


# New Isatin–Indole Conjugates: Synthesis, Characterization, and a Plausible Mechanism of Their *in vitro* Antiproliferative Activity

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**Background:** Cancer remains the leading cause of human morbidity universally. Hence, we sought to assess the *in vitro* antiproliferative activity of new isatin-based conjugates (**5a–s**) against three human cancer cell lines.

**Methods:** The antiproliferative activities of compounds **5a–s** were evaluated *in vitro* and their ADME (absorption, distribution, metabolism and excretion) was carried out using standard protocols. Subsequently, Western blot analysis was conducted to elucidate the potential antiproliferative mechanism of compounds **5a–s**.

**Results:** The *in vitro* antiproliferative activities of compounds **5a–s** against the tested cancer cell lines ranged from 20.3 to 95.9%. Compound **5m** had an IC<sub>50</sub> value of 1.17 μM; thus, its antiproliferative potency was approximately seven-fold greater than that of sunitinib (IC<sub>50</sub> = 8.11 μM). In-depth pharmacological testing was conducted with compound **5m** to gain insight into the potential antiproliferative mechanism of this class of compounds. Compound **5m** caused an increase in the number of cells in the G1 phase, with a concomitant reduction of those in the G2/M and S phases. Additionally, compound **5m** significantly and dose-dependently reduced the amount of phosphorylated retinoblastoma protein detected. Compound **5m** enhanced expression of B cell translocation gene 1, cell cycle-associated proteins (cyclin B1, cyclin D1, and phosphorylated cyclin-dependent kinase 1), and a pro-apoptotic protein (Bcl-2-associated X protein gene), and activated caspase-3. ADME predictions exposed the oral liability of compounds **5a–s**.

**Conclusion:** Herein, we revealed the antiproliferative activity and ADME predictions of the newly-synthesized compounds **5a–s** and provided a detailed insight into the pharmacological profile of compound **5m**. Thus, compounds **5a–s** can potentially be exploited as new antiproliferative lead compounds for cancer chemotherapeutic.

**Keywords:** synthesis, isatin, indole, antiproliferative, cancer cell line

## Introduction

Cancer, the second major source of mortality in humans after cardiovascular diseases, remains a considerable health obstacle worldwide.<sup>1</sup> Although substantial advances have been made with cancer-diagnosis tools and many chemotherapeutic agents are currently available as treatments, successful management of cancer remains a medical challenge. Because current cancer-chemotherapeutic agents show a lack of selectivity and can elicit multiple-drug resistance, there exists a constant need to derive newer and safer anticancer agents.<sup>2–4</sup>

Creating novel anticancer chemotherapeutic agents based on naturally occurring bioactive substances has been gaining increased interest.<sup>5,6</sup> 2,3-Dioxindole (isatin, **I**,

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Figure 1) is a natural substance found in some plant species, which can also be present in humans, due to endogenous consumption. Compound **I** has a distinguished pharmacological profile and exhibits a wide spectrum of biological actions, including anti-oxidant,<sup>7</sup> anti-inflammatory,<sup>8</sup> antibacterial,<sup>9</sup> and anticancer<sup>10,11</sup> activities. The United States Federal Drug Administration approved sunitinib and nintedanib as isatin-bearing anticancer drugs,<sup>10,12</sup> and many compounds with an isatin scaffold such as SU6668 and SU5416 have displayed outstanding anticancer activity.<sup>13–15</sup> Therefore, isatin-bearing drugs might exhibit extensive cytotoxicity against cancer cells.

Indole (1*H*-benzo[*b*]pyrrole, **II**, Figure 1) has a bicyclic heterocycle scaffold that is widely distributed in animals, microbial hormones, and plants.<sup>16,17</sup> It is considered to be a beneficial motif in drug-discovery programs because of its biodiversity, versatility, and anticancer activity.<sup>18–21</sup> Previous evidence suggests that myriad indole-based compounds exert anticancer activities through various mechanisms,<sup>22–27</sup> suggesting the possibility of using the indole scaffold in anticancer drugs. Notably, anticancer indole-based molecules target several pathways, such as those involving tubulin polymerization, DNA topoisomerase, histone deacetylase, proto-oncogenes, and serine/threonine (PIM) kinase.<sup>28–31</sup>

Screening the literature exposed that a number of bioactive molecules bear carbohydrazide moiety. They display a wide spectrum of biological activities including anticancer activity.<sup>32–34</sup>

Combining the structural features of two differing pharmacophores is an effective tactic in medicinal chemistry, as new bioactive, chemotherapeutic chemical entities characterized by a reduced risk of drug–drug interactions and improved anticancer profiles can be obtained.<sup>35–37</sup> Considering the structural characteristics of isatin and indole, merging these two bioactive moieties into a single compound through a carbohydrazide bridge is predicted to result in new promising anticancer leads. Accordingly, we aimed to synthesize and evaluate the

in vitro antiproliferative activities of the molecular hybrids **5a–s**. The most active antiproliferative candidate **5m** was also examined in an in-depth pharmacological-profiling study to understand the potential mechanism whereby these compounds exert their antiproliferative effect.

## Materials and Methods

### Chemistry

#### General

The melting points of compounds **5a–s** were measured using the Gallenkamp melting point equipment and are presented as uncorrected values. Compounds **5a–s** were dissolved in DMSO-*d*<sub>6</sub> for nuclear magnetic resonance (NMR) measurements at 500 and 125 MHz for <sup>1</sup>H and <sup>13</sup>C, respectively, using a Bruker NMR spectrometer (Bruker, Reinstetten, Germany) at the Research Center (College of Pharmacy, King Saud University, Saudi Arabia). TMS was used as an internal standard for the NMR measurements. The measured chemical shifts are presented as δ-values in parts per million (ppm). Compounds **5a–s** were subjected to microanalyses at the Microanalysis Laboratory (Cairo University, Cairo, Egypt), and the results aligned well with the proposed structures (i.e., within ± 0.4% of the theoretical values). A 6120 LC/MS Agilent Quadrupole system with an electrospray-ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA) was used to obtain the mass spectra of compounds **5a–s**. Compound **3** and compounds **4a–n** were prepared as described previously.<sup>38</sup>

#### General Procedure for Synthesizing Carbohydrazides **5a–s**

The acid hydrazide **3** (0.18 g, 1 mmol) was dissolved in absolute ethanol (15 mL), and the appropriate isatin derivative **4a–n** (1 mmol) was added to the stirred ethanolic solution containing drops of glacial acetic acid. The reaction mixture was refluxed under continuous stirring for 4 h, and then the hot alcoholic reaction mixture was filtered. The collected solids were re-crystallized from the ethanol/

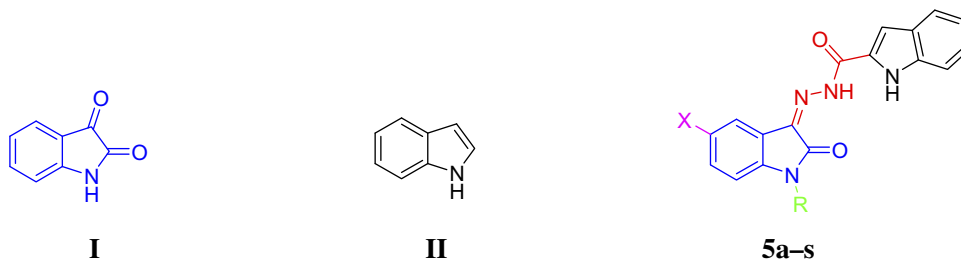


Figure 1 Chemical structures of compounds **I**, **II**, and **5a–s**.

DMF mixture (3:1) to yield the corresponding target carbohydrazides **5a–s** with yields of 40 to 95%.

*N'*-[(3*Z*)-2-Oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5a**): Yellow powder, melting point (m.p.) > 300 °C (yield 89%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 6.95 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.09–7.16 (m, 2H, H<sub>ar.</sub>), 7.29 (d, *J* = 7.0 Hz, 1H, H<sub>ar.</sub>), 7.42 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.61 (s, 1H, H<sub>ar.</sub>), 7.73 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.05 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 10.88 (s, 1H, NH), 11.69 (s, 1H, NH), 11.97 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 104.2, 111.1, 112.9, 116.1, 120.7, 122.3, 122.7, 123.2, 125.1, 127.2, 127.5, 129.3, 133.2, 137.6, 144.4 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 160.9, 165.3 (2 × C=O); MS m/z: 303 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-5-Bromo-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5b**): Yellow powder, m.p. > 300 °C (yield 76%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 6.90 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.09–7.14 (m, 1H, H<sub>ar.</sub>), 7.28 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.59 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.65 (s, 1H, H<sub>ar.</sub>), 7.73 (d, *J* = 8 Hz, 1H, H<sub>ar.</sub>), 8.34 (s, 1H, H<sub>ar.</sub>), 11.01 (s, 1H, NH), 11.87 (s, 1H, NH), 11.95 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 104.8, 108.6, 112.9, 113.8, 117.5, 120.7, 121.0, 122.8, 125.2, 127.5, 129.2, 135.2, 136.4, 137.7, 143.5 (C<sub>ar.</sub> and CH<sub>ar.</sub>, C=N), 160.7, 164.8 (2 × C=O); MS m/z: 381 [M-H]<sup>-</sup>, 383 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-5-Chloro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5c**): Yellow powder, m.p. > 300 °C (yield 91%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 6.95 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.09–7.14 (m, 1H, H<sub>ar.</sub>), 7.27–7.32 (m, 1H, H<sub>ar.</sub>), 7.43–7.47 (m, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.66 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.22 (s, 1H, H<sub>ar.</sub>), 11.00 (s, 1H, NH), 11.86 (s, 1H, NH), 11.96 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 102.1, 109.3, 112.4, 112.9, 117.1, 120.7, 122.8, 125.2, 126.2, 126.6, 127.5, 132.4, 137.6, 138.9, 143.0 (C<sub>ar.</sub> and CH<sub>ar.</sub>, C=N), 163.5, 165.1 (2 × C=O); MS m/z: 337 [M-H]<sup>-</sup>; 339 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-5-Fluoro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5d**): Yellow powder, m.p. > 300 °C (yield 95%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 6.93 (dd, *J* = 4, 8.5 Hz, 1H, H<sub>ar.</sub>), 7.10 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.25–7.30 (m, 2H, H<sub>ar.</sub>), 7.50 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.66 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.02 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 10.89 (s, 1H, NH), 11.79 (s, 1H, NH), 11.95 (s, 1H,

NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 108.4, 111.8, 112.8, 112.9, 113.1, 114.3, 120.7, 120.9, 122.7, 122.8, 125.3, 125.5, 137.5, 139.1 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 157.9 (C<sub>1'-F</sub>, *J* = 235.0 Hz, C<sub>ar.</sub>), 163.7, 165.4 (2 × C=O); MS m/z: 321 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-5-Methoxy-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5e**): Orange powder, m.p. > 300 °C (yield 75%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.82 (s, 3H, OCH<sub>3</sub>), 6.86 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.02 (dd, *J* = 2.0, 8.5 Hz, 1H, H<sub>ar.</sub>), 7.09 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.28 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.63 (s, 1H, H<sub>ar.</sub>), 7.70–7.74 (m, 2H, H<sub>ar.</sub>), 10.69 (s, 1H, NH), 11.81 (s, 1H, NH), 11.95 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 56.3 (OCH<sub>3</sub>), 102.6, 111.6, 112.6, 112.9, 113.1, 116.4, 118.7, 120.7, 122.8, 125.1, 127.5, 137.5, 138.1, 154.9, 155.9 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 163.8, 165.4 (2 × C=O); MS m/z: 333 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-Methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5f**): Yellow powder, m.p. 258–260 °C (yield 41%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.23 (s, 3H, NCH<sub>3</sub>), 7.13 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.17–7.21 (m, 2H, H<sub>ar.</sub>), 7.28 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.47–7.53 (m, 2H, H<sub>ar.</sub>), 7.61 (s, 1H, H<sub>ar.</sub>), 7.73 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.09 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.76 (s, 1H, NH), 11.98 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 26.6 (NCH<sub>3</sub>), 109.8, 110.5, 113.1, 121.0, 122.8, 125.1, 125.4, 126.8, 127.4, 129.3, 132.0, 133.1, 137.6, 144.1, 145.4 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 161.7, 163.9 (2 × C=O); MS m/z: 317 [M-1]<sup>-</sup>.

*N'*-[(3*Z*)-5-Bromo-1-methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5g**): Yellow powder, m.p. 291–293 °C (yield 63%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.21 (s, 3H, NCH<sub>3</sub>), 7.08–7.15 (m, 2H, H<sub>ar.</sub>), 7.28 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.63–7.68 (m, 2H, H<sub>ar.</sub>), 7.73 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.38 (s, 1H, H<sub>ar.</sub>), 11.93 (s, 1H, NH), 11.95 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 26.6 (NCH<sub>3</sub>), 111.6, 112.9, 114.5, 120.6, 122.8, 123.3, 125.2, 127.5, 128.9, 134.1, 135.0, 137.5, 138.1, 143.1, 144.5 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 161.4, 162.1 (2 × C=O); MS m/z: 395 [M-H]<sup>-</sup>, 397 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-5-Chloro-1-methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5h**): Yellow powder, m.p. 292–294 °C (yield 82%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.22 (s, 3H, NCH<sub>3</sub>), 7.10 (d, *J* = 8.0, 1H, H<sub>ar.</sub>), 7.13 (d, *J* = 4.0 Hz, 1H, H<sub>ar.</sub>), 7.27–7.31 (m, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.54 (dd, *J* = 8.5, 2.0 Hz, 1H, H<sub>ar.</sub>), 7.66 (s, 1H, H<sub>ar.</sub>), 7.73 (d, *J* = 8.0, 1H, H<sub>ar.</sub>), 8.26 (s, 1H, H<sub>ar.</sub>), 11.92 (s, 1H, NH), 11.95 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)

ppm: 26.6 (NCH<sub>3</sub>), 109.3, 111.1, 112.0, 112.9, 120.7, 122.8, 125.2, 126.3, 126.8, 127.5, 127.9, 132.2, 137.6, 142.7, 144.1 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 159.4, 161.5 (2 × C=O); MS m/z: 351 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-5-Fluoro-1-methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5i**): Yellow powder, m.p. 283–285 °C (yield 54%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.22 (s, 3H, NCH<sub>3</sub>), 7.09–7.14 (m, 2H, H<sub>ar.</sub>), 7.27–7.30 (m, 1H, H<sub>ar.</sub>), 7.37 (ddd, *J* = 2.5, 9.0, 9.0, 1H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.67 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.07 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.86 (s, 1H, NH), 11.96 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 26.7 (NCH<sub>3</sub>), 109.4, 110.6, 113.1, 114.2, 115.7, 118.9, 120.7, 122.8, 125.2, 125.4, 127.5, 129.0, 137.6, 141.8 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 158.3 (C<sub>1'-F</sub>, *J* = 235.6 Hz, C<sub>ar.</sub>), 161.8, 163.9 (2 × C=O); MS m/z: 335 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-5-Methoxy-1-methyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5j**): Orange powder, m.p. 279–281 °C (yield 86%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.19 (s, 3H, NCH<sub>3</sub>), 3.84 (s, 3H, OCH<sub>3</sub>), 7.04 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.08–7.12 (m, 2H, H<sub>ar.</sub>), 7.27–7.30 (m, 1H, H<sub>ar.</sub>), 7.63 (s, 1H, H<sub>ar.</sub>), 7.66 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.72–7.77 (m, 2H, H<sub>ar.</sub>), 11.86 (s, 1H, NH), 11.96 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 26.6 (NCH<sub>3</sub>), 56.4 (OCH<sub>3</sub>), 110.2, 111.3, 112.9, 113.4, 115.8, 117.9, 120.7, 122.8, 125.1, 127.5, 129.2, 137.5, 139.2, 155.5 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 161.7, 163.9 (2 × C=O); MS m/z: 347 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-Benzyl-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5k**): Yellow powder, m.p. 249–251 °C (yield 56%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.02 (s, 2H, CH<sub>2</sub>), 7.06 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.10–7.13 (m, 1H, H<sub>ar.</sub>), 7.16–7.21 (m, 1H, H<sub>ar.</sub>), 7.28–7.32 (m, 2H, H<sub>ar.</sub>), 7.35–7.38 (m, 3H, H<sub>ar.</sub>), 7.42–7.45 (m, 2H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.65 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 8.14 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.80 (s, 1H, NH), 12.00 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 43.2 (CH<sub>2</sub>), 110.4, 113.0, 115.7, 120.7, 120.9, 121.3, 122.7, 123.0, 127.5, 127.7, 127.9, 128.0, 129.2, 134.1, 136.7, 137.7, 144.3, 146.8, 147.5 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 162.4, 164.2 (2 × C=O); MS m/z: 393 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-Benzyl-5-bromo-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5l**): Yellow powder, m.p. 268–270 °C (yield 51%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.02 (s, 2H, CH<sub>2</sub>), 7.01 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.10 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.29 (d, *J* = 7.5 Hz, 2H, H<sub>ar.</sub>), 7.36–7.38 (m, 3H, H<sub>ar.</sub>), 7.42 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>),

7.52 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.59 (s, 1H, H<sub>ar.</sub>), 7.71–7.77 (m, 2H, H<sub>ar.</sub>), 8.44 (s, 1H, H<sub>ar.</sub>), 11.99 (s, 1H, NH), 12.20 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 43.3 (CH<sub>2</sub>), 110.3, 112.1, 113.1, 114.8, 120.7, 127.7, 127.9, 128.2, 129.2, 134.1, 135.8, 136.4, 136.6, 137.6, 138.1, 142.1, 143.3, 148.7, 156.5 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 161.5, 162.6 (2 × C=O); MS m/z: 471 [M-H]<sup>-</sup>; 473 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-1-Benzyl-5-chloro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5m**): Yellow powder, m.p. 268–270 °C (yield 54%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.02 (s, 2H, CH<sub>2</sub>), 7.06 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.11–7.13 (m, 1H, H<sub>ar.</sub>), 7.28–7.31 (m, 2H, H<sub>ar.</sub>), 7.35–7.37 (m, 3H, H<sub>ar.</sub>), 7.43 (d, *J* = 7.0 Hz, 1H, H<sub>ar.</sub>), 7.46–7.47 (m, 1H, H<sub>ar.</sub>), 7.52 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.71 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.32 (s, 1H, H<sub>ar.</sub>), 11.97 (s, 1H, NH), 12.18 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 43.3 (CH<sub>2</sub>), 111.7, 112.5, 112.9, 113.1, 120.7, 121.6, 127.5, 127.7, 127.9, 128.0, 128.2, 129.2, 135.9, 136.4, 137.6, 138.1, 141.7, 144.4, 149.6 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 159.7, 161.6 (2 × C=O); MS m/z: 427 [M-H]<sup>-</sup>; 429 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-1-Benzyl-5-fluoro-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5n**): Yellow powder, m.p. 251–253 °C (yield 49%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.02 (s, 2H, CH<sub>2</sub>), 7.04–7.06 (m, 1H, H<sub>ar.</sub>), 7.09–7.13 (m, 1H, H<sub>ar.</sub>), 7.28–7.32 (m, 3H, H<sub>ar.</sub>), 7.34–7.39 (m, 4H, H<sub>ar.</sub>), 7.29 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.72 (s, 1H, H<sub>ar.</sub>), 7.75 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.13 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.91 (s, 1H, NH), 11.99 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 43.3 (CH<sub>2</sub>), 110.9, 111.1, 113.0, 114.4, 119.0, 120.7, 122.8, 125.3, 127.5, 127.7, 127.9, 128.0, 128.9, 129.2, 136.5, 137.6, 139.9, 140.6 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 158.4 (C<sub>1'-F</sub>, *J* = 237.9 Hz, C<sub>ar.</sub>), 161.9, 164.2 (2 × C=O); MS m/z: 411 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-Benzyl-5-methoxy-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5o**): Orange powder, m.p. 268–270 °C (yield 64%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 3.81 (s, 3H, OCH<sub>3</sub>), 4.99 (s, 2H, CH<sub>2</sub>), 6.95 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.02 (dd, *J* = 2.0, 8.5 Hz, 1H, H<sub>ar.</sub>), 7.10 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.26–7.31 (m, 2H, H<sub>ar.</sub>), 7.34–7.38 (m, 4H, H<sub>ar.</sub>), 7.52 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.68 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.78 (s, 1H, H<sub>ar.</sub>), 11.92 (s, 1H, NH), 11.98 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 43.1 (CH<sub>2</sub>), 56.4 (OCH<sub>3</sub>), 110.9, 113.0, 116.1, 120.7, 122.8, 125.2, 127.6, 127.7, 127.9, 128.0, 129.2, 129.3, 136.7, 136.8, 137.7, 138.0, 138.1, 144.1, 155.6 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 156.5, 164.1 (2 × C=O); MS m/z: 423 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-(4-Chlorobenzyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5p**): Yellow powder, m.p. 261–263 °C (yield 65%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.02 (s, 2H, CH<sub>2</sub>), 7.06 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.10–7.14 (m, 1H, H<sub>ar.</sub>), 7.19–7.21 (m, 1H, H<sub>ar.</sub>), 7.28–7.32 (m, 1H, H<sub>ar.</sub>), 7.40–7.44 (m, 3H, H<sub>ar.</sub>), 7.51 (d, *J* = 8.0 Hz, 2H, H<sub>ar.</sub>), 7.65 (s, 1H, H<sub>ar.</sub>), 7.72–7.75 (m, 1H, H<sub>ar.</sub>), 7.77 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.15 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.81 (s, 1H, NH), 11.99 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 42.6 (CH<sub>2</sub>), 110.9, 113.0, 119.9, 123.1, 125.2, 125.4, 127.5, 129.2, 129.7, 129.9, 131.9, 132.6, 132.8, 135.2, 135.7, 137.7, 138.0, 142.9, 144.1 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 161.8, 164.2 (2 × C=O); MS *m/z*: 427 [M-H]<sup>-</sup>; 429 [M-H+2]<sup>-</sup>.

*N'*-[(3*Z*)-1-(4-Cyanobenzyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5q**): Yellow powder, m.p. 266–268 °C (yield 57%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 5.13 (s, 2H, CH<sub>2</sub>), 7.05 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 7.10–7.13 (m, 1H, H<sub>ar.</sub>), 7.20 (ddd, *J* = 2.0, 7.5, 7.5 Hz, 1H, H<sub>ar.</sub>), 7.39–7.43 (m, 1H, H<sub>ar.</sub>), 7.56 (d, *J* = 8.5 Hz, 2H, H<sub>ar.</sub>), 7.63–7.65 (m, 3H, H<sub>ar.</sub>), 7.77 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.84 (d, *J* = 3.0 Hz, 1H, H<sub>ar.</sub>), 7.85 (d, *J* = 3.0 Hz, 1H, H<sub>ar.</sub>), 8.17 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.82 (s, 1H, NH), 11.99 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 42.9 (CH<sub>2</sub>), 110.8, 110.9, 113.0, 115.8, 119.9, 120.7, 122.7, 123.2, 125.4, 127.1, 127.5, 128.6, 128.8, 133.1, 133.2, 137.7, 138.0, 141.9, 142.5, 142.8 (C<sub>ar.</sub>, CH<sub>ar.</sub>, 2 × CN), 161.9, 164.2 (2 × C=O); MS *m/z*: 418 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-1-(4-Methylbenzyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5r**): Yellow powder, m.p. 256–258 °C (yield 40%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 2.27 (s, 3H, CH<sub>3</sub>), 4.97 (s, 2H, CH<sub>2</sub>), 7.04 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.10–7.13 (m, 1H, H<sub>ar.</sub>), 7.16 (d, *J* = 8.5 Hz, 2H, H<sub>ar.</sub>), 7.27 (d, *J* = 8.5 Hz, 2H, H<sub>ar.</sub>), 7.29–7.34 (m, 1H, H<sub>ar.</sub>), 7.41–7.44 (m, 1H, H<sub>ar.</sub>), 7.52 (d, *J* = 8.5 Hz, 1H, H<sub>ar.</sub>), 7.65 (s, 1H, H<sub>ar.</sub>), 7.74 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.13 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.80 (s, 1H, NH), 12.00 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 21.1 (CH<sub>3</sub>), 43.0 (CH<sub>2</sub>), 110.4, 113.0, 115.7, 120.7, 122.7, 123.0, 125.2, 127.0, 127.5, 127.7, 127.9, 129.1, 129.2, 129.8, 132.9, 133.6, 137.2, 137.7, 144.3 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 162.5, 164.1 (2 × C=O); MS *m/z*: 407 [M-H]<sup>-</sup>.

*N'*-[(3*Z*)-2-Oxo-1-phenyl-1,2-dihydro-3*H*-indol-3-ylidene]-1*H*-indole-2-carbohydrazide (**5s**): Yellow powder, m.p. 277–279 °C (yield 55%); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) ppm: 6.84 (d, *J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 7.11–7.14 (m, 1H, H<sub>ar.</sub>), 7.24–7.31 (m, 2H, H<sub>ar.</sub>), 7.44–7.47 (m, 1H, H<sub>ar.</sub>), 7.50–7.54 (m, 4H, H<sub>ar.</sub>), 7.61–7.64 (m, 2H, H<sub>ar.</sub>), 7.58 (s, 1H, H<sub>ar.</sub>), 7.74 (d,

*J* = 8.0 Hz, 1H, H<sub>ar.</sub>), 8.23 (d, *J* = 7.5 Hz, 1H, H<sub>ar.</sub>), 11.89 (s, 1H, NH), 12.01 (s, 1H, NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) ppm: 110.1, 113.0, 115.7, 120.7, 122.8, 123.5, 125.2, 127.2, 127.4, 127.5, 128.9, 130.2, 133.1, 134.2, 135.5, 137.3, 137.7, 141.8, 145.1 (C<sub>ar.</sub>, CH<sub>ar.</sub>, C=N), 162.9, 163.4 (2 × C=O); MS *m/z*: 379 [M-H]<sup>-</sup>.

## Pharmacological Evaluation

Pharmacological assessment of the title compounds **5a–s** was carried out using previously reported protocols<sup>39,40</sup> and they are provided as [Supporting materials](#). The human cancer cell lines were procured commercially from the American Type Culture Collection.

## Western Blot Analysis

### Materials

Immunoblotting detection was performed with antibodies from Abcam against cyclin E1 (clone number HE12, catalog number ab3927), cyclin D1 (clone number EP272Y, catalog number ab40754), cyclin B1 (clone number Y106, catalog number ab32053), β actin (clone number mAbcam8226, catalog number ab8226), BTG1 (clone number EPR8274(2), catalog number ab151740), BAX (clone number 2D2, catalog number ab77566), bcl-2 (clone number E17, catalog number ab32124), and caspase-3 (catalog number ab13847).

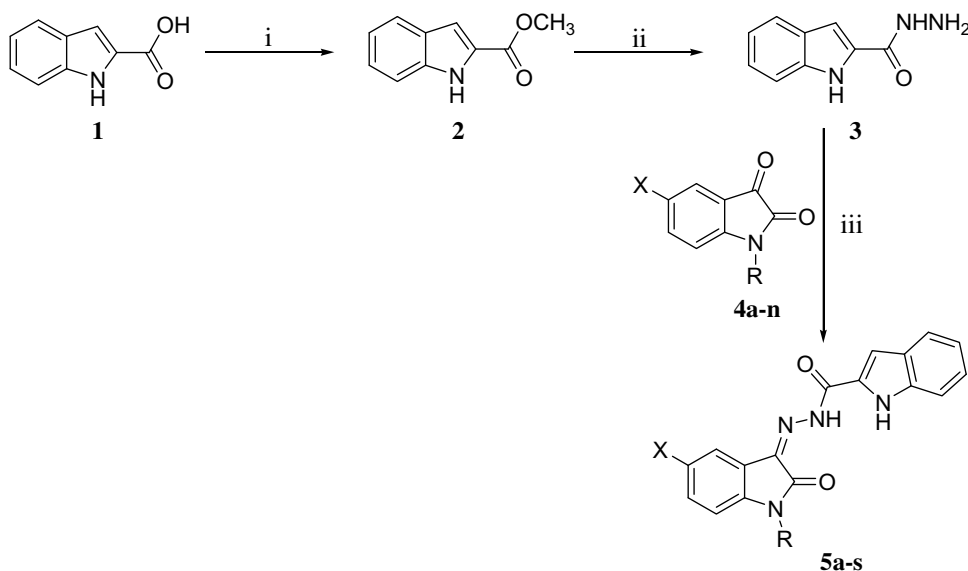
### Methods

A-549 non-small cell lung cancer (NSCLC) cells were plated in 25-cm<sup>2</sup> culture dishes at a seeding density of 5 × 10<sup>4</sup>/dish before treatment with compound **5m** for 24, 48, and 72 h. Whole cell lysates were prepared using radio-immunoprecipitation assay buffer (Sigma-Aldrich, MO, USA). Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, CA, USA). Twenty micrograms of treated and control lysates were loaded in each well of 4–20% Mini-Protean TGX Gels (Bio-Rad) for electrophoresis and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% (w/v) skimmed milk prepared in Tris-buffered saline and Tween 20, incubated with primary antibodies overnight at 4 °C, washed, and then incubated with a secondary, horseradish peroxidase-coupled anti-mouse or anti-rabbit antibody. Bands were visualized with a Western Bright ECL Kit for 5000 cm<sup>2</sup> Membrane and Blue Basic Autoradiography Film (BioExpress, CA, USA).

## Results and Discussion

### Chemistry

The chemical structures of the title compounds **5a–s** and their intermediates are shown in [Scheme 1](#). The free carboxylic group of indole-2-carboxylic acid (**1**) was esterified, which resulted in the corresponding methyl ester **2**.<sup>41</sup> Subsequently, compound **2** was subjected to hydrazinolysis with hydrazine hydrate, thereby forming the acid hydrazide **3**.<sup>42</sup> The target indole–isatin conjugates **5a–s** were respectively prepared by reacting the acid hydrazide **3** with the appropriate isatin derivatives **4a–n** in ethanol-containing drops of acetic acid. The stereochemistry of the title conjugates **5a–s** was assigned the (*Z*)-configuration, based on the previously documented X-ray results of structurally related analogous compounds.<sup>43,44</sup>



Compound No.	X	R	Compound No.	X	R
<b>5a</b>	H	H	<b>5k</b>	H	$\text{C}_6\text{H}_5\text{-CH}_2$
<b>5b</b>	Br	H	<b>5l</b>	Br	$\text{C}_6\text{H}_5\text{-CH}_2$
<b>5c</b>	Cl	H	<b>5m</b>	Cl	$\text{C}_6\text{H}_5\text{-CH}_2$
<b>5d</b>	F	H	<b>5n</b>	F	$\text{C}_6\text{H}_5\text{-CH}_2$
<b>5e</b>	$\text{OCH}_3$	H	<b>5o</b>	$\text{OCH}_3$	$\text{C}_6\text{H}_5\text{-CH}_2$
<b>5f</b>	H	$\text{CH}_3$	<b>5p</b>	H	$4\text{-Cl-C}_6\text{H}_4\text{-CH}_2$
<b>5g</b>	Br	$\text{CH}_3$	<b>5q</b>	H	$4\text{-CN-C}_6\text{H}_4\text{-CH}_2$
<b>5h</b>	Cl	$\text{CH}_3$	<b>5r</b>	H	$4\text{-CH}_3\text{-C}_6\text{H}_4\text{-CH}_2$
<b>5i</b>	F	$\text{CH}_3$	<b>5s</b>	H	$\text{C}_6\text{H}_5$
<b>5j</b>	$\text{OCH}_3$	$\text{CH}_3$			

**Scheme 1** Preparation of compounds **5a–s**. Reagents and conditions: (i) Few drops of concentrated sulfuric acid, absolute methanol, reflux, 4 h; (ii)  $\text{H}_2\text{N-NH}_2 \cdot \text{H}_2\text{O}$ , absolute methanol, reflux, 2 h; (iii) Few drops of glacial acetic acid, absolute ethanol, reflux, 4 h.

## Pharmacological Investigations

### Antiproliferative Activity

The cell growth-inhibitory activities of nineteen conjugates **5a–s** were evaluated with human breast (ZR-75), colon (HT-29) and lung (A-549) tumor cells. Each test compound was assessed at  $30 \mu\text{M}$  in quadruplicate ([Table 1](#)). The title compounds **5a–s** displayed average antiproliferative activities against the ZR-75, HT-29, and A-549 cell lines of 20.3 to 95.9%.

[Table 2](#) shows the  $\text{IC}_{50}$  (inhibitory concentration 50%) values determined for compounds **5a–s** against the tested human cancer cell lines. Compound **5m** had an average  $\text{IC}_{50}$  value of  $1.17 \mu\text{M}$  against the tested human cancer cell lines, making it the most active candidate, with a potency approximately seven-fold greater than that of sunitinib (average  $\text{IC}_{50}$  value of

**Table 1** Antiproliferative Activities (Cell Growth-Inhibitory Activities at a Concentration of 30  $\mu\text{M}$ ) of Compounds **5a–s** and Sunitinib Against the Indicated Cell Lines

Compound No.	HT-29	ZR-75	A-549	Average Growth Inhibition %
5a	75.4 $\pm$ 3.5	57.7 $\pm$ 21.7	90.9 $\pm$ 6.2	80.7
5b	64.5 $\pm$ 6.7	58.9 $\pm$ 13.5	77.0 $\pm$ 9.9	66.8
5c	29.4 $\pm$ 21.4	53.9 $\pm$ 9.5	60.6 $\pm$ 22.3	47.9
5d	13.9 $\pm$ 16.1	40.0 $\pm$ 8.7	6.9 $\pm$ 18.9	20.3
5e	36.5 $\pm$ 8.3	65.9 $\pm$ 8.0	85.3 $\pm$ 11.2	62.6
5f	78.5 $\pm$ 2.8	73.1 $\pm$ 9.8	94.9 $\pm$ 4.7	82.2
5g	95.2 $\pm$ 4.3	90.2 $\pm$ 5.1	97.4 $\pm$ 3.9	94.3
5h	92.4 $\pm$ 5.2	81.8 $\pm$ 8.5	95.8 $\pm$ 4.5	90.0
5i	91.3 $\pm$ 5.4	78.8 $\pm$ 2.3	90.3 $\pm$ 9.3	86.8
5j	90.8 $\pm$ 5.5	82.5 $\pm$ 9.0	97.4 $\pm$ 3.9	90.2
5k	92.7 $\pm$ 2.9	86.6 $\pm$ 3.3	98.4 $\pm$ 2.0	92.6
5l	94.7 $\pm$ 2.9	79.9 $\pm$ 8.8	96.1 $\pm$ 4.6	90.2
5m	94.3 $\pm$ 3.1	83.8 $\pm$ 5.2	99.7 $\pm$ 0.7	92.6
5n	94.3 $\pm$ 1.6	77.5 $\pm$ 8.3	99.1 $\pm$ 1.1	90.3
5o	94.0 $\pm$ 4.8	93.8 $\pm$ 5.1	97.4 $\pm$ 2.9	95.1
5p	93.7 $\pm$ 4.2	89.0 $\pm$ 2.0	96.9 $\pm$ 3.6	93.2
5q	81.6 $\pm$ 5.4	75.4 $\pm$ 12.5	94.6 $\pm$ 2.3	83.9
5r	91.1 $\pm$ 4.2	79.1 $\pm$ 6.4	91.9 $\pm$ 4.6	87.4
5s	97.0 $\pm$ 2.2	92.2 $\pm$ 7.2	98.5 $\pm$ 2.0	95.9
Sunitinib	59.5 $\pm$ 2.3	90.7 $\pm$ 4.5	85.7 $\pm$ 2.7	78.7

**Table 2** The  $\text{IC}_{50}$  Values of Compounds **5a–s** and Sunitinib Against the Tested Cell Lines

Compound No.	$\text{IC}_{50}$ ( $\mu\text{M}$ )			Average $\text{IC}_{50}$ ( $\mu\text{M}$ )
	HT-29	ZR-75	A-549	
5a	19.74 $\pm$ 1.95	22.73 $\pm$ 5.71	12.98 $\pm$ 2.46	18.48
5b	21.93 $\pm$ 1.29	21.92 $\pm$ 4.93	16.01 $\pm$ 2.26	19.95
5c	>30	18.01 $\pm$ 18.47	19.68 $\pm$ 5.20	>22.56
5d	>30	>30	>30	>30
5e	>30	17.87 $\pm$ 7.24	6.68 $\pm$ 1.52	18.18
5f	12.88 $\pm$ 1.03	1.53 $\pm$ 31.5	4.04 $\pm$ 0.83	6.15
5g	3.68 $\pm$ 0.29	1.01 $\pm$ 3.28	1.49 $\pm$ 0.15	2.06
5h	5.67 $\pm$ 0.43	0.84 $\pm$ 7.65	2.13 $\pm$ 0.42	2.88
5i	8.92 $\pm$ 0.67	4.31 $\pm$ 7.98	2.82 $\pm$ 0.30	5.35
5j	5.73 $\pm$ 0.67	1.48 $\pm$ 7.06	1.93 $\pm$ 0.27	3.05
5k	6.19 $\pm$ 1.58	1.12 $\pm$ 3.05	1.06 $\pm$ 0.37	2.79
5l	2.26 $\pm$ 0.33	0.70 $\pm$ 12.04	0.65 $\pm$ 0.12	1.20
5m	2.02 $\pm$ 0.36	0.74 $\pm$ 0.88	0.76 $\pm$ 0.12	1.17
5n	3.50 $\pm$ 0.49	2.10 $\pm$ 3.15	1.41 $\pm$ 0.21	2.34
5o	2.28 $\pm$ 0.22	0.86 $\pm$ 0.76	0.64 $\pm$ 0.24	1.26
5p	2.64 $\pm$ 0.27	1.30 $\pm$ 0.58	1.05 $\pm$ 0.15	1.66
5q	8.11 $\pm$ 1.27	1.51 $\pm$ 5.03	2.13 $\pm$ 0.49	3.92
5r	2.45 $\pm$ 0.32	1.28 $\pm$ 12.44	0.64 $\pm$ 0.61	1.46
5s	6.34 $\pm$ 0.76	1.32 $\pm$ 2.89	2.41 $\pm$ 0.29	3.36
Sunitinib	10.14 $\pm$ 0.8	8.31 $\pm$ 2.4	5.87 $\pm$ 0.3	8.11

**Abbreviation:**  $\text{IC}_{50}$ , inhibitory concentration 50%.

8.11  $\mu\text{M}$ ). Consequently, further pharmacological profiling was conducted with compound **5m** to better understand the potential antiproliferative mechanism of compounds **5a–s**.

## Structure Activity Relationship

The title compounds **5a–s** are structurally classified into five groups namely, *N*-unsubstituted derivatives **5a–e**, *N*-methyl derivatives **5f–j**, *N*-benzyl derivatives **5k–o**, *N*-4-substituted benzyl derivatives **5p–r**, and *N*-phenyl derivative **5s**. In the first group **5a–e**, compound **5e**, bearing a methoxy substituent in the five position of the isatin nucleus, is the most active congener with an average  $\text{IC}_{50}$  value of 18.18  $\mu\text{M}$ . Compound **5g**, in the second group **5f–j**, have a bromo substituent in the five position of the isatin ring and it showed an average  $\text{IC}_{50}$  value of 2.06  $\mu\text{M}$  being nearly four times more potent than sunitinib (average  $\text{IC}_{50}$  value of 8.11  $\mu\text{M}$ ). The third group **5k–o** seems to be the best group with an average  $\text{IC}_{50}$  range of 1.17–2.79  $\mu\text{M}$  in which compound **5m**, bearing 5-chloroisatin fragment, is the most active congener with an average  $\text{IC}_{50}$  value of 1.17  $\mu\text{M}$  being about sevenfold more potent than sunitinib. Moreover, the in vitro antiproliferative activity of the fourth group **5p–r** is in the following order **5r** > **5p** > **5q** with an average  $\text{IC}_{50}$  range of 1.46–3.92  $\mu\text{M}$ . Compound **5s**, bearing *N*-phenylisatin scaffold displayed good in vitro antiproliferative activity with an average  $\text{IC}_{50}$  value of 3.36  $\mu\text{M}$  (Table 2). In conclusion, the *N*-benzyl moiety on the isatin residue seems to be the favorable substituent to get good in vitro antiproliferative activity of this class of compounds.

## Effects on Cell-Cycle Progression

The influence of compound **5m** on different aspects of cell-cycle progression was investigated with the A-549 cell line based on the total DNA content of each cell. Additionally, immunofluorescent imaging of the

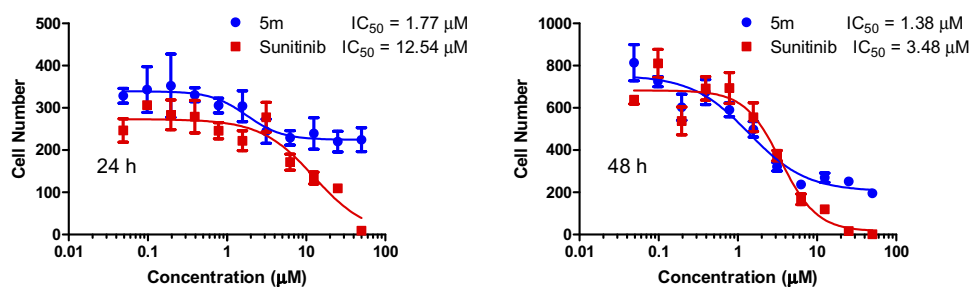
phosphorylated retinoblastoma (Rb) protein was performed over the concentration range, 0.05–100  $\mu\text{M}$ . A significant dose-dependent decrease in the total cell number (Figure 2) was noted after treatment with compound **5m** for 48 h ( $\text{IC}_{50}$  value = 1.38  $\mu\text{M}$ ; Table 3).

In addition, the percentage of cells in G1 phase increased and the percentages of cells in the S and G2/M phases concomitantly decreased after treatment with compound **5m**. This finding suggests that the ability of compound **5m** to influence cell proliferation could be ascribed to decreased cell-cycle progression. Conversely, sunitinib treatment resulted in a reduced percentage of cells in G1 phase and a concomitant increase in cells in S or G2/M phase. G2 arrest could be regarded as a checkpoint blockade. Mitotic arrest may, in some instances, result in mitotic catastrophe and subsequent programmed death of cells with multiple or aberrant nuclei.

Moreover, both sunitinib and **5m** caused a substantial dose-dependent decrease in the extent of Rb protein phosphorylation (Figure 3), suggesting the possibility that the antiproliferative activity of compounds with isatin fragments can be attributed to their effect on cyclin-dependent kinases. Compound **5m** had an  $\text{IC}_{50}$  value of 2.52  $\mu\text{M}$  after 24 h, indicating that it was ~1.7-fold more potent than sunitinib (Table 3). Treatment with compound **5m** did not affect the extent of P-Tyr.

## Western Blot Analysis

Data from a previous study revealed a correlation between the antiproliferative marker, B cell translocation gene 1 (BTG1), and the inhibition of proliferation in lung cancer, which suggests that compound **5m** most likely induces cycle arrest during G1 phase.<sup>45</sup> Treatment with compound **5m** increased BTG1 expression (Figure S1), confirming its potential to act as an antiproliferative agent. To confirm the influence of compound **5m** on cell-cycle progression, the expression levels of different cell cycle-related proteins were examined



**Figure 2** Dose-dependent reductions in the numbers of adherent cells. A-549 NSCLC cells were treated for 24 or 48 h, as indicated, followed by fixation and addition of the fluorescent label. Values are presented as the average number of cells per 4 fields within each well.



**Table 3** IC<sub>50</sub> Values for the Reductions in Total Cell Numbers and the Effects of Compound **5m** and Sunitinib on Cell-Cycle Progression

Compound No.	IC <sub>50</sub> (μM) for Reductions in the Total Cell Numbers		IC <sub>50</sub> (μM) for the Reduction of Rb Phosphorylation		Effects on Cell-Cycle Progression
	24 h	48 h	24 h	48 h	
<b>5m</b>	1.77 ± 1.32	1.38 ± 0.39	2.52 ± 0.20	12.15 ± 3.74	G1 phase increased, G2/Mphases decreased
Sunitinib	12.54 ± 9.83	3.48 ± 0.61	4.37 ± 0.24	6.27 ± 0.36	G1 phase decreased, G2/Mphase increased

**Abbreviation:** IC<sub>50</sub>, inhibitory concentration 50%.

using Western blot analysis. As depicted in [Figure S2](#), compound **5m** regulated cell cycle-related proteins and activated cyclin-dependent kinase 1 (cdc-2) via phosphorylation at Tyr 15.<sup>46</sup> Although cell-cycle analysis revealed an increase in the G1 population after treatment with compound **5m**, the ability of **5m** to upregulate the G2/M phase-related proteins, cyclin B1 and p-cdc2 ([Figure S2](#)), was interesting. Ultimately, these findings suggest that cell-cycle arrest after treatment with **5m** can occur during both G1 and G2 phases.<sup>47,48</sup>

Many anticancer agents are designed to cause DNA damage and, consequently, apoptosis.<sup>49</sup> Hence, the ability of compound **5m** to inhibit cell proliferation by inducing apoptosis was also examined. Compound **5m** activated caspase-3, with increased production of cleaved caspase-3 within 24 h of treatment ([Figure S3](#)). The expression levels of the pro-survival protein encoded by the B-cell lymphoma/leukemia-2 (Bcl-2) gene and the pro-apoptotic protein encoded by the Bcl-2-associated X protein (BAX) gene were also evaluated. Treatment with compound **5m** increase BAX expression but had no obvious effect on Bcl-2 expression ([Figure S3](#)). The altered BAX/BCL-2 ratio after treatment with **5m** suggests the involvement of the intrinsic apoptosis pathway.

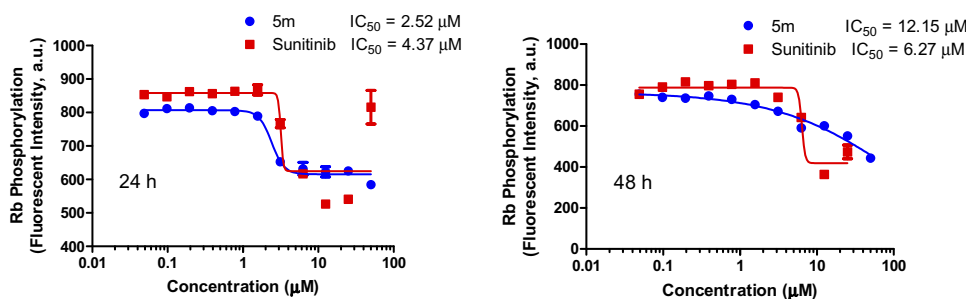
## Selectivity

The selectivity of compound **5m** was tested using three non-tumorigenic cell lines. IEC-6 cells obtained from rat

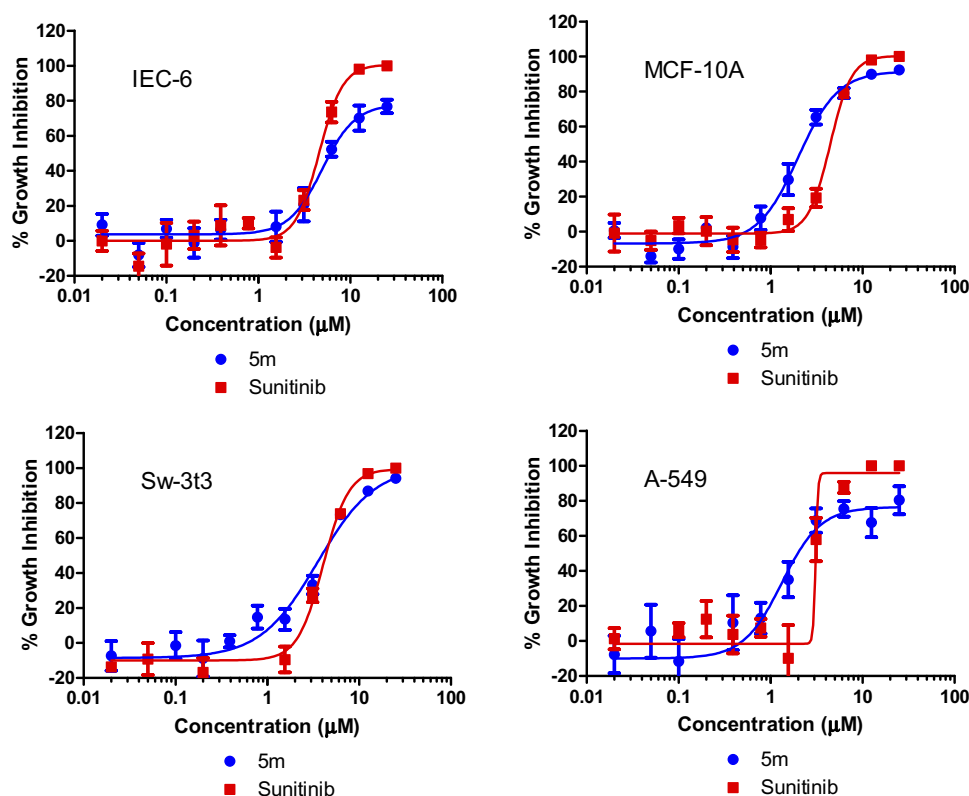
intestines manifested both morphologic and karyotypic properties of normal intestinal epithelial cells.<sup>50</sup> Cultures obtained from human fibrocystic mammary tissue (MCF-10A) are non-tumorigenic and display characteristics similar to primary cultures of breast tissue, including dome formation.<sup>51</sup> Fibroblasts obtained from the embryonic tissue of mice (Swiss 3t3 fibroblasts) are both non-tumorigenic and contact-inhibited.<sup>52</sup> A-549 NSCLC cells were included for comparison. The antiproliferative activities of compound **5m** and sunitinib were assessed in quadruplicate at a maximum concentration of 25 μM and then at 10 serially diluted concentrations. Compound **5m** inhibited cell growth (>50%) in both normal and tumor cell lines. However, compound **5m** exhibited a 2.6-fold selectivity value, whereas sunitinib displayed a 1.4-fold selectivity value ([Figure 4](#) and [Table 4](#)).

## Multidrug-Resistant Lung Cancer Cell Line

The antiproliferative activity of compound **5m** was examined against a sensitive NSCLC cell line (A-549) and a multidrug-resistant lung cancer cell (NCI-H69AR) expressing the ABCC1 efflux pump protein. Both compound **5m** and sunitinib were tested in quadruplicate at a maximum concentration of 25 μM, and then at 10 serially diluted concentrations. Compound **5m** inhibited growth in both cell lines, with an IC<sub>50</sub> value of 1.33 μM in A-549 cells ([Figure 5](#) and [Table 5](#)).



**Figure 3** Dose-dependent reductions in Rb phosphorylation. A-549 NSCLC cells were treated for 24 or 48 h (as indicated), fixed, and subjected to quantitative indirect immunofluorescence and image analysis.



**Figure 4** Tumor selectivity. A-549 NSCLC cells and non-tumorigenic cells derived from intestine, breast, and fibroblasts were treated for 72 h, as indicated, for use in a luminescence-based, growth-inhibition assay.

The H69AR cells were ~19-fold less sensitive, as compound **5m** could be effluxed by the ABCC1 pump protein. Additionally, a lesser degree of sunitinib resistance (1.9-fold less sensitivity) was found in H69AR cells.

### Cell Viability Assay Using HEK 293 Cell Line

Cell viability assay for compound **5m** showed no apparent toxicity towards human embryonic kidney (HEK) 293 cells following 24 hrs exposure to different **5m** concentrations, indicated by percent viability ranging from  $93.72 \pm 0.68$  to  $98.67 \pm 0.39$ . Furthermore, there was no significant change in viability after longer exposure time to **5m** up to 72 hrs.

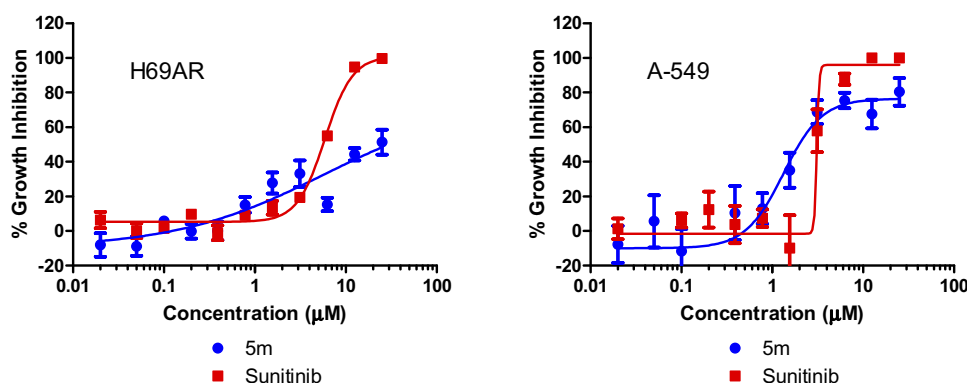
### In Silico Predictions of ADME Properties of Compounds 5a-s

In silico ADME (absorption, distribution, metabolism and excretion) predictions as well as some pharmacokinetic characteristics of the target compounds **5a-s** are presented in [Table S1](#) which are calculated according to the literature methods.<sup>53,54</sup> All the title compounds **5a-s** follow the Lipinski's rule of 5 (RO5) where log *P* values lie between 2.2–4.2 (<5), molecular weight range 304–473 (<500), hydrogen bond acceptor range 3–4 (<5), and hydrogen bond donor range 2–3 (<5), indicating that these compounds might not be expected to cause problems with oral bioavailability. In addition, compound **5a-s** exhibited topological polar surface area (TPSA) range 77.6–101.4 Å<sup>2</sup>

**Table 4** Selectivity of Compound **5m** and Sunitinib Against Tumor and Non-Tumorigenic Cell Lines

Compound No.	IC <sub>50</sub> (µM)				Mean Tumor Selectivity
	Intestine IEC-6	Breast MCF-10A	Fibroblast Swiss 3t3	NSCLC A-549	
<b>5m</b>	4.94 ± 0.82	2.00 ± 0.24	3.49 ± 1.14	1.33 ± 0.47	2.6
Sunitinib	3.06 ± 0.54	4.07 ± 0.23	4.43 ± 0.75	4.56 ± amb	1.4

**Abbreviations:** IC<sub>50</sub>, inhibitory concentration 50%; IEC, intestinal epithelial cell; MCF, Michigan cancer foundation; 3T3, 3-day transfer, inoculums  $3 \times 10^5$  cells; NSCLC, non-small cell lung cancer.



**Figure 5** Evaluation of susceptibility to efflux. A-549 NSCLC cells and NCI-H69AR cells expressing the ABCB1 (MRP1) multi-drug resistance efflux transporter were treated for 72 h, as indicated, for use in a luminescence-based, growth-inhibition assay.

(<140 Å<sup>2</sup>), denoting good transport through cellular plasma membranes. In summary, compounds **5a–s** displayed no violations to RO5 criteria suggesting their liability to be new orally bioavailable antiproliferative candidates.

## Conclusion

Herein, we identified the newly synthesized isatin-indole molecular hybrids **5a–s** with various spectroscopic approaches and estimated their potentials as antiproliferative agents in vitro. Compound **5m** displayed promising in vitro antiproliferative potential against three human cancer cell lines. With an average IC<sub>50</sub> value of 1.17 µM, this compound had a seven-fold greater potency than sunitinib (IC<sub>50</sub> = 8.11 µM). By deriving its pharmacological profile, we sought to characterize the mechanisms underlying the antiproliferative activities of compounds **5a–s**. We found reduced percentages of cells in S and G2/M phases, an increased percentage of cells in G1 phase, and a significant dose-dependent decrease in the extent of Rb protein phosphorylation. Furthermore, Western blot analysis confirmed the extents of BTG1, cyclin B1, cyclin D1, and p-cdc2 upregulation caused by compound **5m**. Within 24 h of cell treatment, compound **5m** increased BAX expression and activated caspase-3, as

indicated by the observed increase in cleaved caspase-3 levels. ADME predictions for compounds **5a–s** might help in further development of new anticancer agents with favorable oral bioavailability. Based on the findings presented herein, these lead compounds can be utilized in preclinical studies in the field of cancer chemotherapy.

## Supporting Materials

Detailed protocols for the pharmacological evaluation of the title compounds **5a–s**, Western blot results for compound **5m** (Figures S1–S3), and ADME Table (Table S1) are provided as [Supporting materials](#).

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## Disclosure

Dr Adam B Keeton reports support of this project through the Deanship of Scientific Research at King Saud University through Research Group Project Number RG-1440-140. The authors declare no other conflicts of interest.

**Table 5** Antiproliferative Activities of Compound **5m** and Sunitinib Against Sensitive (a-549) and Resistant (NCI-H69AR) Cell Lines

Compound No.	IC <sub>50</sub> (µM)		Fold-Resistance
	Sensitive A-549	Resistant NCI-H69AR	
<b>5m</b>	1.33 ± 0.47	>25	>19.2
Sunitinib	4.56	5.8 ± 0.52	1.9

**Abbreviation:** IC<sub>50</sub>, inhibitory concentration 50%.

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