Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Antiproliferative activity and possible mechanism of action of certain 5-methoxyindole tethered C-5 functionalized isatins

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

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Background: Cancer is one of the most dreaded human diseases, that has become an ever-increasing health problem and is a prime cause of death globally. The potential antiproliferative activity of certain indole–isatin molecular hybrids **5a-w** was evaluated in vitro against three human cancer cell lines.

Methods: Standard protocols were adopted to examine the antiproliferative potential and mechanisms of compounds **5a-w**. Western blot analysis was carried out on compound **5o**.

Results: Compounds **5a-w** demonstrated in vitro antiproliferative activity in the range of 22.6–97.8%, with compounds **5o** and **5w** being the most active antiproliferative compounds with IC_{50} values of 1.69 and 1.91 μM, which is fivefold and fourfold more potent than sunitinib (IC_{50}=8.11 μM), respectively. Compound **5o** was selected for in-depth pharmacological testing to understand its possible mechanism of antiproliferative activity. It caused a lengthening of the G1 phase and a reduction in the S and G2/M phases of the cell cycle and had an IC_{50} value of 10.4 μM with the resistant NCI-H69AR cancer cell line. Moreover, compound **5o** significantly decreased the amount of phosphorylated Rb protein in a dose-dependent fashion, which was confirmed via Western blot analysis.

Conclusion: The current investigation highlighted the potential antiproliferative activity of compounds **5a-w** as well as the antiproliferative profile of compound **5o**. These compounds can be harnessed as new lead antiproliferatives in the preclinical studies of cancer chemotherapy.

Keywords: isatin, indole, synthesis, antiproliferative, apoptosis

Introduction

Cancer is one of the most terrifying diseases of humanity and has become a fundamental health problem and a principal cause of death globally. One in four deaths in the United States is a result of cancer.[1] More than ten million new cases of cancer occur every year, approximately half of which occur in developed countries, with the disease causing more than six million deaths every year.[2–4] A molecularly targeted approach has recently been utilized for the management of disseminated cancer which depends on the study of oncogenes and tumor suppressors which are involved in the emergence of human cancers.[5] Consequently, there has been an advancement in the specificity of cancer management, progressing from the use of general cytotoxic agents such as nitrogen mustard in the 1940s, the development of chemotherapeutic agents such as anthracyclines and *Vinca* alkaloids from natural resources in the 1960s and finally the use of specific monoclonal antibodies[6] and

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http://doi.org/10.2147/DDDT.S208241

specific chemotherapeutic agents which inhibit protein tyrosine kinases (PTKs) as advanced approaches.[7–9] These targeted chemotherapeutic agents usually attenuate signaling pathways which control the cancer cell cycle and alter its microenvironment, blocking tumor cell proliferation, cell apoptosis and/or hindering tumor mass growth.[10] These developments led to a reduction of anticancer side effects and ameliorated the response rate. Therefore, the study of the mechanisms by which cancers resist chemotherapeutic agents gave rise to a deep understanding of the reasons for the failure of cancer therapies.

Indole (**I**, Figure 1) is a privileged bicyclic structure which was first synthesized in 1866. The indole scaffold is incorporated into a large number of biologically active molecules endowed with a wide range of bioactivities and is naturally occurring in *Vinca* and ergot alkaloids, fungal metabolites and marines.[11] In recent years, indole and its functionalized derivatives have been embedded in myriad bioactive pharmaceuticals including anti-inflammatories, analgesics, antimicrobials and antitumors.[12–18] Furthermore, 5-methoxyindole is the fundamental fragment in the natural hormone melatonin (MLT, **II**, Figure 1). MLT and its derivatives have a broad spectrum of

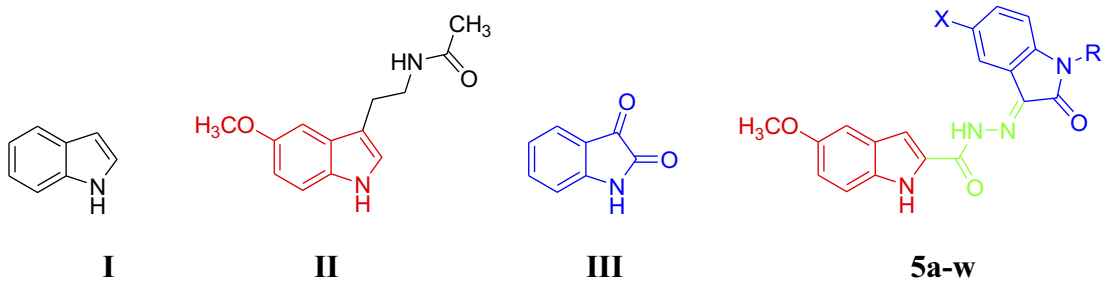


Figure 1 Chemical structures of compounds **I-III** and **5a-w.**

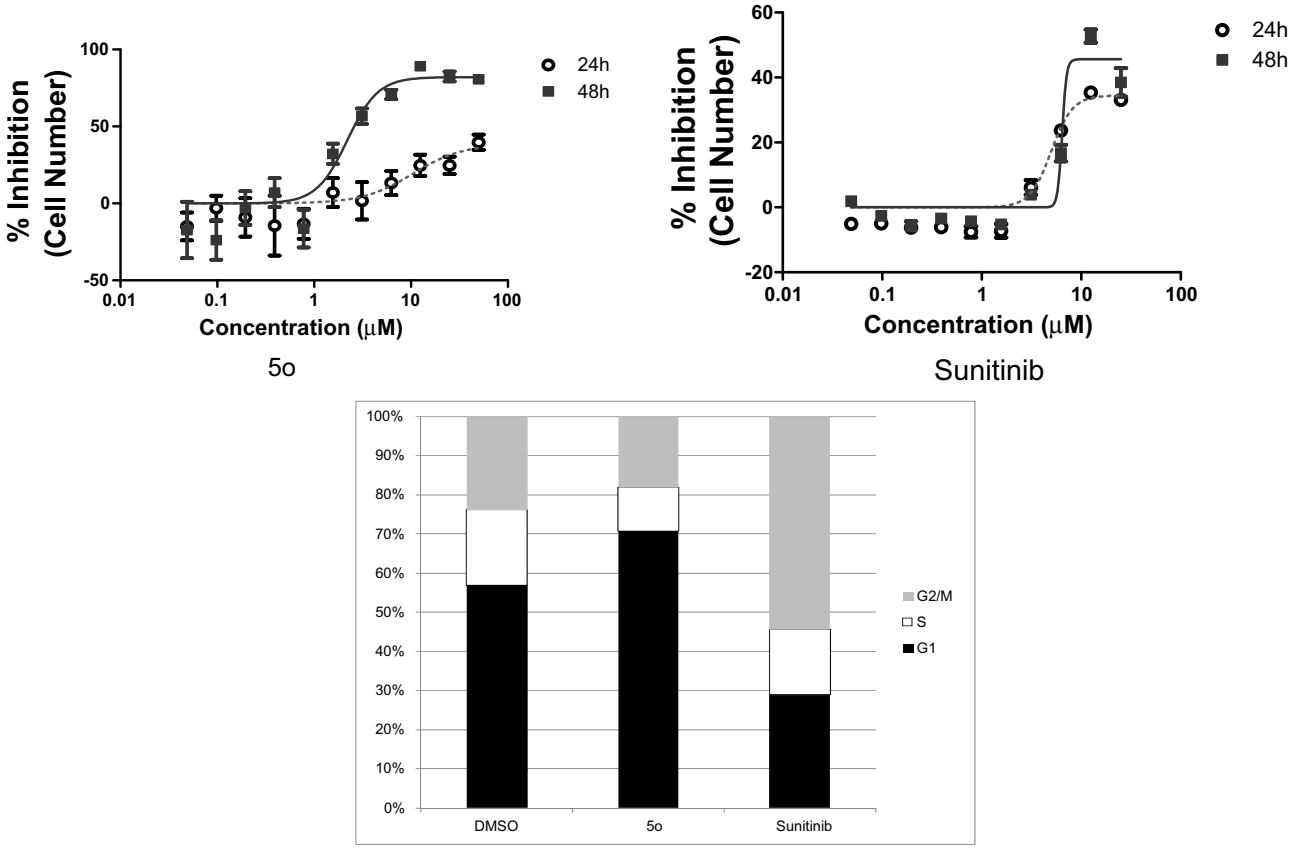


Figure 2 Cell cycle influences of compound **5o** after incubation for 24 and 48 hrs.

pharmaceutical applications, particularly for the treatment of headache, depression and sleep disorders, and for the management of certain types of cancer.[19–21]

On the other hand, isatin (2,3-dioxindole, **III**) is considered an oxidized form of indole and has been recognized to be an endogenous multifunctional molecule in human beings and other mammals.[22] The special electronic properties of isatin along with its proper molecular size give rise to several different valuable biological characteristics. Therefore, isatin was embedded into the backbone of various bioactive molecules including anticonvulsants,[23] antifungals,[24] antibacterials,[25] anti-HIV agents[24,26] and anticancer agents.[27–31]

According to the aforementioned premises, it was our interest to prepare the indole–isatin conjugates **5a-w** as hybrid molecules tailored from indole and isatin pharmacophore fragments for biological evaluation. The isatin moiety of the target conjugates **5a-w** is functionalized on their C-5 position and bears various *N*-aralkyl substitutions that alter the electronic and lipophilic environment, allowing us to explore their impact on the biological activity of compounds **5a-w**. Compounds **5a-w**displayed moderate antimicrobial potential.[32,33] The current report deals with the assessment of their in vitro antiproliferative potential. The most active antiproliferative candidates were subjected to deep pharmacological testing to gain insight into the possible mechanism of their antiproliferative activity.

Materials and methods

Chemistry

5-Methoxy-1*H*-indole-2-carbohydrazide (**3**) – The acid hydrazide **3** was prepared from the corresponding ester **2**[34] using the documented method.[32] It has a melting point (m.p.) of 266–268°C.

General method for the preparation of 5-methoxy-1*H*-indole-2-carbo hydrazide derivatives 5a-w

Glacial acetic acid (catalytic amount) was added to a mixture of the proper isatin derivative **4a-n** (1 mmol) and the acid hydrazide **3** (1 mmol) in absolute ethyl alcohol (15 mL). The reaction mixture was then stirred under reflux for 4 hrs. The precipitated solid was filtered while hot, and the obtained solid was recrystallized from an ethyl alcohol/dimethylformamide mixture (3:1) to furnish the corresponding compounds **5a-w** in 43–94% yields. The analytical data of compounds **5a-w** are previously documented.[32,33]

Pharmacological evaluation

Pharmacological assessment of the title compounds including antiproliferative activity, selectivity, cell cycle effects and quantitative immunofluorescence of **5a-w** was performed with previously documented methods.[29] Western blot analysis of total cellular proteins enabled detection of P-Rb and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using antibodies obtained from Cell Signaling Technology (Boston, MA, USA). Western blots were imaged by direct imaging of chemiluminescent blots (ChemiDoc Imaging System; BioRad, Hercules, CA, USA). Quantitation was carried out using NIH ImageJ public domain image analysis software. The cell lines were purchased commercially from the American Type Culture Collection (ATCC).

Results and discussion

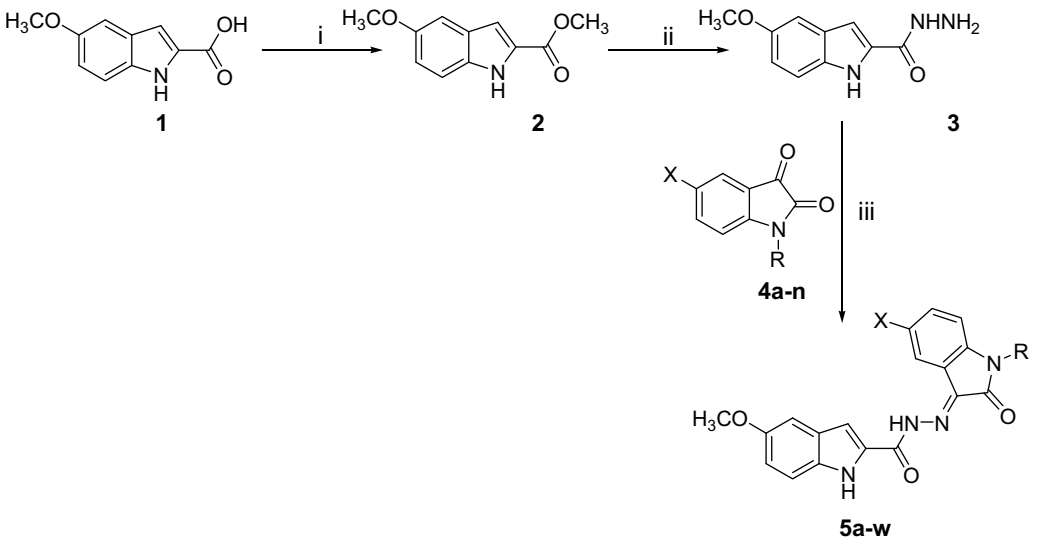
Chemistry

Compounds **5a-w** were prepared as illustrated in Scheme 1. Thus, the commercially available 5-methoxy indole-2-carboxylic acid (**1**) was esterified in absolute methanol and a catalytic amount of concentrated sulfuric acid, followed by hydrazinolysis, to prepare the hydrazide **3**. Subsequently, compound **3** was reacted with the isatin derivatives **4a-n**[33] to achieve the respective title compounds **5a-w** in moderate yields.

Pharmacological investigations

Antiproliferative activity

The isatin nucleus is incorporated into various anticancer candidates.[28,29,31,35,36] The preliminary antiproliferative potential of compounds **5a-w** was tested using A-549 (lung), HT-29 (colon) and ZR-75 (breast) human cancer cell lines, and the obtained data are presented in Table 1. Sunitinib was used against the same human cancer cell lines as a reference drug for the experiments. The results are expressed as an average percent growth inhibition at 30 μM concentration for each compound tested in quadruplicate. The title compounds **5a-w** exhibited an average growth inhibition of 22.6–97.8% in the antiproliferative assay against the tested human cancer cell lines, except for compound **5e**, which stimulated the growth of the ZR-75 cell line. It seems that the *N*-unsubstituted isatin (compounds **5b** and **5c**), *N*-methyl (compounds **5g-i**), *N*-benzyl (compound **5o**) or *N*-phenyl (compound **5w**) moieties are



Scheme 1 Synthesis of compounds **5a-w**. Reagents and conditions: (**I**) absolute methanol, H_2SO_4 (few drops), reflux, 4 hrs; (ii) absolute methanol, H_2N-$NH_2.H_2O$, reflux, 2 hrs; (iii) absolute ethanol, acetic acid (few drops), reflux, 4 hrs.

Compound No.	X	R	Compound No.	X	R
5a	H	H	**5m**	Cl	C_6H_5-CH_2
5b	Br	H	**5n**	F	C_6H_5-CH_2
5c	Cl	H	**5o**	OCH_3	C_6H_5-CH_2
5d	F	H	**5p**	H	4-F-C_6H_4-CH_2
5e	OCH_3	H	**5q**	Br	4-F-C_6H_4-CH_2
5f	H	CH_3	**5r**	Cl	4-F-C_6H_4-CH_2
5g	Br	CH_3	**5s**	F	4-F-C_6H_4-CH_2
5h	Cl	CH_3	**5t**	H	4-Cl-C_6H_4-CH_2
5i	F	CH_3	**5u**	H	4-CN-C_6H_4-CH_2
5j	OCH_3	CH_3	**5v**	H	4-CH_3-C_6H_4-CH_2
5k	H	C_6H_5-CH_2	**5w**	H	C_6H_5
5l	Br	C_6H_5-CH_2			

the preferred fragments at the isatin nitrogen, as they induced average growth inhibition of 96.0, 91.3, 94.5, 95.3, 91.8, 97.8 and 97.6%, respectively. Also, halogen substitution at isatin C-5 is the favored substituent, except for compounds **5o** and **5w** which bear methoxy and hydrogen functionalities, respectively.

Compounds displaying an average growth inhibition of more than 90% toward ZR-75, HT-29, and A-549 cell lines were subjected to median growth inhibitory concentration (IC_{50}) determination. Table 2 illustrates the IC_{50} values of compounds **5b, 5c, 5g-i, 5o, 5w** and sunitinib toward ZR-75, HT-29 and A-549 cell lines. The most active candidates are **5o** (bearing an *N*-benzylisatin moiety) and **5w** (bearing an *N*-phenylisatin moiety) with IC_{50} values of 1.69 and 1.91 μM, respectively, which are about fivefold and fourfold more potent than sunitinib (IC_{50}=8.11 μM). Therefore, detailed pharmacological studies were carried on compound **5o**, aiming to gain insight into the integrated pharmacological profile of this compound, as a representative for compounds **5a-w**.

Caspase 3/7 activity

The A-549 cell line was utilized to assess the apoptosis-inducing potential of compound **5o**. Activity assessment of compound **5o** was carried out at concentrations equal to its IC_{50} for growth inhibition and at threefold above this concentration over a 2–48 hr time course. Compound **5o**

Table 1 In vitro antiproliferative potential of compounds **5a-w** and sunitinib against HT-29, ZR-75 and A-549 cell lines

Compound No.	HT-29	ZR-75	A-549	Average growth inhibition %
5a	10.2±6.3	43.2±19.3	14.4±5.2	22.6
5b	96.2±4.8	96.6±1.4	95.2±6.2	96.0
5c	89.8±2.4	88.8±7.0	95.2±3.3	91.3
5d	52.5±9.4	77.7±7.5	62.5±13.4	64.2
5e	−7.3±30.1	−37.4±8.9	22.2±8.4	−7.5
5f	82.5±11.9	63.8±7.4	89.2±6.4	78.5
5g	93.5±2.5	94.4±3.9	95.5±2.4	94.5
5h	92.3±2.1	96.1±2.1	97.5±1.0	95.3
5i	92.9±0.9	86.1±6.1	96.5±3.8	91.8
5j	32.2±19.9	54.6±22.3	96.3±3.4	61.0
5k	77.4±4.4	46.0±20.1	80.9±5.3	68.1
5l	70.2±3.8	53.8±16.5	68.5±4.1	64.2
5m	69.9±6.0	75.5±5.8	70.1±4.0	71.8
5n	65.9±3.0	73.7±3.7	58.0±7.6	65.9
5o	97.0±2.8	96.5±2.8	100.0±0.0	97.8
5p	80.8±6.3	63.2±11.9	81.9±2.2	75.3
5q	45.1±5.6	35.9±2.1	55.8±2.8	45.6
5r	40.3±10.2	8.1±15.7	56.1±5.7	34.8
5s	41.6±5.3	52.7±12.0	54.8±4.5	49.7
5t	84.1±7.2	79.2±8.8	100.0±0.0	87.8
5u	79.9±3.9	82.6±12.4	97.6±2.8	86.7
5v	84.4±9.1	54.8±16.4	89.2±4.8	76.1
5w	98.4±1.3	94.8±2.1	99.5±1.1	97.6
Sunitinib	59.5±2.3	90.7±4.5	85.7±2.7	78.7

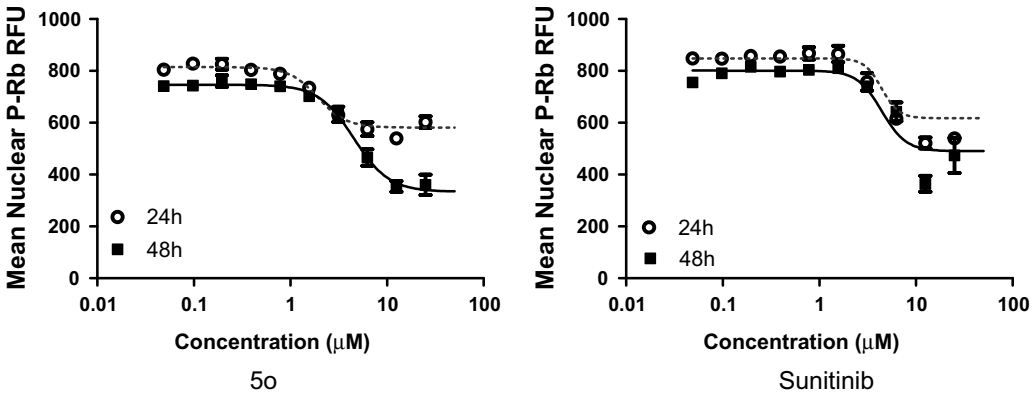
Table 2 Antiproliferative inhibitory concentration 50% (IC_{50}) values of compounds **5b, 5c, 5g-i, 5o, 5w** and sunitinib toward A-549, ZR-75 and HT-29 cell lines

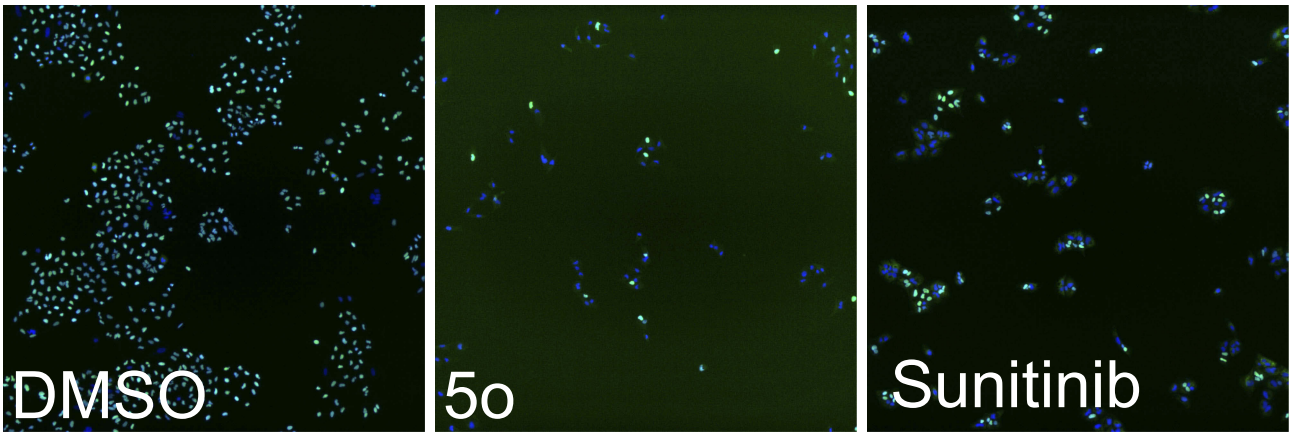
Compound No.	IC_{50} (μM)			Average IC_{50} (μM)
	A-549	ZR-75	HT-29	
5b	23.8	15.0±10.36	>30	>22.9
5c	24.7	16.8±23.36	16.0±16.07	19.2
5g	5.57±0.36	5.29±1.08	5.87±0.93	5.6
5h	6.08±0.86	5.39±1.46	6.29±1.69	5.92
5i	18.60±66.69	21.10	12.80±21.02	17.5
5o	0.54±0.20	1.58±2.97	2.94±0.76	1.69
5w	1.53±0.33	1.93±0.85	2.27±0.26	1.91
Sunitinib	10.14±0.8	8.31±2.4	5.87±0.3	8.11

did not induce any substantial rise in caspase 3/7 activity at any concentration or time point tested.

Cell cycle influences

The A-549 cell line was used to examine the influence of compound **5o** on different features of the cell cycle progression. Activity assessment of compound **5o** was conducted using immunofluorescent imaging of phosphorylated Rb protein as well as by quantification of the total DNA content of each cell to ascertain the phase of the cell cycle. Concentrations of less than 100 μM to 50 nM of compound **5o** were utilized to assess its capability to influence cell cycle distribution as well as Rb phosphorylation. Figure 2 and Table 3 indicated that the total cell number was reduced with an IC50 value of 2.20 μM after a 48 h treatment. Also, the levels of phosphorylated Rb protein were substantially decreased in a dose-dependent manner (Figure 3A).





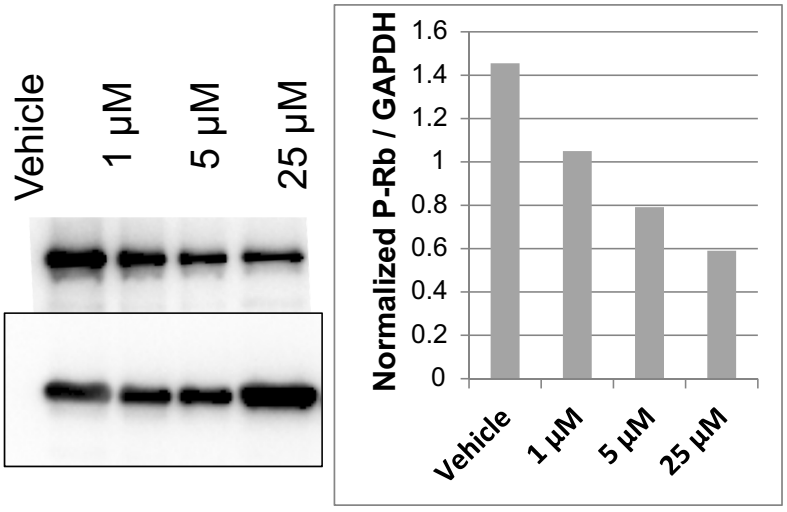


Figure 3 (**A**). Reduction of phosphorylated Rb protein by compound **5o** and sunitinib. Levels of P-Rb in the nuclei were shown by immunofluorescence in cells treated with vehicle, **5o** or sunitinib. Automated image analysis (Molecular Devices) was used to quantitate P-Rb changes and these are presented in the dose–response graphs for each compound after 24 hr or 48 hr treatment. (**B**) Western blot analysis of A-549 NSCLC cells treated with compound **5o** shows the effect of on total cellular levels of P-Rb after 24 hr treatment (left). Densitometric analysis of P-Rb normalized to GAPDH loading control is presented (right).
Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NSCLC, non-small cell lung cancer.

Independent experiments confirmed the effect of **5o** to reduce Rb phosphorylation by Western blot analysis (Figure 3B). Moreover, compound **5o** induced a reduction in the percentage of cells in the S and G2/M phases of the cell cycle, with a concomitant rise in the G1 phase. These results suggest that part of the growth inhibition effect of compound **5o** could be attributed to decreases in the progression rate of the cell cycle, with a concomitant reduction in proliferation. On the

Table 3 Inhibitory concentration 50% (IC_{50}) values for the decreases in the entire cell number and cell cycle influences of compound **5o** and sunitinib

Compound No.	IC_{50} (μM) for the decreases of the entire cell number		IC_{50} (μM) for the decrease of Rb phosphorylation		Cell cycle influences
	24 hrs	48 hrs	24 hrs	48 hrs	
5o	10.47	2.20±1.30	1.92±1.20	3.78±1.10	G1 increased and S, G2/M phases decreased
Sunitinib	12.54±9.82	3.48±0.61	3.18±0.07	6.05±0.61	G1 decreased and S, G2/M phases increased

contrary, sunitinib showed an increase in the percentage of cells in the S or G2/M phases of the cell cycle, with a concomitant reduction in the G1 phase. Mitotic catastrophe followed by programmed death of cells containing aberrant or multiple nuclei may result from mitotic arrest due to arrest in the G2 phase of the cell cycle, which might represent a checkpoint blockade.

It should be mentioned that both compound **5o** and sunitinib substantially reduced the extent of phosphorylated Rb protein in a dose-dependent fashion (Figure 3). Compound **5o** exhibited IC_{50} values of 3.78 and 1.92 μM after 48 and 24 hrs, respectively, which was roughly twofold more potent than sunitinib (Table 3). This may advance the assumption that the growth inhibitory potential of **5o** could be attributed, in part, to its ability to inhibit cyclin-dependent kinases.

Selectivity

Three nontumorigenic cell lines (Table 4) were utilized to examine the growth inhibitory selectivity of compound **5o:** IEC-6 cells which show morphologic and karyotypic characteristics of normal rat intestinal epithelial cells,[37] MCF-10A cells which feature the characteristics of primary cultures of breast tissue with a dome formation[38] and Swiss 3t3 fibroblasts derived from mice embryonic tissue which are both contact inhibited and nontumorigenic.[39] A human non-small cell lung cancer (NSCLCA-549) cell line was used for comparison. Compound **5o** was tested in quadruplicate at a maximum concentration of 25 μM and 10 subsequent serially diluted concentrations.

Figure 4 and Table 4 indicate that compound **5o** was able to inhibit cell growth in both tumor and normal cells. However, it showed threefold selectivity, while sunitinib displayed 1.4-fold selectivity.

Activity against multidrug-resistant cancer cell line

The growth inhibitory potential of compound **5o** was tested against the sensitive lung cancer cell line NSCLC A-549 and the multidrug-resistant lung cancer cell line NCI-H69AR which expresses the ABCC1 efflux pump protein. Compound **5o** was tested in quadruplicate at a maximum concentration of 25 μM and 10 subsequent serially diluted concentrations.

Figure 5 and Table 5 indicate that compound **5o** induced growth inhibition in both lung cancer cell lines, with an IC_{50} value of 0.9 μM in A-549 cells, and being about 12-fold less sensitive toward the NCI-H69AR cell line. This result indicates that compound **5o** might undergo efflux by the ABCC1 efflux pump protein. In contrast, sunitinib was only 1.9-fold less potent toward the NCI-H69AR cell line.

Conclusion

The molecular hybrids **5a-w** were evaluated as new antiproliferative conjugates. Compounds **5o** (bearing an *N*-benzylisatin moiety) and **5w** (bearing an *N*-phenylisatin moiety) were the most active antiproliferative candidates, with IC_{50} values of 1.69 and 1.91 μM, respectively, being about fivefold and fourfold more potent than sunitinib (IC_{50}=8.11 μM).

Table 4 Selectivity of **5o** and sunitinib against nontumorigenic and tumor cell lines

Compound No.	IC_{50} (μM)				Mean tumor selectivity
	Intestine IEC-6	Breast MCF-10A	Fibroblast Swiss 3t3	NSCLCA-549	
5o	3.38±1.21	1.69±1.12	2.78±1.12	0.86±121	3.0
Sunitinib	4.56±0.54	4.43±0.23	4.07±0.75	3.06	1.4

Abbreviations: MCF, Michigan cancer foundation; 3T3, 3-day transfer, inoculum 3×105 cells; IEC, intestinal epithelial cell; IC_{50}, inhibitory concentration 50%; NSCLC, non-small cell lung cancer.

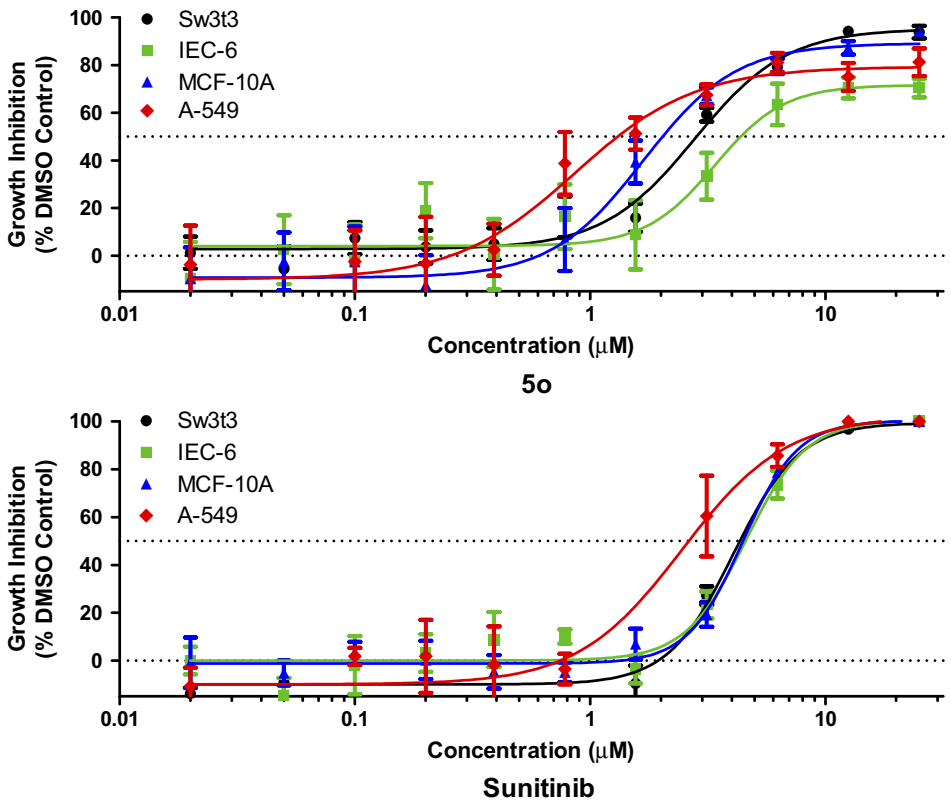


Figure 4 Selectivity characteristics of compound **5o** and sunitinib.

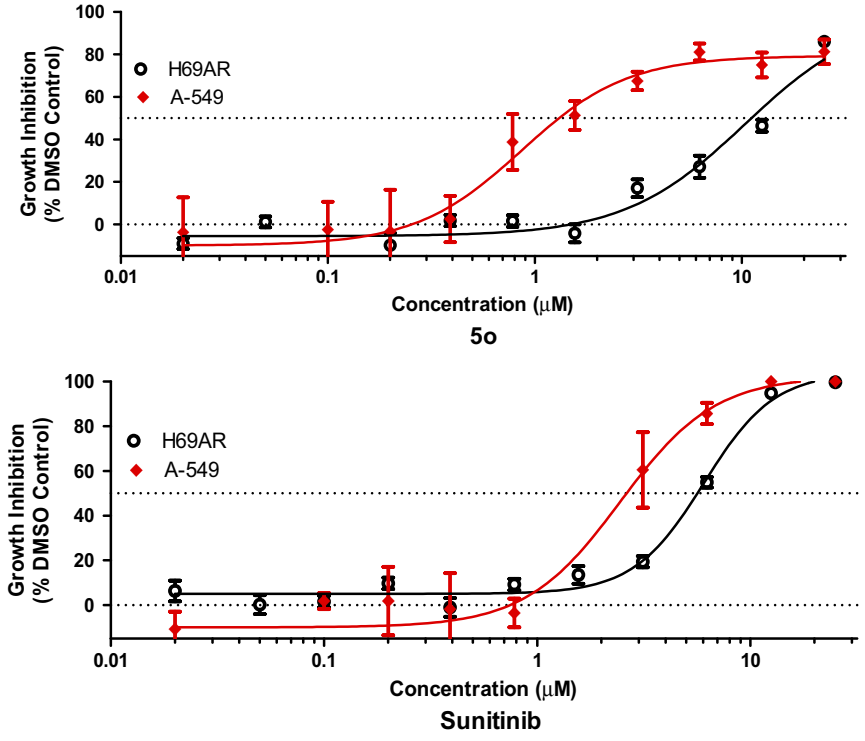


Figure 5 Activity of compound **5o** and sunitinib against A-549 and NCI-H69AR cell lines.

Table 5 Cancer cell growth inhibitory activity of compound **5o** and sunitinib toward sensitive (A-549) and resistant (NCI-H69AR) cell lines

Compound No.	IC_{50} (μM)		Fold resistant
	A-549	NCI-H69AR	
5o	0.86±1.21	10.4±1.82	12.1
Sunitinib	3.06	5.8±0.52	1.9

Abbreviation: IC_{50}, inhibitory concentration 50%.

Detailed pharmacological studies were conducted on compound **5o**, a promising antiproliferative candidate, for a better understanding of its pharmacological properties. Compound **5o** did not show any significant rise in caspase 3/7 activity at any concentration or time point tested. Moreover, it exhibited an increase in the G1 phase and a reduction in the S and G2/M phases of the cell cycle, and it presented an IC_{50}value of 10.4 μM toward the resistant NCI-H69AR cancer cell line. Furthermore, the extent of phosphorylated Rb protein was substantially decreased in a dose-dependent fashion by compound **5o** which was further confirmed via Western blot analysis. This promotes the assumption that inhibition of cyclin-dependent kinases by compound **5o** plays a role in its growth inhibitory potential.

Overall, the current investigation indicates that the new antiproliferative potential of the chemical entities **5a-w**, compound **5o** in particular, can support the development of new antiproliferative leads to be harnessed in preclinical studies of cancer chemotherapy.

Acknowledgment

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project no. RGP-196.

Disclosure

Dr Adam B Keeton is a shareholder for ADT Pharmaceuticals, LLC, outside the submitted work. Prof. Dr. Gary A Piazza is a co-founder, shareholder, and Chief Scientist for ADT Pharmaceuticals LLC and founder and president of PDEi Pharmaceuticals LLC. The authors report no other conflicts of interest in this work.

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