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RESEARCH ARTICLE

Synthesis and evaluation of in vitro cytotoxic effects of triazol/spiroindolinequinazolinedione, triazol/indolin-3-thiosemicarbazone and triazol/thiazol-indolin-2-one conjugates

Saeed Nazari[1] · Fatemeh Safari[2] · Mohammad Barasm Mamaghani[1] · Ayoob Bazgir[1]

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Abstract

Purpose Cancer as one of the major diseases with high mortality rates threats human life in the world. Subsequently, the design new potent anticancer agents has attracted much attention in the area of synthetic and medicinal chemistry. In this study, new triazol-linked spiroindolinequinazolinone, thiazol-oxindole and oxindole-thiosemicarbazone conjugates were synthesized and evaluated for their in vitro cytotoxic activity toward different cancer lines.

Methods Some new triazol-linked oxindoles and spirooxindoles conjugates were synthesized. The synthesized compounds were tested for their in vitro cytotoxic activity toward cancer lines including A375, PC3, LNCaP, MDA MB231 and normal cells HDF (human dermal fibroblast).

Results Among all synthesized compounds, the triazol-linked oxindol-thiosemicarbazone conjugate **10b** showed the highest cytotoxic activity against different cancer cells. By using quantitative real time PCR (qRT-PCR), it was found that **10b** is able to induce apoptosis by alteration of Bax, Bcl2 balance (i.e. by up regulation of Bax and down regulation of Bcl-2 mRNA expression levels). The DAPI staining was used to show the death of cancer cells in the presence of **10b**. Interestingly, **10b** suppressed the migration of LNCaP cancer cells by up-regulation of epithelial markers (E-cadherin) and down-regulation of mesenchymal markers (vimentin).

Conclusion Our findings revealed that the compound **10b** may be a new potent candidate with multiple biological activities to design therapeutic agents against different cancers.

Keywords Spirooxindole containing triazole · Spiroindolinequinazoline-dione · Oxindole containing thiazole · Isatin · Apoptosis · Anticancer activity

Introduction

Cancer as an uncontrollable growth of abnormal cells is considered as one of the major diseases with high mortality rates which threats human life in the world [1]. Most of the clinically available anticancer chemotherapeutic agents cannot distinguish between the normal and cancerous cells. Moreover, they have unwanted side effects and drug resistance [2, 3]. Subsequently, the design, development and synthesize new potent anticancer agents has attracted much attention in the area of synthetic and medicinal chemistry.

Isatin is endogenously found in human and other mammalian tissues and has been observed as a privileged scaffold in many natural products and alkaloids [4]. Isatin has become known as an important moiety that is endowed with many

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✉ Fatemeh Safari
fsafari@guilan.ac.ir

✉ Ayoob Bazgir
a_bazgir@sbu.ac.ir

[1] Department of Chemistry, Shahid Beheshti University, General Campus, Tehran 1983963113, Iran

[2] Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran

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promising biological properties [4–8], mainly anticancer activity [9]. Spirotryprostatin A (**I**), Spirotryprostatin B (**II**) and Strychnofoline are natural spirooxindoles possessing anticancer activities (Fig. 1) [10]. The FDA has been approved Sunitinib (Sutent®) (**III**), a 5-fluoro-3-substituted oxindole derivative, for treating advanced renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GIST) [11]. Nintedanib (Ofev®) (**IV**) in combination with docetaxel for the patients with non-small cell lung cancer is in first-line chemotherapy [12]. Nintedanib was tested against colorectal cancer [13] and breast cancer [14].

Recently, molecular conjugation and hybridization have reported as efficient and applicable tools for development of new potentially active molecules [15]. The strategy is extremely interesting and can minimize the drug resistance and risk of drug-drug interactions [16]. Therefore, a lot of efforts have been focused on anticancer potential of isatin-based hybrid or conjugate molecules such as isatin-thiazol hybrids (**V**) [17], isatin–benzothiazole analogs (**VI**) [18], isatin-thiazolidinone (**VII**) [19], isatin-benzimidazole (**VIII**) [20] and N-indolylmethyl spiroindoline-quinazolines (**IX**) [21]. The outstanding structural properties of 1,2,3-triazoles like hydrogen bonding ability, moderate dipole character, and stability and rigidity under in vivo conditions make their enhanced biological activities [22]. Likewise, thiosemicarbazones have significant antitumor activities [23]. Their antitumor activity is because of an inhibition of DNA formation produced by the moderation in the reductive reaction of ribonucleotides to deoxyribonucleotides [23]. In the recent years the attention of organic and medicinal chemists has attracted to design the 1,2,3-triazoles or thiosemicarbazone conjugate or hybrid with isatin derivatives as new anticancer candidate drugs [24–30]. For example, the 1,2,3-triazole tethered nitroimidazole-isatin conjugates [25], and spirooxindole-derived morpholine-fused-1,2,3-triazoles [26] displayed a good anti-proliferative activity against selected human cancers. The potential anticancer activity of triazole-linked indole and oxindole glycoconjugates was evaluated by Babu's research group [27]. The oxindole-thiosemicarbazones (**X**) with anti-cancer activity was synthesized and the effectiveness of aromatic/hydrophobic properties at the N4 position of the thiosemicarbazone was investigated [28]. The 4-thiazolidinone-indoles showed anti-proliferative activities against human tumor cells [29]. Inspired by the above reports and as a continuation of our previous work on the development of new methods for oxindole and spirooxindole synthesis [31–33], we report herein the synthesis and in vitro cytotoxic evaluation of 1,2,3-triazol/spiroindolinequinazolinedione, 1,2,3-triazol/thiazol-indolin-2-one and 1,2,3-triazol/indolin-3-thiosemicarbazone conjugates (Fig. 1).

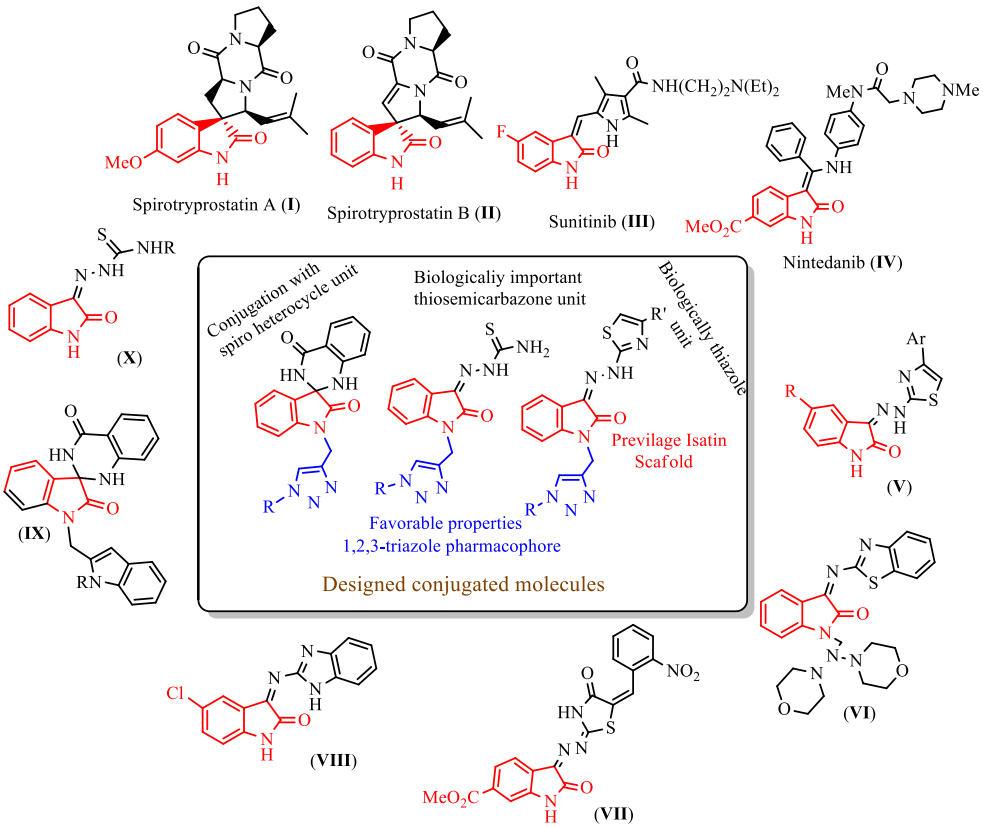


Fig. 1 Design of target molecules based on reported isatin-based molecules with cytotoxic potential

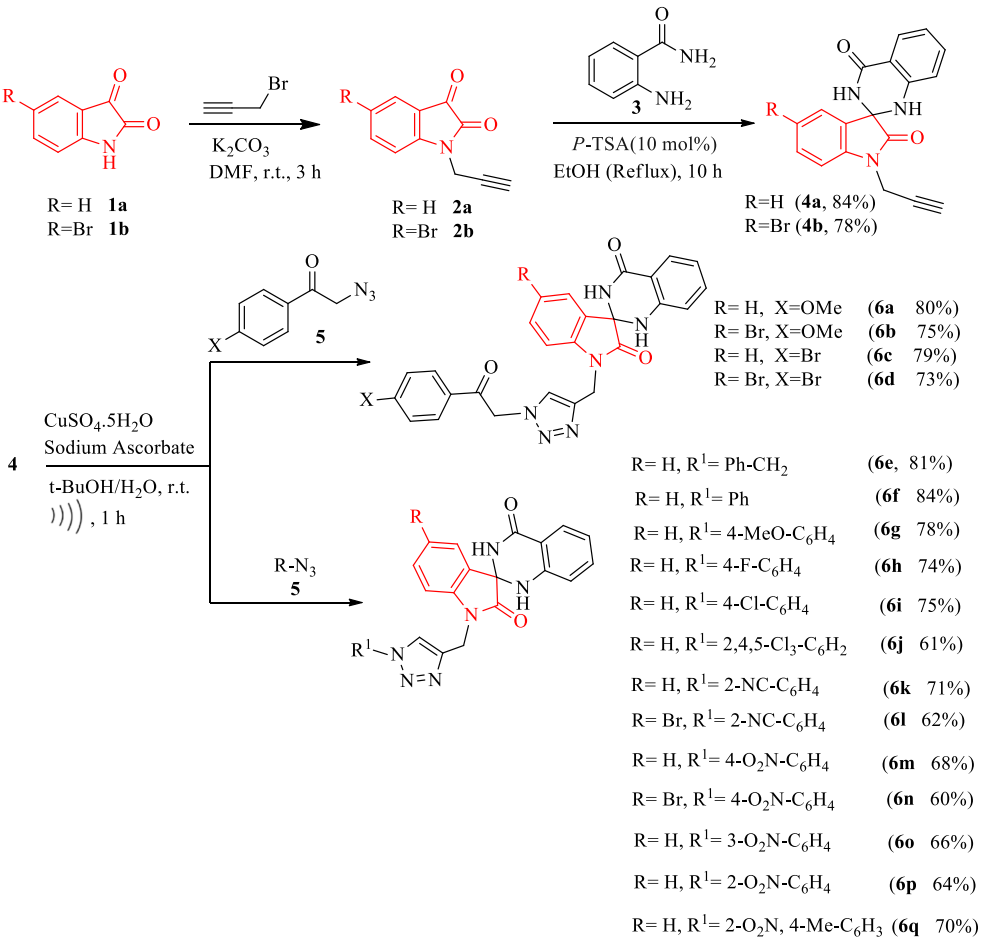
Results and discussion

Chemistry

The synthesis pathway of 1,2,3-triazol/spiro[indoline-3,2′-quinazoline]-dione conjugate **6** has been shown in Scheme 1. The product **6** was synthesized by a sequential reaction starting from isatin **1**. The cyclization and Cu-catalyzed click chemistry are the key steps for the operationally simple synthesis of **6**. First, N-propargyl isatins **2** were synthesized by the nucleophilic reaction of isatins **1** and propargyl bromide in the presence of anhydrous potassium carbonate. The subsequent *P*-toluenesulfonic acid (*p*-TSA) catalyzed nucleophilic condensation reaction of **2** with 2-aminobenzamide **3** in EtOH under reflux conditions afforded 1-(prop-2-yn-1-yl)-1′H-spiro[indoline-3,2′-quinazoline]-2,4′(3′H)-diones **4** [31]. The target triazol-liked spiro[indoline-quinazoline]-diones **6** were obtained by the Cu-catalyzed click reaction of **4** and aryl or alkyl azids **5** under ultrasonic irradiation in t-BuOH-H_2O for 1 h (Scheme 1). To delineate the role of ultrasound, the final step of **6a** synthesis was done without ultrasonic irradiation at the same temperature in t-BuOH-H_2O. The product **6a** was obtained in 72% isolated yield after 5 h. It confirms the use of ultrasound irradiation leads to the higher yield at shorter reaction time (Scheme 1). Therefore, ultrasonic irradiation conditions was selected for the final step.

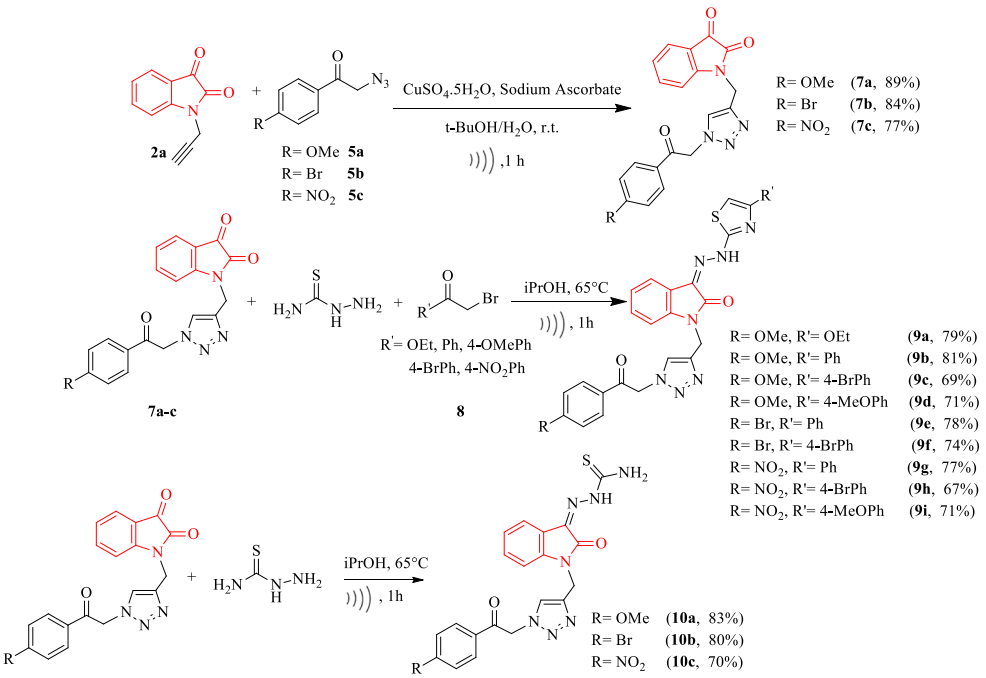
The potential anticancer activities of thiazole skeleton [34] encouraged us to synthesize a series of 1,2,3-triazol-linked thiazole-oxindole conjugates **9** (Scheme 2). The 1H-1,2,3-triazol-indoline-2,3-diones **7** were obtained by the Cu-catalyzed click reaction of **2** and phenacyl azides **5** under ultrasonic irradiation in t-BuOH-H_2O at room temperature. Then, 1,2,3-triazol-linked thiazole-oxindole conjugates **9** were synthesized by a three-component reaction of **7**, thiosemicarbazid and phenacyl bromides **8** in isopropyl alcohol under ultrasonic irradiation at 65 °C for 1 h.

So far, there is not any report to evaluate the possible cytotoxic effects of indolinone and thiosemicarbazone conjugate molecule. Therefore, the 1,2,3-triazol-linked oxindol-thiosemicarbazone conjugates **10** were synthesized by the reaction of **7** and thiosemicarbazid in isopropyl alcohol under ultrasonic irradiation at 65 °C for 1 h (Scheme 2). All compounds **6**, **9** and **10** are stable solids whose structures were established by IR, Mass, and ^1H, and ^{13}C NMR spectroscopy.



Scheme 1 Synthesis of 1,2,3-triazol/spiro[indoline-3,2′-quinazoline]-diones **6**

Scheme 2 Synthesis of triazol-linked thiazole-oxindole conjugates **9**



In vitro cytotoxic evaluation

In an initial step, the MTT colorimetric assay was used to evaluate the cytotoxic potential of synthesized compounds **6a-q**, **7a-c**, **9a-i** and **10a-c** against cancer cell lines such as A375 cells, PC3 cells, LNCaP cells, MDA-MB-231 cells, and normal cell line HDF (human dermal fibroblast).

The results is indicated in term of inhibitory concentration of 50% growth (IC_{50}) at 48 h (Table 1) The Etoposide and DMSO (1%) were used as a positive and a negative control, respectively. As can be seen in Table 1, the most of compounds **6** showed inhibitory effects against A375 cancer cell line. The compounds **6f**, **6i** and **6m** showed cytotoxicity against two cancer cell lines. However, their IC_{50} values were higher than IC_{50} values of Etoposide. Notably, the compounds **6q** was able to inhibit cell growth in A375 and LNCaP cancer cell lines with IC_{50} values that was close to the response rates for Etoposide. The results revealed that the type of substituent on isatin and phenyl ring of triazol moiety considerably influences cytotoxic potential of conjugate molecules **6**. As evident from the activity results, on in vitro evaluation of the intermediates 1H-1,2,3-triazol-indoline-2,3-diones **7**, compounds **7a** and **7c** having 4-methoxy or nitro group in the phenyl ring of triazol moiety showed moderate to good activity against tested cancer cell lines. The cytotoxicity comparison of the triazol-linked thiazole-oxindoles **9** with triazol-indoline-2,3-dione intermediate **7** showed that the conjugation of hydrazino thiazole moiety to triazol-indoline-2,3-dione **7** did not improve the cytotoxicity effect in the most of compounds **9** and they were inactive against cancer cell lines. Compared to compounds 7, the compounds **9a** and **9d** showed better activity against LNCaP and PC3, respectively. The cytotoxicity evaluation of triazol-linked oxindol-thiosemicarbazone conjugates **10a-c** suggested that the compounds activity is strongly influenced by the nature of the substituents in the phenyl ring of triazole moiety. The compound **10a** with methoxy group was inactive while **10c** with nitro group showed good activity against MDA-MB231 and PC3. Interestingly, compound **10b** having Br demonstrated the highest cytotoxic activity against A375 cell line (IC_{50} = 25.91 μM), MDA-MB-231 cell line (IC_{50} = 18.42 μM), PC3 cell line (IC_{50} = 15.32 μM) and LNCaP cell line (IC_{50} = 29.23 μM). Therefore, **10b** was selected for further study.

Then, DAPI staining was used to show clear morphological changes and chromatin fragmentation within the nucleus of A375, MDA-MB-231, PC3 and LNCaP treated cells with **10b**. It was found, their morphology is not altered in untreated cells (or controls). The images of inverted fluorescence microscopy reveal that compound **10b** led to cell death of cancer cells (Fig. 2).

To prove the cancer cell death, we used the qRT-PCR method to test the expression levels of Bax and Bcl-2 mRNAs. The balance of Bcl-2 protein and pro-apoptotic Bax prevents cytochrome c translocation from the mitochondria to cytoplasm and represents the apoptosis resistance. This balance is compromised by down-regulation of Bcl-2 and/or up-regulation of Bax and contributes to apoptosis. The results showed the apoptosis of LNCaP cancer cells (Fig. 3a and b).

We used wound healing assay to find out the effect of **10b** on the cancer cells migration. In this regard, the LNCaP cells were scratched with a pipette tip and then treated with **10b**. The cells migration into wound was assessed at 0 and 24 h

Table 1 In vitro cytotoxic activities of **6**, **7**, **9**, and **10** against A375, MDA-MB-231, LNCaP, PC3 cells and normal cell HDF. Data represent mean ± SD of three independent experiments

Compounds	IC_{50} (μM)				
	A375	MDA-MB231	PC3	LNCaP	HDF
6a	**42.40 ± 0.002**	>100	>100	>100	>100
6b	**50.26 ± 0.008**	>100	>100	>100	>100
6c	**50.11 ± 0.005**	>100	>100	>100	>100
6d	>100	>100	>100	>100	>100
6e	>100	>100	>100	>100	>100
6f	**50.01 ± 0.006**	>100	>100	**47.50 ± 0.007**	>100
6g	>100	>100	>100	>100	>100
6h	>100	>100	>100	>100	>100
6i	**37.95 ± 0.005**	>100	**52.20 ± 0.008**	>100	>100
6j	**56.54 ± 0.005**	>100	>100	>100	>100
6k	**50.78 ± 0.006**	>100	>100	>100	>100
6l	**50.27 ± 0.001**	>100	>100	>100	>100
6m	**57.32 ± 0.001**	**58.45 ± 0.004**	>100	>100	>100
6n	>100	>100	>100	>100	>100
6o	**50.05 ± 0.001**	>100	>100	>100	>100
6p	**56.80 ± 0.008**	>100	>100	>100	>100
6q	**27.74 ± 0.001**	>100	>100	**28.07 ± 0.004**	>100
7a	**35.86 ± 0.002**	**51.76 ± 0.001**	>100	**59.42 ± 0.001**	>100
7b	>100	>100	>100	>100	>100
7c	**37.69 ± 0.002**	**51.05 ± 0.002**	**72.01 ± 0.003**	>100	>100
9a	**46.33 ± 0.001**	>100	>100	**45.01 ± 0.012**	>100
9b	>100	**55.10 ± 0.005**	>100	>100	>100
9c	>100	>100	>100	>100	>100
9d	>100	>100	**62.95 ± 0.010**	>100	>100
9e	>100	>100	>100	>100	>100
9f	>100	>100	>100	>100	>100
9g	>100	>100	>100	>100	>100
9h	>100	>100	>100	>100	>100
9i	>100	>100	>100	**51.34 ± 0.006**	>100
10a	>100	>100	>100	>100	>100
10b	**25.91 ± 0.005**	**18.42 ± 0.002**	**15.32 ± 0.002**	**29.23 ± 0.003**	>100
10c	>100	**32.74 ± 0.003**	**57.98 ± 0.012**	>100	>100
Etoposide	**24.46 ± 0.019**	**31.02 ± 0.051**	**30 ± 0.037**	**31.21 ± 0.005**	>100

after treatment. It was found, **10b** has potential inhibitory activity on LNCaP cells migration (compared to untreated cells as control) (Fig. 4).

Eventually, we examined the expression rate for mRNAs of two genes as potent markers of EMT (E-cadherin, vimentin) to validate the impact of **10b** on cancer cell migration [35, 36] (Fig. 5a). The expression levels of E-cadherin and vimentin mRNA were evaluated by qRT-PCR method. As can be seen in Fig. 5b and c, the E-cadherin expression was significantly increased, while vimentin expression was significantly reduced in **10b**-treated LNCaP cells. This indicates that **10b** is able to inhibit EMT.

Previously, the anti-tyrosine kinase activity of indolinones [37, 38] and inhibitory effects of thiosemicarbazones on DNA replication by suppression of ribonucleotide reductase activity were reported [23, 39, 40]. Notably, it seems that **10b** may involve to inhibit tyrosine kinases activity or/and DNA synthesis process. Moreover, we showed inhibition effects of **10b** on cell migration. Thus, it supports this idea that **10b** may have anti tyrosine activity. Most of tyrosine kinases regulate the cell motility. There are some potent candidates as mitogen-activated protein kinase (MAPK) cascade activated by epidermal growth factor receptor (EGFR). Proliferation and

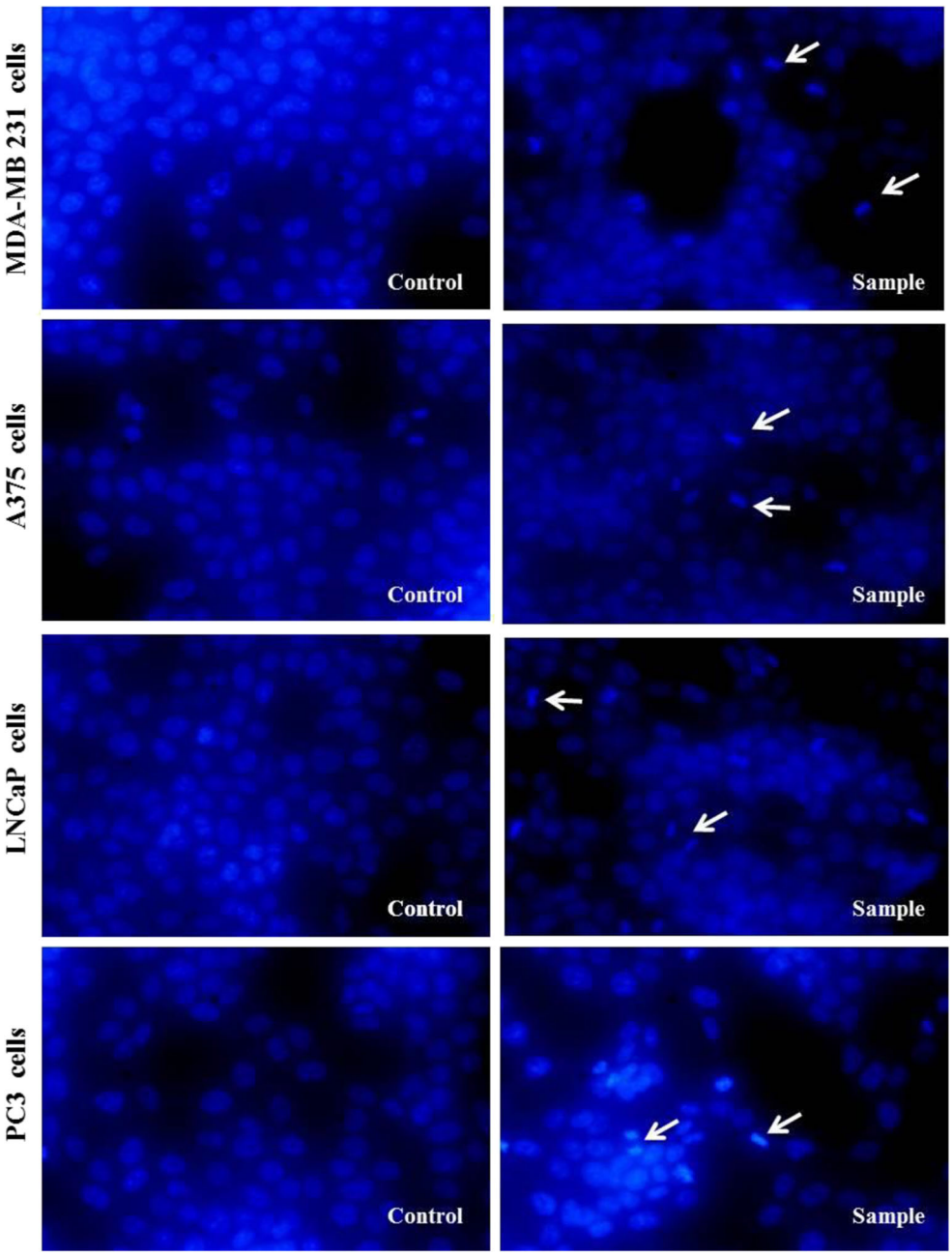


Fig. 2 Inverted fluorescent microscopy images of chromatin damages occurrence in the nucleus of treated cells with **10b** compound and DMSO (1%), which have been stained with DAPI in MDA-MB 231, A375, LNCaP, and PC3 cancer cell lines. The experiments were performed three times (original microscope magnification, 40X, Scale bar, 10 μm)

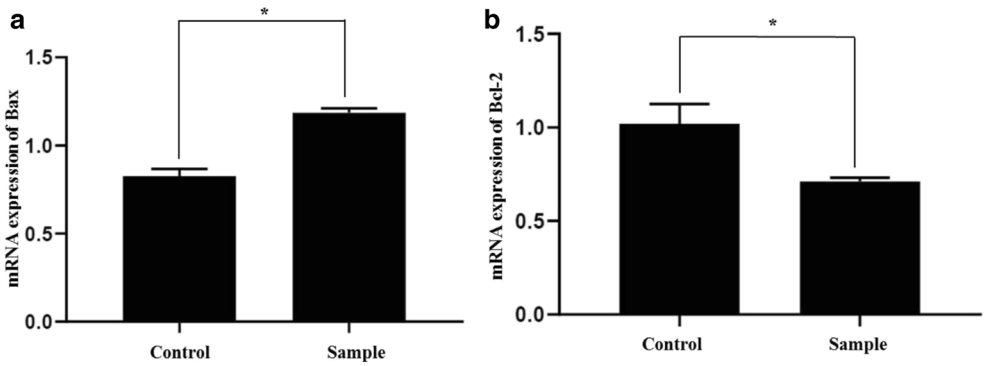


Fig. 3 (**a**), (**b**): In treated LNCaP cells with **10b** compound (as sample) and no treated cells (as control), relative expression of Bax mRNA and relative expression of Bcl-2 mRNA were shown. Data represent mean ± SD of three independent experiments. $p < 0.05$ was considered to be statistically significant

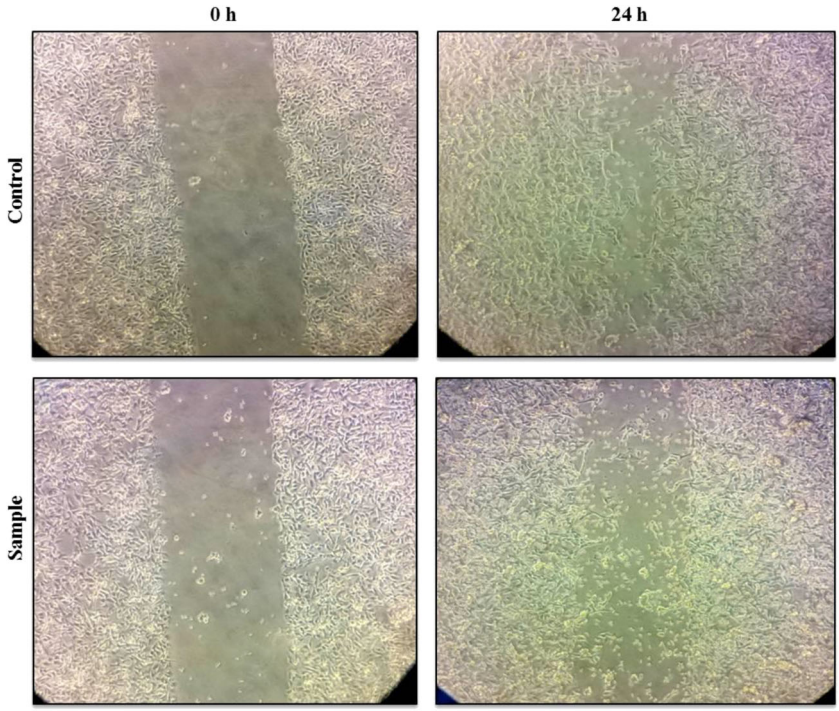


Fig. 4 Effects of compounds **10b** on the migration of LNCaP cells in different time (0 and 24 h after treatment; concentration of **10b** compound was 29.23 μM). Images were obtained using phase-contrast microscopy. Scale bars represent 50 μm

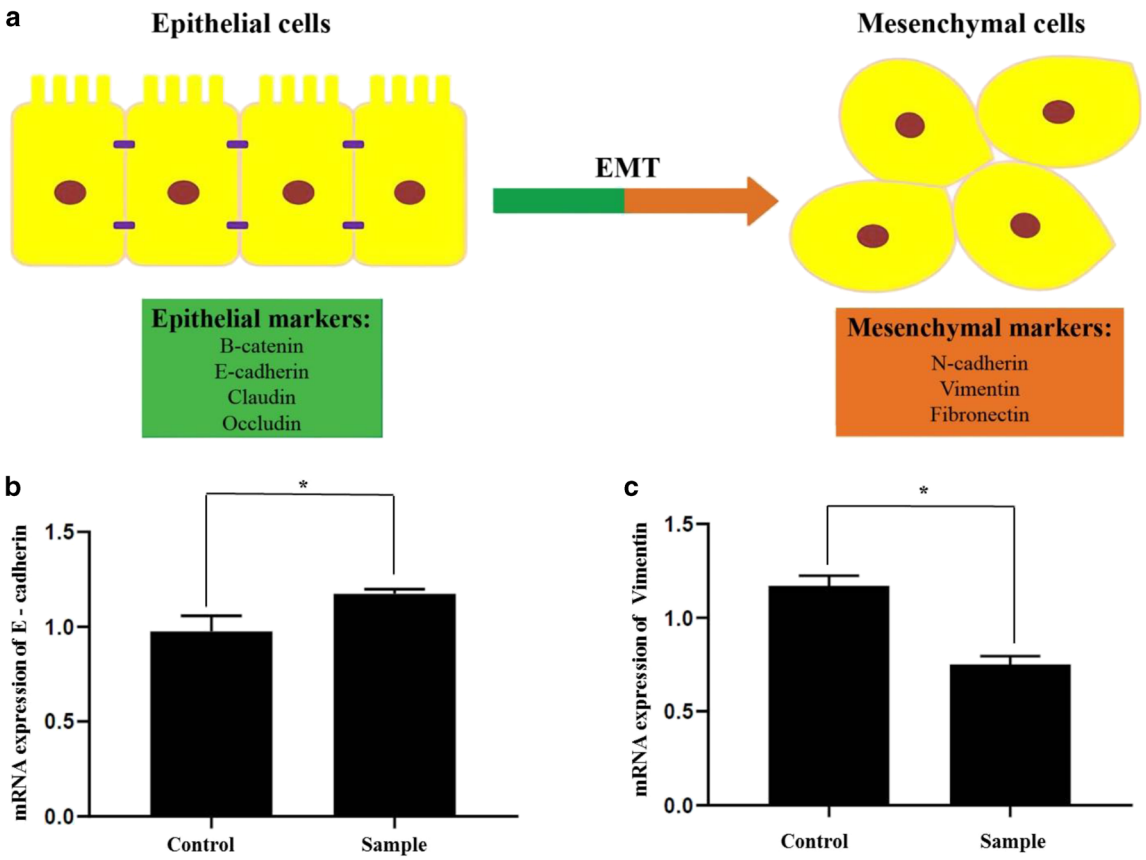


Fig. 5 (**a**)**:** Schematic model of epithelial-mesenchymal transition (EMT). (**b**, **c**): Relative expression of E-cadherin and Vimentin mRNAs as two potential markers of EMT in treated LNCaP cells with **10b** compound (compared to control) were shown. Data represent mean ± SD of three independent experiments. $p < 0.05$ was considered to be statistically significant

migration are outcomes of EGFR activity that are tightly regulated by MAPKs. Thus, the exploring of the possible effects of **10b** on related tyrosine kinases in MAPK cascade was suggested. Also, in vivo experiments will be required to find the side effects of **10b**.

Conclusion

In conclusion, in vitro cytotoxic activity of some new synthesized triazol-linked spiroindolinequinazolinone **6**, thiazol-oxindole **7** and oxindole-thiosemicarbazone conjugates **9** were evaluated toward different cancer lines. The synthesized compounds **6**, **7**, and **9** showed IC_{50} values higher than IC_{50} value of Etoposide as positive control. Among three synthesized triazol-linked oxindol-thiosemicarbazone **10a-c**, compound **10b** displayed promising antitumor activity against all tested cancer cell lines (IC_{50} = 15.32–29.23 μ) compared to the Etoposide. It showed the importance of bromophenyl group substitution in triazol/indolin-3-thiosemicarbazone series **10** in inducing more cytotoxic effects on cancer cells. The results revealed that the compound **10b** induces apoptosis and has EMT inhibition activity. Therefore, the compound **10b** as an indolinone and thiosemicarbazone conjugate molecule could be a novel active agent with multiple activities to design anticancer therapeutic strategies. Our current study reports that conjugation of indolinone, thiosemicarbazone and triazole moieties can be considered as a new therapeutic agent against different cancer cells.

Experimental

General information

Melting points were determined on a melting point apparatus and are uncorrected. IR spectra were taken with a Bomem FT-IR MB spectrometer. The NMR spectra were recorded on a BRUKERDRX-300AVANCE spectrometer. Mass spectra were recorded on an Agilent 5975C VL MSD with Tripe-Axis Detector operating at an ionization potential of 70 eV. Elemental analyses were performed using a Heraeus CHN–O–Rapid analyzer. All chemicals were purchased from Merck or Aldrich and were used without further purification.

Synthesis of N-propargyl isatins 2

A mixture of isatins (1 mmol), propargyl bromide (1 mmol) and Na_2CO_3 (2 mmol) in DMF (3 mL) was stirred at room temperature for 3 h. Then, water (5 mL) was added and stirred for 15 min. Then, the solid residue was filtered and crystallized with EtOH to afford the pure product.

Synthesis of spiro[indoline-3,2′-quinazoline]-diones 4

A mixture of N-propargyl isatins **2** (1 mmol),2-aminobenzamide **3** (1 mmol) and *P*-TSA (10 mol%) in EtOH (5 mL) was stirred at reflux conditions for 10 h. Then, water (15 mL) was added and stirred for 15 min. The solid residue was filtered and washed with water. Then, the solid was crystallized with EtOH to afford the pure product.

Synthesis of compound 6

A mixture of spiro[indoline-3,2′-quinazoline]-diones **4** (1 mmol), azide **5** (1 mmol), $CuSO_4.5H_2O$ (15 mol%) and sodium ascorbate (30 mol%) in t-BOH/H_2O (2 mL, 1:1) was sonicated at room temperature for 1 h. Then, water (5 mL) was added and stirred for 15 min. The solid residue was filtered and washed with water. Then, the solid was crystallized with DMF/H_2O to afford the pure product.

Synthesis 1H-1,2,3-triazol-indoline-diones 7

A mixture of N-propargyl isatins **2** (1 mmol), azide **5** (1 mmol), $CuSO_4.5H_2O$ (15 mol%) and sodium ascorbate (30 mol%) in t-BOH/H_2O (2 mL, 1:1) was sonicated at room temperature for 1 h. Then, water (3 mL) and EtOAc (3 mL) was added and stirred for 15 min. The aqueous layer was extracted with EtOAc for three times. The combined organic layers were washed with brine, dried over MgSO4 and evaporated to give the pure product.

Synthesis of triazol/thiazol-indolinones 9

A mixture of **7** (1 mmol), thiosemicarbazid (1 mmol) and phenacyl bromides (1 mmol) in iso-PrOH (2 mL) was sonicated at 65 °C for 1 h. Then, water (5 mL) was added and stirred for 15 min. The solid residue was filtered and washed with water and EtOH to afford the pure product.

Synthesis of triazol/indolin-thiosemicarbazones 10

A mixture of **7** (1 mmol) and thiosemicarbazid (1 mmol) in iso-PrOH (2 mL) was sonicated at 65 °C for 1 h. Then, water (5 mL) was added and stirred for 15 min. The solid residue was filtered and washed with water and EtOH to afford the pure product.

Cell lines and cell culture

Human malignant melanoma cells (A375), human prostate cancer cells (PC3 cells, LNCaP cells), human breast cancer cells (MDA-MB-231) and normal cells HDF (human dermal fibroblast) were received from Pasture Institute, Tehran, Iran. A375, PC3, LNCaP and HDF cell lines were grown in

DMEM medium and MDA-MB-231 cell line was grown in RPMI 1640 medium. All media contains 10% fetal bovine serum (FBS; Bioidea BI 201, Iran), penicillin G, streptomycin 100 μg/mL and 1% L-Glutamine. The cells were cultured and incubated under humidified 5% CO_2 atmosphere at 37 °C.

MTT assay

The effect of compounds treatment on the viability of cancer cell lines was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT assay (MTT assay kit, Bio IDEA, CatNo:BI1017, Iran) based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorb at 490 nm as per the manufacturer's instructions [41]. Etoposide (was kindly provided by Dr. A. Foroumadi, Tehran medical Science University, Iran) and DMSO was used as positive and negative controls, respectively. Briefly, cells were plated in 96-well culture plates (5×10^3 cells/well). After 24 h incubation, the cells were treated with different concentrations of the compounds. After 48 h at 37 °C, the medium was removed and 100 μL of MTT reagent (1 mg/mL) was added to each well, and cells were further incubated at 37 °C for 4 h. The MTT solution was removed, 50 μL of DMSO was added to each well to dissolve formazan crystals, and the plates were gently shaken for 10 min, followed by reading with an ELISA plate reader (BiotekELx 800, USA). The 50% inhibition concentration (IC_{50}) was defined as the concentration that inhibited cell proliferation by 50% when compared to DMSO treated cells (as negative control).

DAPI staining assay

DAPI staining assay was used to determine chromatin changes. Related cancer cell lines were seeded in six well plates (5×10^4 cells/well) containing 12 mm cover-slips and subsequently treated for **10b** compound (Sample or treated cells) and DMSO (Control or untreated cells) for 24 h. Cells then were fixed with 3.7% paraformaldehyde, permeabilized in 0.5% (*w*/*v*) Triton X-100, 1% BSA (w/v) for 5 min, washed in PBS, and stained with DAPI (Sigma-Aldrich, USA). All images were taken by an inverted fluorescent microscope (Nikon Eclipse Ti-E).

Wound-healing migration assay

The LNCaP cells were seeded in culture medium onto 6-well plates at a density of 4×10^5 cells per well. The confluent monolayer of cells was scratched with a fine pipette tip, and cell migration into the wound was visualized and scored by measuring the size of the initial wound and comparing it to the size of the wound after 24 h by microscopy.

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

For quantitative real-time RT-PCR analysis, after 48 h of treatment with 29.23 μM of related drug (**10b**), LNCaP cells was lysed and the total RNA was extracted using 500 μL of Trizol® reagent according to the protocol provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA) followed by reverse transcription into cDNA according to manufactures protocol (ReveretAid M-Mulv reverse transcriptase kit, Thermo Fisher Scientific, MA, USA). Real-time RT-PCR was then performed to amplify cDNA using SYBR Green dye universal master mix (Bioron GmbH, Germany), on a Light Cycler 480 (Roche) using the primers for GAPDH-F: 5′-CAA GGT CAT CCA TGA CAA CTTTG-3′, R:5′-GTCCACCACCCTGTTGCTGTAG-3′; Bax-F:5′-GTCG CCCTTTTCTACTTTGCC-3′, R: 5′-CTCC CGCCACAAAGATGGTCA-3′and Bcl-2-F: 5′-CCCC TCGTCCAAGAATGCAA-3′, R: 5′- TCTCCCGG TTATCGTACCCTG-3′ for 40 cycles. Data represent averaged copy number normalized to the GAPDH housekeeping gene. Primer synthesis was done by Pishgam Biotech Co. Tehran, Iran. The negative control reaction was set as a reaction similar to the above but with deionized water instead of cDNA. Thermal conditions of the PCR consisted of primary denaturation at 94 °C for 2 min, 45 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, amplification at 72 °C for 30 s. Primers were used for E-cadherin–F: 5′-GCCG AGAGCTACACGTTCAC-3′, R: 5′- CAGGCGTA GACCAAGAAATG-3′and for vimentin-F: 5′-CTAC GTCCACCCGCACCTAC-3′; R: 5′- CCAGCGAG AAGTCCACCGAG-3′ with the following conditions: 95 °C for 90 s; followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s (E-cadhein) and 57 °C for 30 s (vimentin), and 72 °C for 30 s. All reactions were triplicated.

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Authors' contributions SN and MBM synthesis of the title compounds. FS supervision of the pharmacological part and collaboration in manuscript preparations. AB supervision of the chemistry part, designing of title compounds and manuscript preparation.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Shirinyan VZ, Markosyan AI, Baryshnikova MA, Yaminova LV, L'vov AG, Gabrielyan SA. Synthesis and antiproliferative activity

evaluation of aryl(Hetaryl)cyclopentenone analogs of combretastatin A-4. Pharm Chem J. 2018;51:867–72.
2. Avendano C, Menendez JC. Medicinal chemistry of anticancer drugs. 1st ed. Chapter 1. Amsterdam: Elsevier; 2008.
3. Kathawala RJ, Gupta P, Ashby CR Jr, Chen ZS Jr. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist Update. 2015;18:1–7.
4. El-Naggar M, Eldehna WM, Almahli EA, Fares M, Elaasser MM, Abdel-Aziz HA. Novel thiazolidinone/thiazolo[3,2-a]benzimidazolone-isatin conjugates as apoptotic anti-proliferative agents towards breast cancer: one-pot synthesis and in vitro biological evaluation. Molecules. 2018;23:1420.
5. Sridhar SK, Ramesh A. Synthesis and pharmacological activities of hydrazones, Schiff and Mannich bases of isatin derivatives. Biol Pharm Bull. 2001;24:1149–52.
6. Al-Wabli RI, Zakaria AS, Attia MI. Synthesis, spectroscopic characterization and antimicrobial potential of certain new isatin-indole molecular hybrids. Molecules. 2017;22:1958.
7. Jiang T, Kuhen KL, Wolff K, Yin H, Bieza K, Caldwell J, et al. Design, synthesis, and biological evaluations of novel oxindoles as HIV-1 non-nucleoside reverse transcriptase inhibitors. Part 2. Bioorg Med Chem Lett. 2006;16:2109–12.
8. Igosheva N, Lorz C, O'Connor E, Glover V, Mehmet H. Isatin, an endogenous monoamine oxidase inhibitor, triggers a dose- and time-dependent switch from apoptosis to necrosis in human neuroblastoma cells. Neurochem Int. 2005;47:216–24.
9. Vine KL, Matesic L, Locke JM, Skropeta D. Recent highlights in the development of isatin-based anticancer agents. Adv Anticancer Agents Med Chem. 2013;2:254–312.
10. Al-Rashood ST, Hamed AR, Hassan GS, Alkahtani HM, Almehizia AA, Alharbi A, et al. Antitumor properties of certain spirooxindoles towards hepatocellular carcinoma endowed with antioxidant activity. J Enz Inhibit Med Chem. 2020;35:831–9.
11. Goodman VL, Rock EP, Dagher R, Ramchandani RP, Abraham S, Gobburu JVS, et al. Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. Clin Cancer Res. 2007;13:1367–73.
12. Dhillon S. Nintedanib: a review of its use as second-line treatment in adults with advanced non-small cell lung cancer of adenocarcinoma histology. Target Oncol. 2015;10:303–10.
13. Rossi A, Latiano TP, Parente P, Chiarazzo C, Limosani F, Di Maggio G, et al. The potential role of nintedanib in treating colorectal cancer. Expert Opin Pharmacother. 2017;18:1153–62.
14. Quintela-Fandino M, Urruticoechea A, Guerra J, Gil M, Gonzalez-Martin A, Marquez R, et al. Phase I clinical trial of nintedanib plus paclitaxel in early HER-2- negative breast cancer (CNIO-BR-01-2010/GEICAM-2010-10 study). Br J Cancer. 2014;111:1060–4.
15. Raj R, Gut J, Rosenthal PJ, Kumar V. 1H-1,2,3-Triazole-tethered isatin-7-chloroquinoline and 3-hydroxy-indole-7-chloroquinoline conjugates: synthesis and antimalarial evaluation. Bioorg Med Chem Lett. 2014;24:756–9.
16. Contelles JM, Soriano E. The medicinal chemistry of hybrid-based drugs targeting multiple sites of action. Curr Top Med Chem. 2011;11:2714–5.
17. Eldehna WM, Al-Wabli RI, Almutairi MS, Keeton AB, Piazza GA, Abdel-Aziz HA, et al. Synthesis and biological evaluation of certain hydrazonoindolin-2-one derivatives as new potent antiproliferative agents. J Enz Inhib Med Chem. 2018;33:867–8.
18. Raja Solomona V, Hu C, Lee H. Hybrid pharmacophore design and synthesis of isatin–benzothiazole analogs for their anti-breast cancer activity. Bioorg Med Chem Lett. 2010;17:1563–72.
19. Ramshid PK, Jagadeeshan S, Krishnan A, Mathew M, Nair SA, Pillai MR. Synthesis and in vitro evaluation of some isatin-thiazolidinone hybrid analogues as anti-proliferative agents. Med Chem. 2010;6:306–12.
20. Taher AT, Khalil NA, Ahmed EM. Synthesis of novel isatin-thiazoline and isatin-benzimidazole conjugates as anti-breast cancer agents. Arch Pharm Res. 2011;34:1615–21.
21. Rambabu D, Raja G, Sreenivas BY, Seerap GPK, Lalith Kumar K, Singh Deora G, et al. Spiro heterocycles as potential inhibitors of SIRT1: Pd/C-mediated synthesis of novel N-indolylmethyl spiroindoline-3, 2′-quinazolines. Bioorg Med Chem Lett. 2013;23:1351–7.
22. Kolb HC, Sharpless KB. The growing impact of click chemistry on drug discovery. Drug Discov Today. 2003;8:1128–37.
23. Hu W-X, Zhou W, Xia C-N, Wen X. Synthesis and anticancer activity of thiosemicarbazones. Bioorg Med Chem Lett. 2006;16:2213–8.
24. Singh P, Sharma P, Anand A, Bedi PM, Kaur T, Saxena AK, et al. Azide-alkyne cycloaddition en route to novel 1H-1,2,3-triazole tethered isatin conjugates with in vitro cytotoxic evaluation. Eur J Med Chem. 2012;55:455–61.
25. Kumar S, Saha ST, Gu L, Palma G, Perumal S, Singh-Pillay A, et al. 1H-1,2,3-triazole tethered nitroimidazole-isatin conjugates: synthesis, docking, and anti-proliferative evaluation against breast Cancer. ACS Omega. 2018;3:12106–13.
26. Senwar KR, Sharma P, Reddy TS, Jeengar MK, Nayak VL, Naidu VG, et al. Spirooxindole-derived morpholine-fused-1,2,3-triazoles: design, synthesis, cytotoxicity and apoptosis inducing studies. Eur J Med Chem. 2015;102:413–24.
27. Nagarsenkar A, Prajapti SK, Guggilapu SD, Birineni S, Kotapalli SS, Ummanni R, et al. Investigation of triazole-linked indole and oxindole glycoconjugates as potential anticancer agents: novel Akt/PKB signaling pathway inhibitors. Med Chem Commun. 2016;7:646–53.
28. Hall MD, Salam NK, Hellawell JL, Fales HM, Kensler CB, Ludwig JA, et al. Synthesis, activity, and pharmacophore development for isatin-β-thiosemicarbazones with selective activity toward multidrug-resistant cells. J Med Chem. 2009;52:3191–204.
29. de Oliveira JF, Lima TS, Vendramini-Costa DB, de Lacerda Pedrosa SCB, Lafayette EA, da Silva RMF, et al. Thiosemicarbazones and 4-thiazolidinones indole-based derivatives: synthesis, evaluation of antiproliferative activity, cell death mechanisms and topoisomerase inhibition assay. Eur J Med Chem. 2017;136:305–14.
30. Chen G, Meng M, Zhang Y, Hao X, Wang Y, Mu S. Synthesis, cytoprotective and anti-tumor activities of isatin Schiff bases. Lett Drug Des Discov. 2015;12:802–5.
31. Ahadi S, Khavasi HR, Bazgir A. Highly efficient construction of bisspirooxindoles containing vicinal spirocenters through an organocatalytic modified Feist-Bénary reaction. Chem Eur J. 2013;19:12553–9.
32. Imani Shakibaei G, Bazgir AA. Highly efficient one-pot synthesis of indenopyridine-fused spirocyclic systems. RSC Adv. 2016;6:22306–11.
33. Ghahremanzadeh R, Fereshtehnejad F, Mirzaei P, Bazgir A. Ultrasound-assisted synthesis of 2,2′-(2-oxoindoline-3,3-diyl)bis(1H-indene-1,3(2H)-dione) derivatives. Ultrason Sonochem. 2011;18:415–8.
34. Sarangi PKN, Sahoo J, Paidesetty SK, Mohanta GP. Thiazoles as potent anticancer agents: a review. Indian Drugs. 2016;53:5–11.
35. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2:442–54.
36. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 2009;28:15–33.
37. Fong TA, Shawver LK, Sun L, Tang C, App H, Powell TJ, et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res. 1999;59:99–106.

38. Zhang C, Xu D, Wang J, Kang C. Efficient synthesis and biological activity of novel Indole derivatives as VEGFR-2 tyrosine kinase inhibitors. Russ J Gen Chem. 2017;87:3006–16.
39. Moore EC, Zedeck MS, Agrawal KC, Sartorelli AC. Inhibition of ribonucleoside diphosphate reductase by 1-formylisoquinoline thiosemicarbazone and related compounds. Biochemistry. 1970;9:4492–8.
40. Beraldo H, Gambino D. The wide pharmacological versatility of semicarbazones, thiosemicarbazones and their metal complexes. Mini-Rev Med Chem. 2004;4:31–9.
41. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 1986;89:271–7.