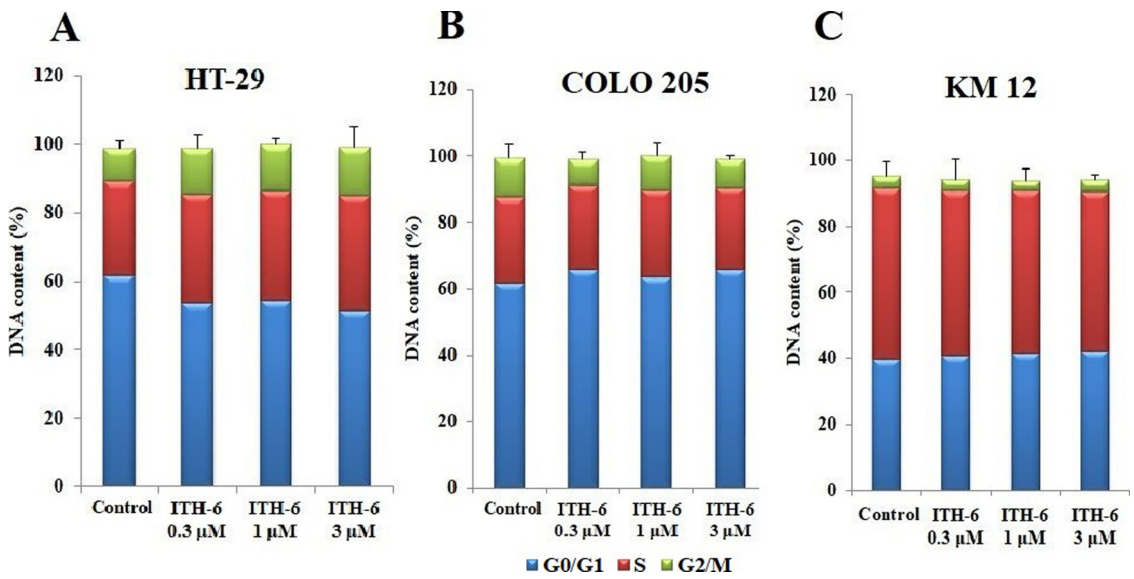


Fig. 3. Effect of ITH-6 on the cell cycle of HT-29, COLO 205, and KM 12 cell lines after washing the drug out. HT-29 (A), COLO 205 (B), and KM 12 (C) cells were treated with ITH-**6** (24 h) in a concentration-dependent manner, incubated in the drug free medium for 24, stained with propidium iodide and then analyzed by flow cytometer. Quantification of the PI staining data is presented as the percentage of distribution through stages of the cell cycle: blue-G0/G1; red- S; green- G2/M (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

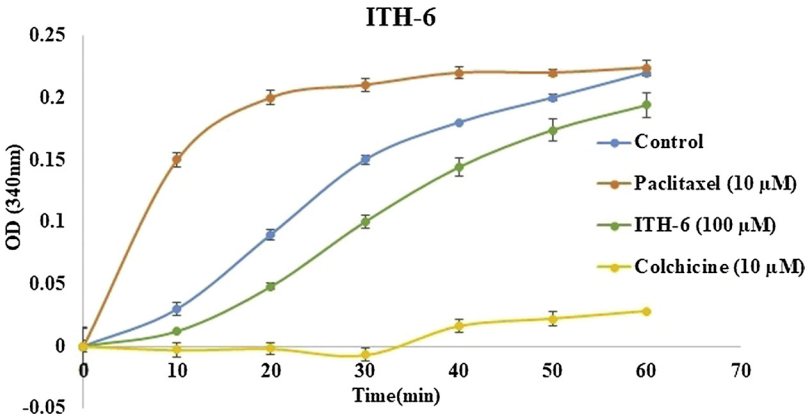


Fig. 4. Effect of ITH-6 on the tubulin polymerization. The tubulin polymerization assay was performed as per manufacturer's protocol. The change in optical density (OD) at 340 nm was plotted against time in min for ITH-**6** at 100 µM (green) was compared with control (blue), paclitaxel at 10 µM (orange), and colchicine at 10 µM (yellow). Points with error bars represent the mean ± SD for independent determinations in triplicate. The figures are representative of three independent experiments (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

were determined at the indicated concentrations. Our results showed that ITH-**6** exhibits a concentration-dependent decrease in intracellular GSH levels. As shown in Fig. 7, the GSH percentage decreased from 93.8% (control) to 23.7% (ITH-**6** at 3 µM) in HT-29, from 96.8% (control) to 34.9% (ITH-**6** at 3 µM) in COLO 205, and from 91.3% (control) to 7.8% (ITH-**6** at 3 µM) in KM 12 cells.

4. Discussion

Despite of the advances in chemotherapy, the mortality rate of colorectal cancer is quite alarming. Patients with colorectal cancer fall into two categories; ones in which the disease is confined to the primary site of origin (Dukes'A and B) and the other where it spreads to the regional lymph nodes (Dukes'C and D). The first category of patients can be surgically cured while for the later ones, surgery has is only palliative role and survival rate is less than 30% (Hampel et al., 2005).

The drugs already approved and being used for the treatment of colon cancer include irinotecan, oxaliplatin, capecitabine and the targeted drugs include bevacizumab, ramucirumab etc. Irinotecan, approved by the USFDA in 1996, is a prodrug which is converted into its active metabolite, SN-38 inside the body. It has long been used as the first line therapy for patients with recurrent and metastatic colorectal cancer however, the dose related toxicities such as vomiting, dehydration, myelosuppression, alopecia, and diarrhea are a serious concern (Rougier et al., 1998). Bevacizumab, a humanized monoclinal antibody was approved by the USFDA in 2004 for the treatment of patients with advanced colorectal cancer. Bevacizumab exhibits some rare serious adverse effects such as bowel perforation, arterial embolic events, and leukoencephalopathy (Glusker et al., 2006; Hurwitz et al., 2004, 2005).

In the present study, we find that the compound ITH-**6** has lower IC_{50} values on the colon cancer cell lines, HT-29, COLO 205 and KM 12 as compared to the conventional anti-cancer drug, irinotecan. Indanone and its derivatives are well known for their wide range of biological activity (Leoni et al., 2000). Studies done in the past have shown that the indanone derivative are potent anti-inflammatory, analgesic, antimicrobial, anticholinergic, anti-cancer, and antimalarial agents. 3-aryl substituted indanone analog was found significantly active against the HeLa and K562 cell lines (Patil and Patil, 2017).The other derivatives, gallic acid based indanone analogs are cytotoxic (IC_{50} of 0.01 µM) on breast cancer cell lines MCF-7 and MDA-MB-231 (Saxena et al., 2008). In addition, 2-substituted indanone analogs are active against non-small lung cancer cell line (Charrier et al., 2007) and 5,6-dimethoxy-1-indanone derivative is significantly cytotoxic on multidrug resistant cell lines, MCF-7/ADR, MES-SA/DX5 and HL-60/ADR (Leoni et al., 2000). The present indanone derivative, ITH-**6** exhibited IC_{50} values of 0.44

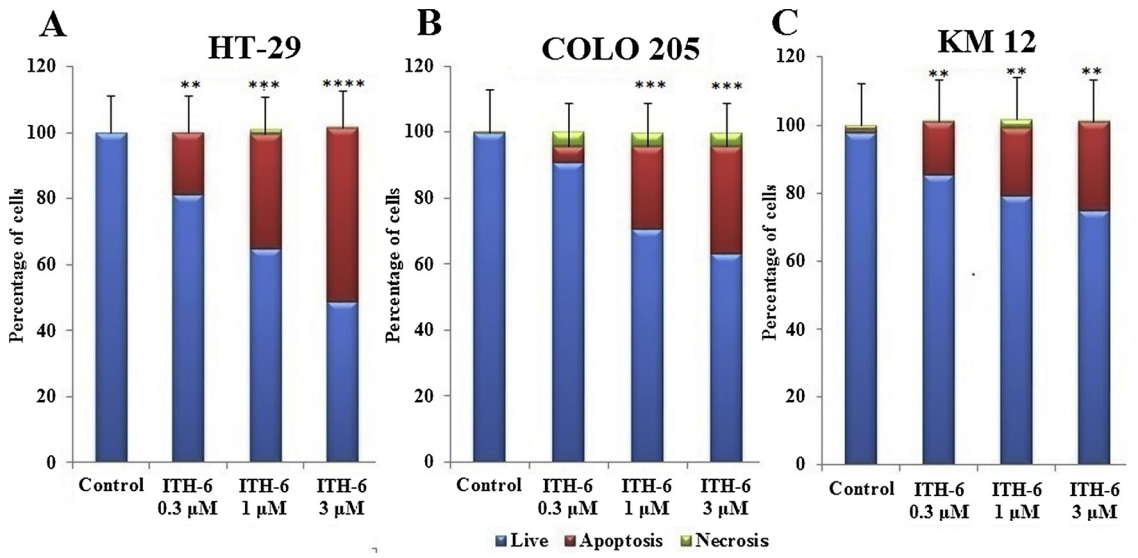
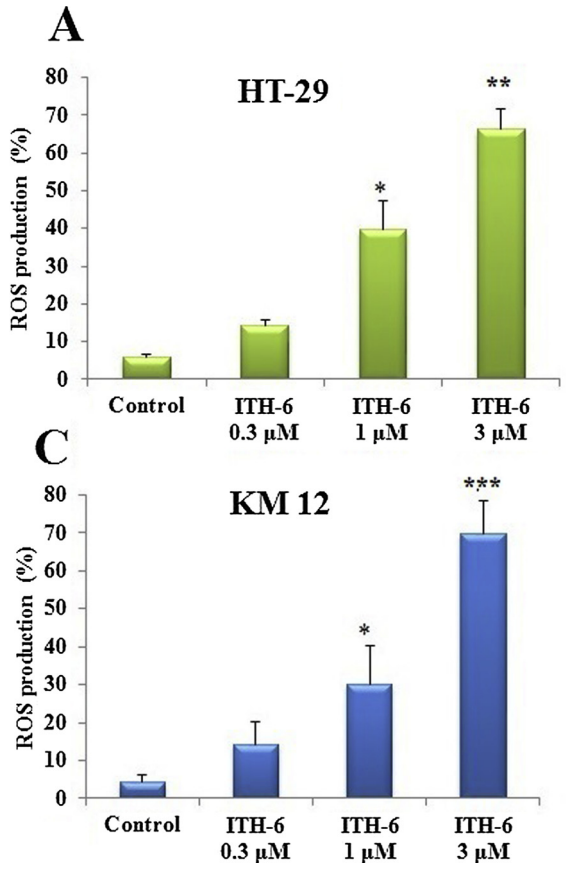


Fig. 5. Effect of ITH-6 on the apoptosis of HT-29, COLO 205, and KM 12 cell lines. HT-29 (A), COLO 205 (B), and KM 12 (C) cells were treated with ITH-**6** (24 h) in a concentration-dependent manner, stained with Annexin-V and PI, and analyzed by flow cytometer. The apoptotic cell population was quantified by flow cytometry. Bar graphs in blue represents live cells, in red represents cells undergoing apoptosis, and in green represents cell undergoing necrosis. Bar graphs represents average cell population of three independent experiments and error bars represents SD. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

μM, 0.98 μM, and 0.41 μM on HT-29, COLO 205 and KM 12 cell lines respectively. The IC_{50} of regorafenib and irinotecan on HT-29, COLO 205 and KM 12 cell lines (22.7 μM, 9.43 μM and 5.02 μM for regorafenib and 8.49 μM, 22.84 μM and 23.15 μM for irinotecan) has shown that ITH-**6** exhibited lower IC_{50} as compared to the newer drugs, regorafenib and irinotecan. The difference in response to different colon cancer cell lines are due to their establishment from different origin and p53 mutation status Barretina, 2012).



D

Treatment	Cell lines		
	HT-29 ROS (%)	COLO 205 ROS (%)	KM 12 ROS (%)
Control	5.98 ± 0.73	1.88 ± 0.98	4.26 ± 1.98
ITH-6 0.3 μM	14.39 ± 1.39	10.13 ± 4.03	14.32 ± 3.1
ITH-6 1 μM	39.6 ± 7.6	26.5 ± 10.7	30.06 ± 10.04
ITH-6 3 μM	66.3 ± 5.3	71.17 ± 6.3	69.57 ± 8.8

Fig. 6. Effect of ITH-6 on the ROS production in HT-29, COLO 205, and KM 12. HT-29 (A), COLO 205 (B), and KM 12 (C) cells were treated with ITH-**6** (24 h) in a time-dependent manner as mentioned in "Materials and Methods". Quantification of DCF (5-chloromethyl-2′-7′-dichlorofluorescein) positive cells (D). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by ANOVA.

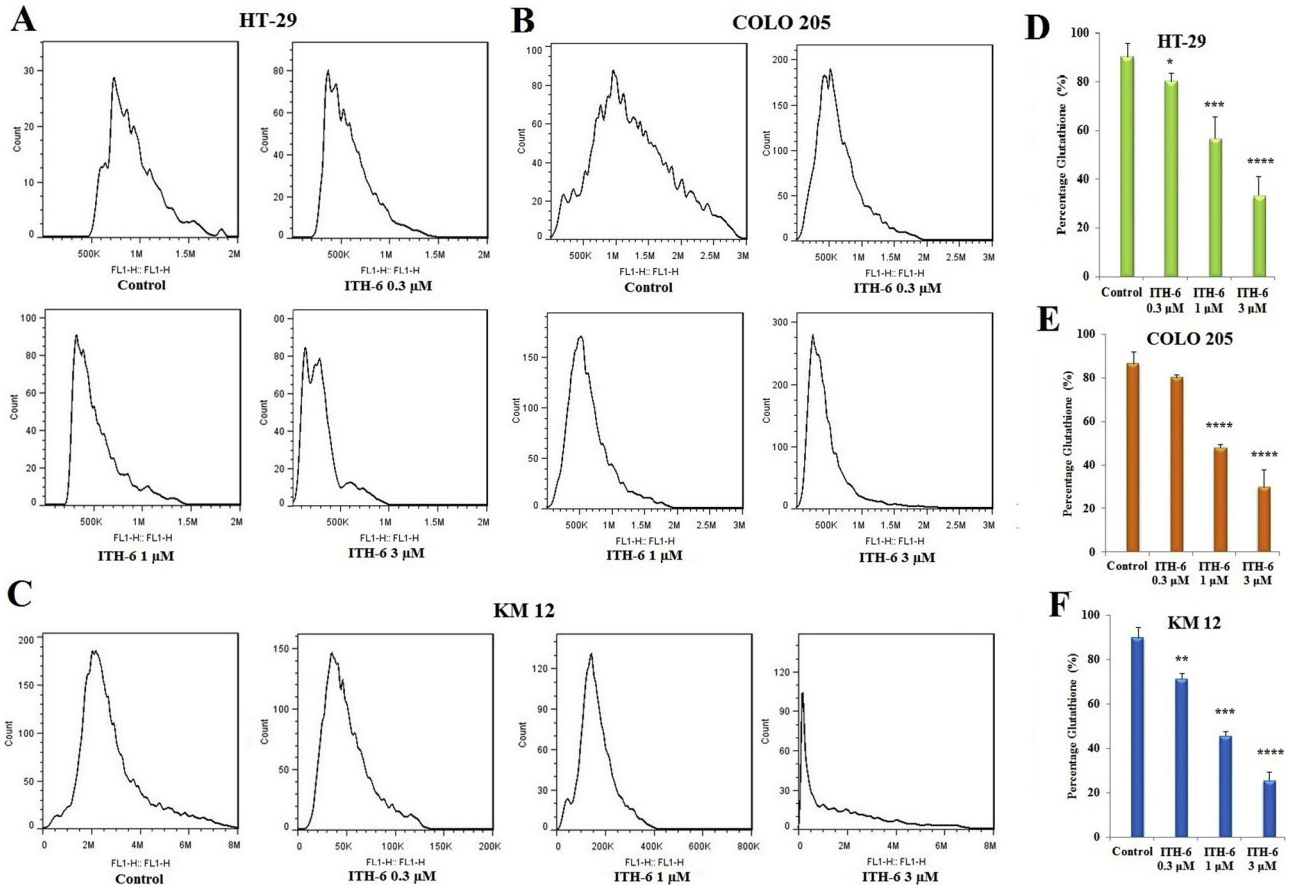


Fig. 7. Effect of ITH-6 on the GSH levels of HT-29, COLO 205, and KM 12 cell lines. The GSH assay was performed as per manufacturer's protocol. HT-29 (A), COLO 205 (B), and KM 12 (C) cells were treated with ITH-**6** (24 h) in a time-dependent manner as mentioned in "Materials and methods". Quantification of percentage of glutathione for HT-29 (D), COLO 205 (E), and KM 12 (F) cells. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ by ANOVA.

Inhibition of the cell proliferation has long been known to be associated with the changes in the cell cycle (Gupta et al., 2016b). The alterations in the cell cycle progression cause tumor growth and proliferation. It has been stated that anti-cancer drugs can arrest the cells in various phases of cell cycle and inhibit the tumor growth (Malumbres et al., 2008). Our cell cycle results indicate that ITH-**6** arrest the cells in G2/M phase and the maximum effect is at high concentration (3 µM) and there is no significant effect on other phases of cell cycle. These cell cycle results show that the test compound is G2/M phase specific. This instigated the idea to investigate the effects of ITH-**6** on tubulin polymerization and mitotic spindle formation, two processes that take place in G2/M phases of cell cycle. The tubulin polymerization assay results show that ITH-**6** at 100 µM inhibits tubulin polymerization for 1 h. Paclitaxel (Taxol), a well-known anti-cancer drug, stabilizes the microtubule against depolymerization, and is hence known as polymerization enhancer (Arnal and Wade, 1995). Colchicine on the other hand, inhibits the microtubule polymerization and is thus known as a polymerization inhibitor (Hastie, 1991). We compared the tubulin polymerization effects of ITH-**6** to that of paclitaxel and colchicine and found that, similar to colchicine, ITH-**6** inhibited the tubulin polymerization. However, the extent of inhibition was not significantly comparable.

Since the cell cycle arrest is related to apoptosis, an apoptotic analysis was carried out using HT-29, COLO 205 and KM 12 cell lines. In all the three cell lines, a substantial number of apoptotic cells were observed in the lower and upper right quadrants, which are the representatives of early and late apoptosis. The results showed an increase in early and late apoptosis in these cell lines with maximum apoptosis seen at the highest concentration of 3 µM. Cellular studies have shown that an increase in the level of ROS causes an oxidative stress which results in oxidative damage to the cellular components (Ngamchuea et al., 2017). It enters into the cells, gets converted into the fluorescent (5-chloromethyl-20-7′-dichlorofluorescein (DCF)) product by the action of intracellular peroxides, hence, the ROS analysis is conducted in all the cell lines (Circu and Aw, 2010; Matés and Sánchez-Jiménez, 2000). We found that ITH-**6** at the highest concentration (3 µM) induced intracellular ROS production in HT-29, COLO 205 and KM 12 cell lines. The mitochondrial GSH maintains the integrity of mitochondrial proteins and lipids and modulates ROS production. Oxidative damage is associated with an increase in mitochondrial ROS production and a decrease in GSH which in turn triggers apoptosis (Circu and Aw, 2008). Therefore, intracellular GSH assay was performed in all the three colon cancer cell lines. A significant decrease in GSH levels was also observed with compound ITH-**6** in all the three cell lines with the maximum decrease at the highest concentration of 3 µM.

5. Conclusion

Anti-cancer drug discovery and development are considered as the grand challenges for the pharmaceutical industry. Extremely dynamic mitotic-spindle microtubules indeed remain the most successful and promising targets for anti-cancer therapy. Microtubule-stabilizing agents are continually playing an important role in anti-cancer drug discovery and development. In this study, we have shown that ITH-**6** is an effective cytotoxic agent against colon cancer cells and exhibits a better cytotoxic effect compared to other drugs approved for colon cancer. Mechanistically, ITH-**6** inhibits tubulin polymerization, alters the cell cycle progression and induces apoptosis by elevating the intra

cellular ROS and decreasing the intracellular GSH levels. Together with its mechanism of action, ITH-**6** could be a potential anti-cancer drug candidate for colorectal cancer treatment.

Author contributions

S.N. and Z.-S.C. designed the experiments and U.N. and M.A. synthesized the compound. S.N., P.G. and N.K. performed the experiments, S.N. and P.G. analyzed the data, S.N., Z.-S.C. and M.A. wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.02.004.

References

Albanes, D., 1990. Energy balance, body size, and cancer. Crit. Rev. Oncol. Hematol. 10 (3), 283–303.
Anreddy, N., Gupta, P., Kathawala, R.J., Patel, A., Wurpel, J.N., Chen, Z.S., 2014. Tyrosine kinase inhibitors as reversal agents for ABC transporter mediated drug resistance. Molecules 19 (9), 13848–13877.
Arnal, I., Wade, R.H., 1995. How does taxol stabilize microtubules? Curr. Biol. 5 (8), 900–908.
Arnold, M., Sierra, M.S., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2016. Global patterns and trends in colorectal cancer incidence and mortality. Gut 66 (4), 683–691.
Barretina, J., 2012. The cancer cell line Encyclopedia enables predictive modeling of anticancer drug sensitivity. Nature 483 (7391), 603–607.
Cepowicz, D., Zaręba, K., Pryczynicz, A., Dawidziuk, T., Żurawska, J., Hołody-Zaręba, J., Gryko, M., Kędra, B., 2017. Blood serum levels of E-cadherin in patients with colorectal cancer. Prz. Gastroenterol. 12 (3), 186–191.
Charrier, C., Roche, J., Gesson, J.P., Bertrand, P., 2007. Antiproliferative activities of a library of hybrids between indanones and HDAC inhibitor SAHA and MS-275 analogues. Bioorg. Med. Chem. Lett. 17 (22), 6142–6146.
Circu, M.L., Aw, T.Y., 2008. Glutathione and apoptosis. Free Radic. Res. 42 (8), 689–706.
Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic. Biol. Med. 48 (6), 749–762.
Compton, C.C., 2003. Colorectal carcinoma: diagnostic, prognostic, and molecular features. Mod. Pathol. 16 (4), 376–388.
Ganellin, C., 1967. Indane and indene derivatives of biological interest. Adv. Drug Res. 4, 163.
Glusker, P., Recht, L., Lane, B., 2006. Reversible posterior leukoencephalopathy syndrome and bevacizumab. N. Engl. J. Med. 354 (9), 980–982 discussion 980–982.
Gryfe, R., Kim, H., Hsieh, E.T., Aronson, M.D., Holowaty, E.J., Bull, S.B., Redston, M., Gallinger, S., 2000. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N. Engl. J. Med. 342 (2), 69–77.
Gupta, P., Jani, K.A., Yang, D.H., Sadoqi, M., Squillante, E., Chen, Z.S., 2016a. Revisiting the role of nanoparticles as modulators of drug resistance and metabolism in cancer. Expert Opin. Drug Metab. Toxicol. 12 (3), 281–289.
Gupta, P., Kathawala, R.J., Wei, L., Wang, F., Wang, X., Druker, B.J., Fu, L.W., Chen, Z.S., 2016b. PBA2, a novel inhibitor of imatinib-resistant BCR-ABL T315I mutation in chronic myeloid leukemia. Cancer Lett. 383 (2), 220–229.
Gupta, P., Xie, M., Narayanan, S., Wang, Y.J., Wang, X.Q., Yuan, T., Wang, Z., Yang, D.H., Chen, Z.S., 2017a. GSK1904529A, a potent IGF-IR inhibitor, reverses MRP1-mediated multidrug resistance. J. Cell. Biochem. 118 (10), 3260–3267.
Gupta, P., Xie, M., Narayanan, S., Wang, Y.J., Wang, X.Q., Yuan, T., Wang, Z., Yang, D.H., Chen, Z.S., 2017b. GSK1904529A, a potent IGF-IR inhibitor, reverses MRP1-mediated multidrug resistance. J. Cell. Biochem. 118 (10), 3260–3267.
Gupta, P., Zhang, Y.K., Zhang, X.Y., Wang, Y.J., Lu, K.W., Hall, T., Peng, R., Yang, D.H., Xie, N., Chen, Z.S., 2018. Voruciclib, a potent CDK4/6 inhibitor, antagonizes ABCB1 and ABCG2-mediated multi-drug resistance in cancer cells. Cell. Physiol. Biochem. 45 (4), 1515–1528.
Hampel, H., Frankel, W.L., Martin, E., Arnold, M., Khanduja, K., Kuebler, P., Nakagawa, H., Sotamaa, K., Prior, T.W., Westman, J., Panescu, J., Fix, D., Lockman, J., Comeras, I., de la Chapelle, A., 2005. Screening for the Lynch syndrome (hereditary non-polyposis colorectal cancer). N. Engl. J. Med. 352 (18), 1851–1860.
Hastie, S.B., 1991. Interactions of colchicine with tubulin. Pharmacol. Ther. 51 (3), 377–401.
Hisamuddin, I.M., Yang, V.W., 2004. Genetics of colorectal cancer. MedGenMed 6 (3), 13.
Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., Kabbinavar, F., 2004. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N. Engl. J. Med. 350 (23), 2335–2342.
Hurwitz, H.I., Fehrenbacher, L., Hainsworth, J.D., Heim, W., Berlin, J., Holmgren, E., Hambleton, J., Novotny, W.F., Kabbinavar, F., 2005. Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. J. Clin. Oncol. 23 (15), 3502–3508.
Kathawala, R.J., Gupta, P., Ashby, C.R., Chen, Z.S., 2015. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist. Updat. 18, 1–17.
Kim, E.H., Jang, H., Roh, J.L., 2016. A novel polyphenol conjugate sensitizes cisplatin-resistant head and neck cancer cells to cisplatin via Nrf2 inhibition. Mol. Cancer Ther. 15 (11), 2620–2629.
Klaus, M., 1983. Tetrahydronaphthalene and indane compounds useful as anti-tumor agents. Google Patents.
Kono, S., Handa, K., Hayabuchi, H., Kiyohara, C., Inoue, H., Marugame, T., Shinomiya, S., Hamada, H., Onuma, K., Koga, H., 1999. Obesity, weight gain and risk of colon adenomas in Japanese men. Jpn. J. Cancer Res. 90 (8), 805–811.
Leoni, L.M., Hamel, E., Genini, D., Shih, H., Carrera, C.J., Cottam, H.B., Carson, D.A., 2000. Indanocine, a microtubule-binding indanone and a selective inducer of apoptosis in multidrug-resistant cancer cells. J. Natl. Cancer Inst. 92 (3), 217–224.
Malumbres, M., Pevarello, P., Barbacid, M., Bischoff, J.R., 2008. CDK inhibitors in cancer therapy: what is next? Trends Pharmacol. Sci. 29 (1), 16–21.
Matés, J.M., Sánchez-Jiménez, F.M., 2000. Role of reactive oxygen species in apoptosis: implications for cancer therapy. Int. J. Biochem. Cell Biol. 32 (2), 157–170.
Mirone, G., Perna, S., Shukla, A., Marfe, G., 2016. Involvement of notch-1 in resistance to regorafenib in colon cancer cells. J. Cell. Physiol. 231 (5), 1097–1105.
Mokhtari, S., Mosaddegh, M., Moghadam, M.H., Soleymani, Z., Ghafari, S., Kobarfard, F., 2012. Synthesis and cytotoxic evaluation of novel 3-substituted derivatives of 2-indolinone. Iran. J. Pharm. Res.: IJPR 11 (2), 411.
Moore, J.R., LaMont, J.T., 1984. Colorectal cancer. Risk factors and screening strategies. Arch. Intern. Med. 144 (9), 1819–1823.
Ngamchuea, K., Batchelor-McAuley, C., Compton, R.G., 2017. Rapid method for the quantification of reduced and oxidized glutathione in human plasma and saliva. Anal. Chem. 89 (5), 2901–2908.
O'Brien, J.M., 2000. Environmental and heritable factors in the causation of cancer: analyses of cohorts of twins from Sweden, Denmark, and Finland, by P. Lichtenstein, N.V. Holm, P.K. Verkasalo, A. Iliadou, J. Kaprio, M. Koskenvuo, E. Pukkala, A. Skytthe, and K. Hemminki. N Engl J Med 343:78–84, 2000. Surv. Ophthalmol. 45 (2), 167–168.
Patil, S.A., Patil, R., 2017. Recent developments in biological activities of indanones. Eur. J. Med. Chem. 138, 182–198.
Rougier, P., Van Cutsem, E., Bajetta, E., Niederle, N., Possinger, K., Labianca, R., Navarro, M., Morant, R., Bleiberg, H., Wils, J., Awad, L., Herait, P., Jacques, C., 1998. Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. Lancet 352 (9138), 1407–1412.
Saxena, H.O., Faridi, U., Srivastava, S., Kumar, J.K., Darokar, M.P., Luqman, S., Chanotiya, C.S., Krishna, V., Negi, A.S., Khanuja, S.P., 2008. Gallic acid-based indanone derivatives as anticancer agents. Bioorg. Med. Chem. Lett. 18 (14), 3914–3918.
Schneider, Y., Chabert, P., Stutzmann, J., Coelho, D., Fougerousse, A., Gossé, F., Launay, J.F., Brouillard, R., Raul, F., 2003. Resveratrol analog (Z)-3,5,4'-trimethoxystilbene is a potent anti-mitotic drug inhibiting tubulin polymerization. Int. J. Cancer 107 (2), 189–196.
Schuell, B., Gruenberger, T., Kornek, G.V., Dworan, N., Depisch, D., Lang, F., Schneeweiss, B., Scheithauer, W., 2005. Side effects during chemotherapy predict tumour response in advanced colorectal cancer. Br. J. Cancer 93 (7), 744–748.
Siegel, R.L., Miller, K.D., Jemal, A., 2017. Cancer statistics, 2017. CA Cancer J. Clin. 67 (1), 7–30.
Śliwka, L., Wiktorska, K., Suchocki, P., Milczarek, M., Mielczarek, S., Lubelska, K., Cierpiał, T., Łyżwa, P., Kiełbasiński, P., Jaromin, A., Flis, A., Chilmonczyk, Z., 2016. The comparison of MTT and CVS assays for the assessment of anticancer agent interactions. PLoS One 11 (5), e0155772.
Vilums, M., Heuberger, J., Heitman, L.H., IJzerman, A.P., 2015. Indanes—properties, preparation, and presence in ligands for G protein coupled receptors. Med. Res. Rev. 35 (6), 1097–1126.
Wang, Y.J., Zhang, Y.K., Zhang, G.N., Al Rihani, S.B., Wei, M.N., Gupta, P., Zhang, X.Y., Shukla, S., Ambudkar, S.V., Kaddoumi, A., Shi, Z., Chen, Z.S., 2017. Regorafenib overcomes chemotherapeutic multidrug resistance mediated by ABCB1 transporter in colorectal cancer: in vitro and in vivo study. Cancer Lett. 396, 145–154.
Wiela-Hojeńska, A., Kowalska, T., Filipczyk-Cisarż, E., Łapiński, Ł., Nartowski, K., 2015. Evaluation of the toxicity of anticancer chemotherapy in patients with colon cancer. Adv. Clin. Exp. Med. 24 (1), 103–111.
Wynder, E.L., Shigematsu, T., 1967. Environmental factors of cancer of the colon and rectum. Cancer 20 (9), 1520–1561.
Yao, S.-W., Lopes, V., Fernández, F., Garcıa-Mera, X., Morales, M., Rodrıguez-Borges, J., Cordeiro, M., 2003. Synthesis and QSAR study of the anticancer activity of some novel indane carbocyclic nucleosides. Bioorg. Med. Chem. 11 (23), 4999–5006.