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ORIGINAL RESEARCH

Isatin-benzoazine molecular hybrids as potential antiproliferative agents: synthesis and in vitro pharmacological profiling

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Hatem A Abdel-Aziz[1]
Wagdy M Eldehna[2]
Adam B Keeton[3]
Gary A Piazza[3]
Adnan A Kadi[4]
Mohamed W Attwa[4]
Ali S Abdelhameed[4]
Mohamed I Attia[4,5]

[1]Department of Applied Organic Chemistry, National Research Centre, Giza, [2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kafrelsheikh University, Kafrelsheikh, Egypt; [3]Department of Oncologic Sciences and Pharmacology, Drug Discovery Research Center, Mitchell Cancer Institute, University of South Alabama, Mobile, AL, USA; [4]Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia; [5]Medicinal and Pharmaceutical Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Centre, Giza, Egypt

Correspondence: Hatem A Abdel-Aziz
Department of Applied Organic Chemistry, National Research Centre, Dokki, Giza, PO Box 12622, Egypt
Tel +2 12 216 8624
Fax +2 23 337 0931
Email hatem_741@yahoo.com

Mohamed I Attia
Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia
Tel +966 1 467 3764
Fax +966 1 467 6220
Email mattia@ksu.edu.sa

Abstract: In continuation of our endeavor with respect to the development of potent and effective isatin-based anticancer agents, we adopted the molecular hybridization approach to design and synthesize four different sets of isatin-quinazoline (**6a–f** and **7a–e**)/phthalazine (**8a–f**)/quinoxaline (**9a–f**) hybrids. The antiproliferative activity of the target hybrids was assessed towards HT-29 (colon), ZR-75 (breast) and A-549 (lung) human cancer cell lines. Hybrids **8b–d** emerged as the most active antiproliferative congener in this study. Compound **8c** induced apoptosis via increasing caspase 3/7 activity by about 5-fold in the A-549 human cancer cell line. In addition, it exhibited an increase in the G1 phase and a decrease in the S and G2/M phases in the cell cycle effect assay. Furthermore, it displayed an inhibitory concentration 50% value of 9.5 µM against multidrug-resistant NCI-H69AR lung cancer cell line. The hybrid **8c** was also subjected to in vitro metabolic investigations through its incubation with rat liver microsomes and analysis of the resulting metabolites with the aid of liquid chromatography-mass spectrometry.

Keywords: isatins, hybridization approach, antiproliferative, apoptosis

Introduction

In the current medical era, molecular hybridization approach has stood out as a valuable and important structural modification tool useful for the discovery and development of better therapies for diverse human diseases, mostly for cancer.[1] The growing endeavors to discover hybrid drugs resulting from the combination of two or more haptophoric moieties of different bioactive substances have brought a new hope for the treatment of multifactorial disorders in recent years. Moreover, hybrid drugs can potentially overcome most of the pharmacokinetic drawbacks encountered by conventional anticancer drugs as well as provide combination therapies in a single multifunctional therapeutic agent at the target molecule conferring a more powerful, selective and safer drug compared to conventional classic treatments.[2–4]

Isatin (1*H*-indole-2,3-dione) is an endogenous compound found in many organisms, which was first isolated in 1988.[5] As a privileged scaffold, isatin has emerged as an attractive and promising nucleus in the development of novel anticancer agents.[6] Sunitinib (**I**) (Sutent™, Figure 1), granted the US Food and Drug Administration (FDA) approval in 2006, is an isatin-based orally active multi-targeted tyrosine kinase inhibitor used for the management of imatinib-resistant gastrointestinal stromal tumors and metastatic renal-cell carcinoma.[7–9] By 2014, Nintedanib (**II**, Vargatef™, Figure 1), an orally available triple angiokinase inhibitor, was approved in the US for the treatment of idiopathic pulmonary fibrosis. One year later, the European Medicines

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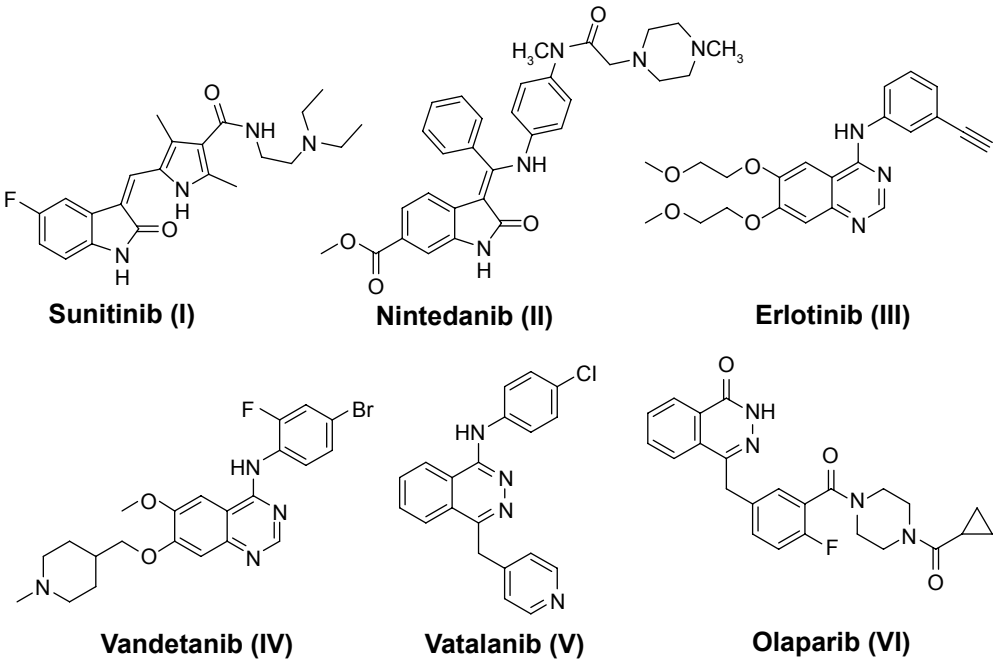


Figure 1 Chemical structures of clinically used anticancer agents.

Agency approved Nintedanib (**II**) as a second-line treatment in combination with docetaxel for non-small cell lung cancer of adenocarcinoma.[10,11] Also, semaxanib and orantinib are other examples for isatin-based anticancer agents that are being used in clinical trials and possess multiple tyrosine kinase receptor inhibitory activities.[12]

Over the last decade, several studies suggested the significance of developing isatin-based hybrids as promising anticancer agents;[13] among them, isatin-chromene **VII**,[14] isatin-pyridine **VIII**,[15] bis-isatin **IX**,[16,17] isatin-benzoxazole **X**,[18] isatin-benzimidazole **XI**,[19] isatin-benzothiazole,[20] isatin-thiazolidine/thiazolidinone,[21–25] isatin-4-piperazinylquinoline[26] and isatin-pyrazoline[27] hybrids were reported (Figure 2).

On the other hand, quinazolines constitute the cornerstone for a number of tyrosine kinase inhibitors such as the reversible EGFR-inhibitor. Erlotinib (**III**, Tarceva™,

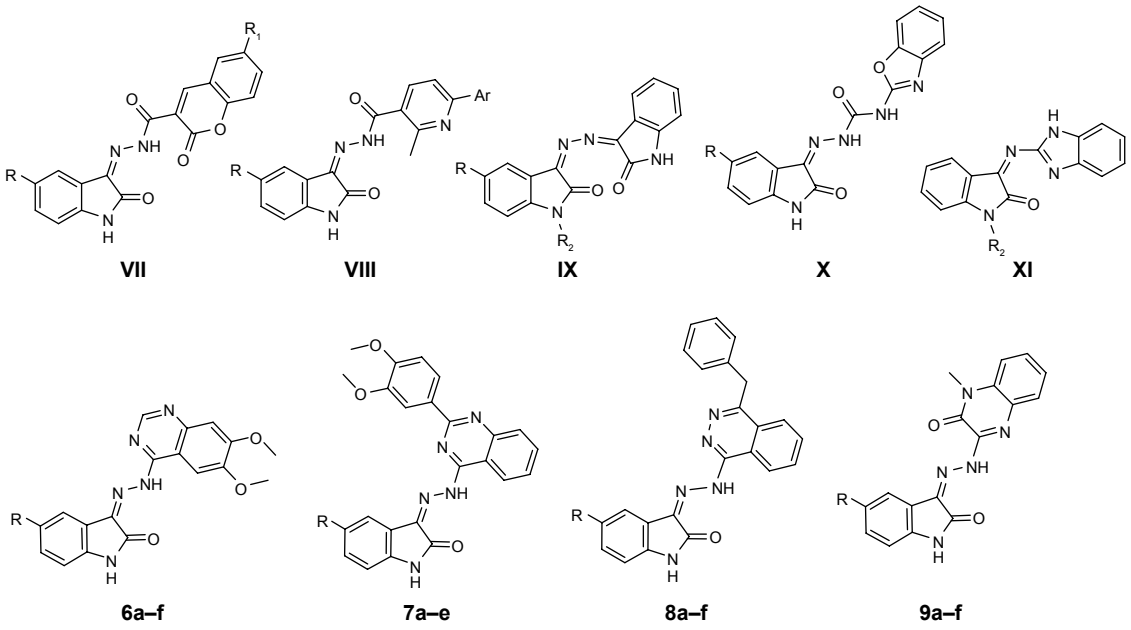


Figure 2 Structures of some reported isatin-based hybrids with promising anticancer activity and structures of the target hybrids **6a–f**, **7a–e**, **8a–f** and **9a–f**.

Figure 1), as well as the dual VEGFR-2-EGFR inhibitor Vandetanib (**IV**, Caprelsa™, Figure 1), is indicated for the treatment of symptomatic or progressive medullary thyroid cancer in patients with unresectable locally advanced or metastatic disease.[28,29] Also, phthalazine is another attractive scaffold forming the backbone of certain promising antitumor lead candidates. Among them, Vatalanib (**V**, PTK787, Figure 1) is an orally active VEGFR-1 and VEGFR-2 inhibitor undergoing phase III clinical trials for the treatment of colorectal cancer.[30,31] Olaparib (**VI**, Lynparza, Figure 1), the first approved phthalazine-based anticancer drug, is an oral small molecule poly (ADP-ribose) polymerase inhibitor that was approved in 2014 for the treatment of BRCA-mutated ovarian cancer.[32] Recently, much attention has been paid to anticancer drug discovery based on the quinoxaline nucleus as an important heterocyclic one that exhibited interesting biological activities.[33] It has been documented that several isatin-quinazoline and isatin-phthalazine hybrids displayed promising anticancer activities.[34,35]

In the light of the aforementioned findings and in continuation of our endeavor with respect to the development of potent and effective isatin-based anticancer agents,[35,36] we adopted the molecular hybridization approach to design and synthesize four different sets of isatin-quinazolines (**6a–f** and **7a–e**)/phthalazines (**8a–f**)/quinoxaline (**9a–f**) hybrids (Figure 2). All the synthesized hybrids (**6a–f**, **7a–e**, **8a–f** and **9a–f**) were in vitro evaluated for their antiproliferative activity against three human cancer cell lines, namely human colon cancer HT-29 cell line, breast cancer ZR-75 cell line and lung cancer A-549 cell line. Moreover, the most active congeners were further assessed for their apoptosis induction potential using human cancer A-549 cell line, via evaluation of their effects on the expression of caspase 3/7 as well as on the cell cycle progression, to obtain mechanistic insights into their anticancer activity. Furthermore, their antiproliferative activity against multidrug-resistant lung cancer NCI-H69AR cell line was evaluated. Finally, the most active candidates were subjected to in vitro metabolic investigations through their incubation with rat liver microsomes (RLMs) and analysis of the resulting metabolites with the aid of liquid chromatography-mass spectrometry (LC-MS).

Materials and methods

General

Melting points of the synthesized compounds were measured with a Stuart melting point apparatus (Staffordshire, UK) and were uncorrected. Infrared (IR) spectra were recorded as KBr disks using FT-IR Spectrum BX apparatus (Perkin Elmer, Shelton, CT, USA). Mass spectra were recorded using Agilent Quadrupole 6120 LC-MS with electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). NMR spectra were recorded on a Bruker NMR spectrometer (Bruker Biospin, Billerica, MA, USA). ^{1}H spectra were run at 500 or 700 MHz, and ^{13}C spectra were run at 125 or 175 MHz in deuterated dimethyl sulfoxide (DMSO-d_6). Chemical shifts are expressed in δ values (ppm) using the solvent peak as internal standard. All coupling constant (J) values are given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Elemental analyses were carried out at Microanalytical Centre, Cairo University, Egypt. High-resolution mass spectrometry (HRMS) measurements were performed on an LTQ-Orbitrap XL coupled to matrix-assisted laser desorption ionization (MALDI). Reaction courses and product mixtures were routinely monitored by thin layer chromatography on silica-gel precoated F_{254} Merck plates (Merck Millipore, Billerica, MA, USA). Unless otherwise noted, all solvents and reagents were commercially available and used without further purification. Compounds **1**,[37] **2**,[35] **3**,[38] and **4**[39] were prepared according to the reported method. All cell lines were purchased from the American Type Culture Collection (ATCC) as follows: HT-29: (ATCC® HTB-38™); ZR75: (ATCC® CRL-1500™); A549: (ATCC® CRM-CCL-185™); IEC-6: (ATCC® CRL-1592™); 3T3-Swiss albino (ATCC® CCL-92™); MCF 10A (ATCC® CRL-10317™); H69AR (ATCC® CRL-11351™).

Chemistry

General procedure for preparation of the target hybrids 6a–f, 7a–e, 8a–f and 9a–f

The appropriate indoline-2,3-dione derivative **5a–f** (1 mmol) was added to a suspension of each hydrazinyl intermediate **1–4** (1 mmol) in ethanol (10 mL) and a catalytic amount of glacial acetic acid. The reaction mixture was refluxed for 1 h. The precipitate formed was collected by filtration while hot, washed with hot ethanol, dried and re-crystallized from DMF/ethanol to furnish the desired hybrids.

3-(2-(6,7-Dimethoxyquinazolin-4-yl)hydrazono) indolin-2-one (**6a**) – orange powder (yield 75%), m.p. 297°C–299°C; IR (KBr, ν cm^{-1}): 3,411 (NH) and 1,699 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.96 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 6.87–6.89 (m, 2H, Ar-H), 7.17 (t, 1H, Ar-H, J=8.9 Hz), 7.21 (s, 1H, Ar-H), 7.74 (s, 1H, Ar-H), 8.06 (s, 1H, Ar-H), 8.25 (d, 1H, Ar-H, J=8.8 Hz), 10.61 (s, 1H, NH), 12.36 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 55.8 (OCH_3), 56.58 (OCH_3), 104.2, 108.6, 111.1, 111.2, 112.9, 114.4, 114.5, 117.4, 118.9, 139.6, 144.3, 149.6, 155.4, 157.2,

158.5, 166.4 (C=O); MS (ESI) *m/z*: 350.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{15}N_5O_3$ (349.12): C, 61.89; H, 4.33; N, 20.05; found C, 62.13; H, 4.28; N, 20.11; HRMS (MALDI) calcd. for $C_{18}H_{15}N_5O_3$: 350.1253, found: 350.1224 $[M+H]^+$.

3-(2-(6,7-Dimethoxyquinazolin-4-yl)hydrazono)-5-fluoroindolin-2-one (**6b**) – orange powder (yield 73%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,420 (NH) and 1,700 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 4.01 (s, 3H, OCH_3), 4.03 (s, 3H, OCH_3), 7.00–7.39 (m, 3H, Ar-H), 7.73 (s, 1H, Ar-H), 8.07 (s, 1H, Ar-H), 8.72 (s, 1H, Ar-H), 11.33 (s, 1H, NH), 13.65 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 56.0, 56.5, 104.4, 108.5, 110.4 ($^3J_{F-C}$ =9.0 Hz), 111.9, ($^2J_{F-C}$ =27.0 Hz), 113.0 ($^2J_{F-C}$ =25.5 Hz), 118.4 ($^2J_{F-C}$ =8.0 Hz), 120.4, 122.3, 128.0, 131.5, 133.4, 144.4, 149.5, 153.8 ($^1J_{F-C}$ =238.0 Hz), 155.5, 166.2; MS (ESI) *m/z*: 368.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{14}FN_5O_3$ (367.11): C, 58.85; H, 3.84; N, 19.07; found C, 59.09; H, 3.77; N, 19.13; HRMS (MALDI) calcd. for $C_{18}H_{14}FN_5O_3$: 368.1159, found: 368.1151 $[M+H]^+$.

5-Chloro-3-(2-(6,7-dimethoxyquinazolin-4-yl)hydrazono)indolin-2-one (**6c**) – orange powder (yield 80%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,421 (NH) and 1,706 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.96 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 6.90 (d, 1H, Ar-H, *J* =8.3 Hz), 7.21 (s, 1H, Ar-H), 7.36 (d, 1H, Ar-H, *J* =8.3 Hz), 7.72 (s, 1H, Ar-H), 8.08 (s, 1H, Ar-H), 8.57 (s, 1H, Ar-H), 10.71 (s, 1H, NH), 12.45 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 55.7 (OCH_3), 56.6 (OCH_3), 104.0, 108.5, 111.78, 112.9, 119.7, 125.7, 127.4, 130.5, 141.9, 142.5, 144.0, 149.5, 152.4, 153.7, 155.4, 166.0 (C=O); MS (ESI) *m/z*: 384.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{14}ClN_5O_3$ (383.08): C, 56.33; H, 3.68; N, 18.25; found C, 56.21; H, 3.72; N, 18.17; HRMS (MALDI) calcd. for $C_{18}H_{14}ClN_5O_3$: 384.0863, found: 384.0853 $[M+H]^+$.

5-Bromo-3-(2-(6,7-dimethoxyquinazolin-4-yl)hydrazono)indolin-2-one (**6d**) – orange powder (yield 85%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,420 (NH) and 1,717 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 3.96 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 6.86 (d, 1H, Ar-H, *J* =8.3 Hz), 7.21 (s, 1H, Ar-H), 7.48 (d, 1H, Ar-H, *J*=8.3 Hz), 7.72 (s, 1H, Ar-H), 8.08 (s, 1H, Ar-H), 8.73 (s, 1H, Ar-H), 10.73 (s, 1H, NH), 12.45 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 55.9 (OCH_3), 56.6 (OCH_3), 104.0, 108.4, 112.3, 112.9, 113.5, 120.2, 130.3, 133.3, 142.3, 142.9, 143.6, 144.3, 149.6, 155.5, 155.8, 165.9 (C=O); MS (ESI) *m/z*: 428.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{14}BrN_5O_3$ (427.03): C, 50.48; H, 3.30; N, 16.35; found C, 50.61; H, 3.26; N, 16.28; HRMS (MALDI) calcd. for $C_{18}H_{14}BrN_5O_3$: 428.0358, found: 428.0353 $[M+H]^+$.

3-(2-(6,7-Dimethoxyquinazolin-4-yl)hydrazono)-5-methoxyindolin-2-one (**6e**) – red powder (yield 72%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,413 (NH) and 1,699 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 3.81 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.80 (d, 1H, Ar-H, *J* =8.4 Hz), 6.90 (d, 1H, Ar-H, *J* =8.4 Hz), 7.19 (s, 1H, Ar-H), 7.76 (s, 1H, Ar-H), 8.06 (s, 1H, Ar-H), 8.71 (s, 1H, Ar-H), 10.40 (s, 1H, NH), 12.23 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 55.6 (OCH_3), 55.7 (OCH_3), 56.5 (OCH_3), 104.2, 108.6, 111.1, 112.5, 113.0, 118.0, 118.7, 137.2, 143.2, 143.7, 144.1, 149.4, 154.1, 154.9, 155.2, 166.4 (C=O); MS (ESI) *m/z*: 380.0 $[M+H]^+$; Anal. calcd. for $C_{19}H_{17}N_5O_4$ (379.13): C, 60.15; H, 4.52; N, 18.46; found C, 59.91; H, 4.58; N, 18.59; HRMS (MALDI) calcd. for $C_{19}H_{17}N_5O_4$: 380.1359, found: 380.1345 $[M+H]^+$.

3-(2-(6,7-Dimethoxyquinazolin-4-yl)hydrazono)-5-methylindolin-2-one (**6f**) – orange powder (yield 75%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,421 (NH) and 1,700 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 2.31 (s, 3H, CH_3), 3.95 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 6.77 (d, 1H, Ar-H, *J* =7.5 Hz), 7.12 (d, 1H, Ar-H, *J* =7.0 Hz), 7.18 (s, 1H, Ar-H), 7.77 (s, 1H, Ar-H), 8.02 (s, 1H, Ar-H), 8.69 (s, 1H, Ar-H), 10.48 (s, 1H, NH), 12.32 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 21.3 (CH_3), 55.7 (OCH_3), 56.5 (OCH_3), 104.2, 108.6, 110.1, 113.1, 118.6, 128.9, 130.8, 131.8, 141.2, 143.8, 144.8, 149.4, 153.3, 155.1, 156.3, 166.4 (C=O); MS (ESI) *m/z*: 364.0 $[M+H]^+$; Anal. calcd. for $C_{19}H_{17}N_5O_3$ (363.13): C, 62.80; H, 4.72; N, 19.27; found C, 63.03; H, 4.66; N, 19.15; HRMS (MALDI) calcd. for $C_{19}H_{17}N_5O_3$: 364.1409, found: 364.1422 $[M+H]^+$.

3-(2-(2-(3,4-Dimethoxyphenyl)quinazolin-4-yl)hydrazono)indolin-2-one (**7a**).[35]

3-(2-(2-(3,4-Dimethoxyphenyl)quinazolin-4-yl)hydrazono)-5-fluoroindolin-2-one (**7b**).[35]

5-Chloro-3-(2-(2-(3,4-dimethoxyphenyl)quinazolin-4-yl)hydrazono)indolin-2-one (**7c**).[35]

5-Bromo-3-(2-(2-(3,4-dimethoxyphenyl)quinazolin-4-yl)hydrazono)indolin-2-one (**7d**) – red powder (yield 80%), m.p. 295°C–297°C; IR (KBr, ν cm^{-1}): 3,421 (NH) and 1,718 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.87 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.96 (d, 1H, Ar-H, *J* =8.0 Hz), 7.15 (d, 1H, Ar-H, *J* =8.0 Hz), 7.55 (d, 1H, Ar-H, *J* =8.5 Hz), 7.73 (t, 1H, Ar-H, *J*=7.0 Hz), 7.85 (s, 1H, Ar-H), 7.94–7.97 (m, 2H, Ar-H), 8.12 (s, 1H, Ar-H), 8.18 (d, 1H, Ar-H, *J* =8.5 Hz), 8.52–8.53 (m, 1H, Ar-H), 11.49 (s, 1H, NH), 13.81 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 56.2 (OCH_3), 56.3 (OCH_3), 110.7, 110.9, 112.1, 118.1, 120.6, 122.4, 125.1, 125.3, 127.7, 128.0, 128.4, 132.3, 132.5, 134.9, 143.3, 146.0, 147.4, 149.3, 152.5, 166.2 (C=O); MS (ESI) *m/z*: 504 $[M+H]^+$; Anal. calcd. for $C_{24}H_{18}BrN_5O_3$ (503.06):

C, 57.16; H, 3.60; N, 13.89; found C, 57.29; H, 3.63; N, 13.79; HRMS (MALDI) calcd. for $C_{24}H_{18}BrN_5O_3$: 504.0671, found: 504.0653 $[M+H]^+$.

3-(2-(2-(3,4-Dimethoxyphenyl)quinazolin-4-yl) hydrazono)-5-methoxyindolin-2-one (**7e**) – orange powder (yield 79%), m.p. 263°C–265°C; IR (KBr, ν cm^{-1}): 3,412 (NH) and 1,718 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.76 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.83 (d, 1H, Ar-H, J=8.5 Hz), 6.96 (d, 1H, Ar-H, J=8.5 Hz), 7.14 (s, 1H, Ar-H), 7.19 (d, 1H, Ar-H, J =9.0 Hz), 7.81 (t, 1H, Ar-H, J =7.5 Hz), 7.96 (s, 1H, Ar-H), 8.06–8.09 (m, 4H, Ar-H), 11.31 (s, 1H, NH), 13.08 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 55.8 (OCH_3), 56.2 (OCH_3), 56.3 (OCH_3), 110.0, 110.8, 111.5, 113.3, 114.4, 117.6, 120.9, 123.1, 124.8, 125.8, 127.1, 127.9, 129.1, 131.8, 133.0, 135.2, 142.5, 144.7, 148.2, 149.1, 154.8, 166.3 (C=O); MS (ESI) *m/z*: 456 $[M+H]^+$; Anal. calcd. for $C_{25}H_{21}N_5O_4$ (455.16): C, 65.93; H, 4.65; N, 15.38; found C, 66.17; H, 4.59; N, 15.52; HRMS (MALDI) calcd. for $C_{25}H_{21}N_5O_4$: 456.1672, found: 456.1662 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)indolin-2-one (**8a**) – orange powder (yield 75%), m.p. 259°C–261°C; IR (KBr, ν cm^{-1}): 3,412 (NH) and 1,700 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 4.36 (s, 2H, CH_2), 6.89 (d, 1H, Ar-H, J =7.5 Hz), 7.06 (t, 1H, Ar-H, J =7.5 Hz), 7.19 (t, 1H, Ar-H, J =7.5 Hz), 7.29–7.36 (m, 5H, Ar-H), 7.86–7.98 (m, 3H, Ar-H), 8.45 (d, 1H, Ar-H, J =7.5 Hz), 8.63 (d, 1H, Ar-H, J =7.5 Hz), 10.63 (s, 1H, NH), 12.89 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 38.1 (CH_2), 111.6, 115.6, 118.3, 120.3, 122.3, 126.2, 127.0, 127.6, 127.8, 128.9, 129.1, 131.4, 132.9, 134.0, 138.6, 143.3, 144.0, 148.4, 156.5, 166.7; MS (ESI) *m/z*: 380.0 $[M+H]^+$; Anal. calcd. for $C_{23}H_{17}N_5O$ (379.14): C, 72.81; H, 4.52; N, 18.46; found C, 73.01; H, 4.48; N, 18.55; HRMS (MALDI) calcd. for $C_{23}H_{17}N_5O$: 380.1511, found: 380.1525 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)-5-fluoroindolin-2-one (**8b**) – orange powder (yield 79%), m.p. 275°C–277°C; IR (KBr, ν cm^{-1}): 3,411 (NH) and 1,705 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 4.39 (s, 2H, CH_2), 6.88–7.36 (m, 7H, Ar-H), 7.90–8.01 (m, 3H, Ar-H), 8.19 (d, 1H, Ar-H, J=8.5 Hz), 8.61 (d, 1H, Ar-H, J=7.0 Hz), 10.62 (s, 1H, NH), 12.93 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 38.1 (CH_2), 111.1 ($^3J_{F\text{-}C}$ =8.8 Hz), 114.0 ($^2J_{F\text{-}C}$ =24.5 Hz), 117.3 ($^2J_{F\text{-}C}$ =25.0 Hz), 118.7 ($^3J_{F\text{-}C}$ =8.8 Hz), 125.9, 126.2, 126.6, 127.0, 127.6, 128.9, 129.1, 133.1, 134.1, 138.6, 139.4, 143.3, 148.9, 152.8, 157.3 ($^1J_{F\text{-}C}$ =234.5 Hz), 166.5; MS (ESI) *m/z*: 398.0 $[M+H]^+$; Anal. calcd. for $C_{23}H_{16}FN_5O$ (397.13): C, 69.51; H, 4.06; N, 17.62; found C, 69.32; H, 4.13; N, 17.51; HRMS (MALDI) calcd. for $C_{23}H_{16}FN_5O$: 398.1417, found: 398.1405 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)-5-chloroindolin-2-one (**8c**) – orange powder (yield 80%), m.p. 295°C–297°C; IR (KBr, ν cm^{-1}): 3,410 (NH) and 1,700 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 4.39 (s, 2H, CH_2), 6.91 (d, 1H, Ar-H, J =8.5 Hz), 7.21 (t, 1H, Ar-H, J =7.5 Hz), 7.30–7.36 (m, 5H, Ar-H), 7.90–8.02 (m, 3H, Ar-H), 8.42 (s, 1H, Ar-H), 8.56 (d, 1H, Ar-H, J =7.0 Hz), 10.73 (s, 1H, NH), 12.96 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 38.1 (CH_2), 111.8, 119.5, 125.7, 126.3, 126.6, 126.7, 127.0, 127.7, 128.9, 129.0, 129.1, 130.5, 133.1, 134.2, 138.6, 141.8, 142.7, 149.0, 152.8, 166.2 (C=O); MS (ESI) *m/z*: 414.0 $[M+H]^+$; Anal. calcd. for $C_{23}H_{16}ClN_5O$ (413.10): C, 66.75; H, 3.90; N, 16.92; found C, 66.97; H, 3.83; N, 17.05; HRMS (MALDI) calcd. for $C_{23}H_{16}ClN_5O$: 414.1122, found: 414.1146 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)-5-bromoindolin-2-one (**8d**) – orange powder (yield 86%), m.p. 298°C–299°C; IR (KBr, ν cm^{-1}): 3,412 (NH) and 1,716 (C=O); ^{1}H NMR (DMSO-d_6) δppm: 4.39 (s, 2H, CH_2), 6.86 (d, 1H, Ar-H, J =8.5 Hz), 7.21 (t, 1H, Ar-H, J =7.5 Hz), 7.30–7.36 (m, 3H, Ar-H), 7.47 (d, 1H, Ar-H, J =8.5 Hz), 7.90–8.02 (m, 3H, Ar-H), 8.20 (s, 1H, Ar-H), 8.53–8.56 (m, 2H, Ar-H), 10.74 (s, 1H, NH), 12.69 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 38.1 (CH_2), 112.3, 113.5, 120.0, 126.4, 126.6, 127.0, 127.7, 128.8, 128.9, 129.1, 129.5, 133.0, 133.3, 134.2, 138.5, 142.1, 142.6, 149.0, 152.8, 166.0 (C=O); MS (ESI) *m/z*: 458.0 $[M+H]^+$; Anal. calcd. for $C_{23}H_{16}BrN_5O$ (457.05): C, 60.28; H, 3.52; N, 15.28; found C, 60.46; H, 3.47; N, 15.40; HRMS (MALDI) calcd. for $C_{23}H_{16}BrN_5O$: 458.0617, found: 458.0605 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)-5-methoxyindolin-2-one (**8e**) – red powder (yield 74%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,420 (NH) and 1,707 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.79 (s, 3H, OCH_3), 4.37 (s, 2H, CH_2), 6.80 (d, 1H, Ar-H, J =8.5 Hz), 6.91 (d, 1H, Ar-H, J =8.5 Hz), 7.19 (t, 1H, Ar-H, J =7.5 Hz), 7.29–7.36 (m, 4H, Ar-H), 7.87–7.99 (m, 3H, Ar-H), 8.08 (s, 1H, Ar-H), 8.56 (d, 1H, Ar-H, J =8.0 Hz), 10.44 (s, 1H, NH), 12.88 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δppm: 38.1 (CH_2), 55.8 (OCH_3), 110.7, 112.1, 113.5, 116.9, 120.1, 123.7, 126.4, 127.1, 127.6, 128.8, 129.0, 129.2, 131.7, 133.8, 138.5, 141.7, 144.2, 146.2, 149.9, 154.5, 166.5 (C=O); MS (ESI) *m/z*: 410.0 $[M+H]^+$; Anal. calcd. for $C_{24}H_{19}N_5O_2$ (409.15): C, 70.40; H, 4.68; N, 17.10; found C, 70.64; H, 4.63; N, 16.98; HRMS (MALDI) calcd. for $C_{24}H_{19}N_5O_2$: 410.1617, found: 410.1644 $[M+H]^+$.

3-(2-(4-Benzylphthalazin-1-yl)hydrazono)-5-methylindolin-2-one (**8f**) – orange powder (yield 71%), m.p. 253°C–255°C; IR (KBr, ν cm^{-1}): 3,350 (NH) and 1,697 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 2.34 (s, 3H, CH_3), 4.36 (s, 2H, CH_2), 6.78 (d, 1H, Ar-H, *J* =7.5 Hz), 7.12 (d, 1H, Ar-H, *J* =8.0 Hz), 7.21 (t, 1H, Ar-H, *J* =7.5 Hz), 7.29–7.36 (m, 4H, Ar-H), 7.88–7.97 (m, 3H, Ar-H), 8.26 (s, 1H, Ar-H), 8.60 (d, 1H, Ar-H, *J* =7.5 Hz), 10.52 (s, 1H, NH), 12.84 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 21.4 (CH_3), 38.1 (CH_2), 110.1, 118.5, 125.8, 126.1, 126.8, 127.0, 127.5, 128.3, 128.9, 129.1, 130.8, 131.7, 132.8, 133.8, 138.6, 141.2, 144.6, 148.2, 152.1, 166.6 (C=O); MS (ESI) *m/z*: 394.0 $[M+H]^+$; Anal. calcd. for $C_{24}H_{19}N_5O$ (393.16): C, 73.27; H, 4.87; N, 17.80; found C, 73.39; H, 4.91; N, 17.72; HRMS (MALDI) calcd. for $C_{24}H_{19}N_5O$: 394.1668, found: 394.1646 $[M+H]^+$.

1-Methyl-3-(2-(2-oxoindolin-3-ylidene)hydrazinyl) quinoxalin-2(1*H*)-one (**9a**) – orange powder (yield 80%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,347 (NH) and 1,711 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.71 (s, 3H, CH_3), 6.95 (d, 1H, Ar-H, *J* =7.5 Hz), 7.10 (t, 1H, Ar-H, *J* =7.5 Hz), 7.33–7.39 (m, 2H, Ar-H), 7.46 (t, 1H, Ar-H, *J* =7.5 Hz), 7.54 (d, 1H, Ar-H, *J* =8.0 Hz), 7.64 (d, 1H, Ar-H, *J* =7.5 Hz), 7.71 (d, 1H, Ar-H, *J* =7.5 Hz), 10.72 (s, 1H, NH), 11.24 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 29.8 (CH_3), 111.5, 115.3, 120.1, 121.0, 122.9, 124.7, 126.6, 131.5, 132.6, 137.8, 140.5, 142.0, 143.2, 150.5, 162.3, 168.3; MS (ESI) *m/z*: 320.0 $[M+H]^+$; Anal. calcd. for $C_{17}H_{13}N_5O_2$ (319.11): C, 63.94; H, 4.10; N, 21.93; found C, 64.17; H, 4.07; N, 21.84; HRMS (MALDI) calcd. for $C_{17}H_{13}N_5O_2$: 320.1148, found: 320.1133 $[M+H]^+$.

3-(2-(5-Fluoro-2-oxoindolin-3-ylidene)hydrazinyl)-1-methylquinoxalin-2(1*H*)-one (**9b**) – red powder (yield 75%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,412 (NH) and 1,701 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.71 (s, 3H, CH_3), 6.94–6.97 (m, 1H, Ar-H), 7.18 (t, 1H, Ar-H, *J* =7.0 Hz), 7.37 (t, 1H, Ar-H, *J* =7.5 Hz), 7.43–7.50 (m, 2H, Ar-H), 7.55 (d, 1H, Ar-H, *J* =8.0 Hz), 7.71 (d, 1H, Ar-H, *J* =7.5 Hz), 11.26 (s, 1H, NH), 13.70 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 29.7 (CH_3), 111.4 ($^3J_{F-C}$ =9.0 Hz), 115.2 ($^2J_{F-C}$ =28.8 Hz), 118.5 ($^2J_{F-C}$ =28.00 Hz), 119.0 ($^3J_{F-C}$ =9.0 Hz), 121.9, 123.9, 125.7, 128.2, 131.5, 138.3, 140.4, 147.1, 150.8, 156.8 ($^1J_{F-C}$ =239.5 Hz), 163.5, 165.9; MS (ESI) *m/z*: 338.0 $[M+H]^+$; Anal. calcd. for $C_{17}H_{12}FN_5O_2$ (337.10): C, 60.53; H, 3.59; N, 20.76; found C, 60.74; H, 3.62; N, 20.67; HRMS (MALDI) calcd. for $C_{17}H_{12}FN_5O_2$: 338.1053, found: 338.1041 $[M+H]^+$.

3-(2-(5-Chloro-2-oxoindolin-3-ylidene)hydrazinyl)-1-methylquinoxalin-2(1*H*)-one (**9c**) – orange powder (yield 81%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,411 (NH) and 1,698 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.71 (s, 3H, CH_3), 6.97 (d, 1H, Ar-H, *J*=8.5 Hz), 7.38–7.41 (m, 2H, Ar-H), 7.50 (t, 1H, Ar-H, *J*=7.5 Hz), 7.56 (d, 1H, Ar-H, *J*=8.5 Hz), 7.62 (s, 1H, Ar-H), 7.72 (d, 1H, Ar-H, *J*=8.0 Hz), 11.37 (s, 1H, NH), 13.65 (s, 1H, NH), ^{13}C NMR (DMSO-d_6) δ ppm: 29.8 (CH_3), 112.1, 115.4, 117.5, 120.3, 122.8, 124.7, 126.1, 128.3, 130.7, 132.1, 134.7, 140.9, 142.7, 150.9, 163.3, 165.5; MS (ESI) *m/z*: 354.0 $[M+H]^+$; Anal. calcd. for $C_{17}H_{12}ClN_5O_2$ (353.07): C, 57.72; H, 3.42; N, 19.80; found C, 57.88; H, 3.38; N, 19.91; HRMS (MALDI) calcd. for $C_{17}H_{12}ClN_5O_2$: 354.0758, found: 354.0757 $[M+H]^+$.

3-(2-(5-Bromo-2-oxoindolin-3-ylidene)hydrazinyl)-1-methylquinoxalin-2(1*H*)-one (**9d**) – orange powder (yield 84%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,411 (NH) and 1,708 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.72 (s, 3H, CH_3), 6.93 (d, 1H, Ar-H, *J*=8.5 Hz), 7.38 (t, 1H, Ar-H, *J*=7.5 Hz), 7.48–7.58 (m, 3H, Ar-H), 7.73–7.74 (m, 2H, Ar-H), 11.44 (s, 1H, NH), 13.64 (s, 1H, NH), ^{13}C NMR (DMSO-d_6) δ ppm: 29.8 (CH_3), 112.6, 115.4, 117.1, 119.7, 122.8, 124.0, 127.6, 128.3, 131.2, 133.4, 141.2, 143.1, 147.8, 150.8, 163.1, 165.4; MS (ESI) *m/z*: 398.0 $[M+H]^+$; Anal. calcd. for $C_{17}H_{12}BrN_5O_2$ (397.02): C, 51.27; H, 3.04; N, 17.59; found C, 51.09; H, 3.07; N, 17.70; HRMS (MALDI) calcd. for $C_{17}H_{12}BrN_5O_2$: 398.0253, found: 398.0243 $[M+H]^+$.

3-(2-(5-Methoxy-2-oxoindolin-3-ylidene)hydrazinyl)-1-methylquinoxalin-2(1*H*)-one (**9e**) – red powder (yield 78%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,413 (NH) and 1,707 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.69 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 6.80–6.94 (m, 2H, Ar-H), 7.14–7.51 (m, 4H, Ar-H), 7.69 (d, 1H, Ar-H, *J*=8.0 Hz), 10.53 (s, 1H, NH), 13.70 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 38.1 (CH_3), 55.8 (OCH_3), 110.8, 113.6, 116.5, 118.9, 125.5, 126.8, 127.6, 128.9, 129.1, 132.9, 133.9, 137.0, 138.6, 148.4, 154.9, 166.5; MS (ESI) *m/z*: 350.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{15}N_5O_3$ (349.12): C, 61.89; H, 4.33; N, 20.05; found C, 62.11; H, 4.29; N, 19.94; HRMS (MALDI) calcd. for $C_{18}H_{15}N_5O_3$: 350.1253, found: 350.1239 $[M+H]^+$.

1-Methyl-3-(2-(5-methyl-2-oxoindolin-3-ylidene) hydrazinyl)quinoxalin-2(1*H*)-one (**9f**) – orange powder (yield 77%), m.p. >300°C; IR (KBr, ν cm^{-1}): 3,411 (NH) and 1,700 (C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 3.70 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 6.82 (d, 1H, Ar-H, *J* =7.5 Hz), 7.14–7.16 (m, 2H, Ar-H), 7.36 (d, 1H, Ar-H, *J*=7.0 Hz), 7.44 (s, 1H, Ar-H), 7.52 (d, 1H, Ar-H, *J* =7.5 Hz), 7.68 (d, 1H, Ar-H, *J*=7.0 Hz), 11.11 (s, 1H, NH), 13.63 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ ppm: 21.0 (CH_3), 29.8 (CH_3), 111.3, 115.4, 118.1, 121.4, 124.6, 127.2, 131.8, 132.5, 134.6, 137.4, 140.7, 146.3, 152.4, 157.8, 162.5, 164.1; MS (ESI) *m/z*: 334.0 $[M+H]^+$; Anal. calcd. for $C_{18}H_{15}N_5O_2$ (333.12): C, 64.86; H, 4.54; N, 21.01; found C, 64.69; H, 4.61; N, 21.12; HRMS

(MALDI) calcd. for $C_{18}H_{15}N_5O_2$: 334.1304, found: 350. 334.1322 $[M+H]^+$.

Pharmacological evaluation

The details of the experimental protocols are provided in Supplementary materials.

Metabolic investigations

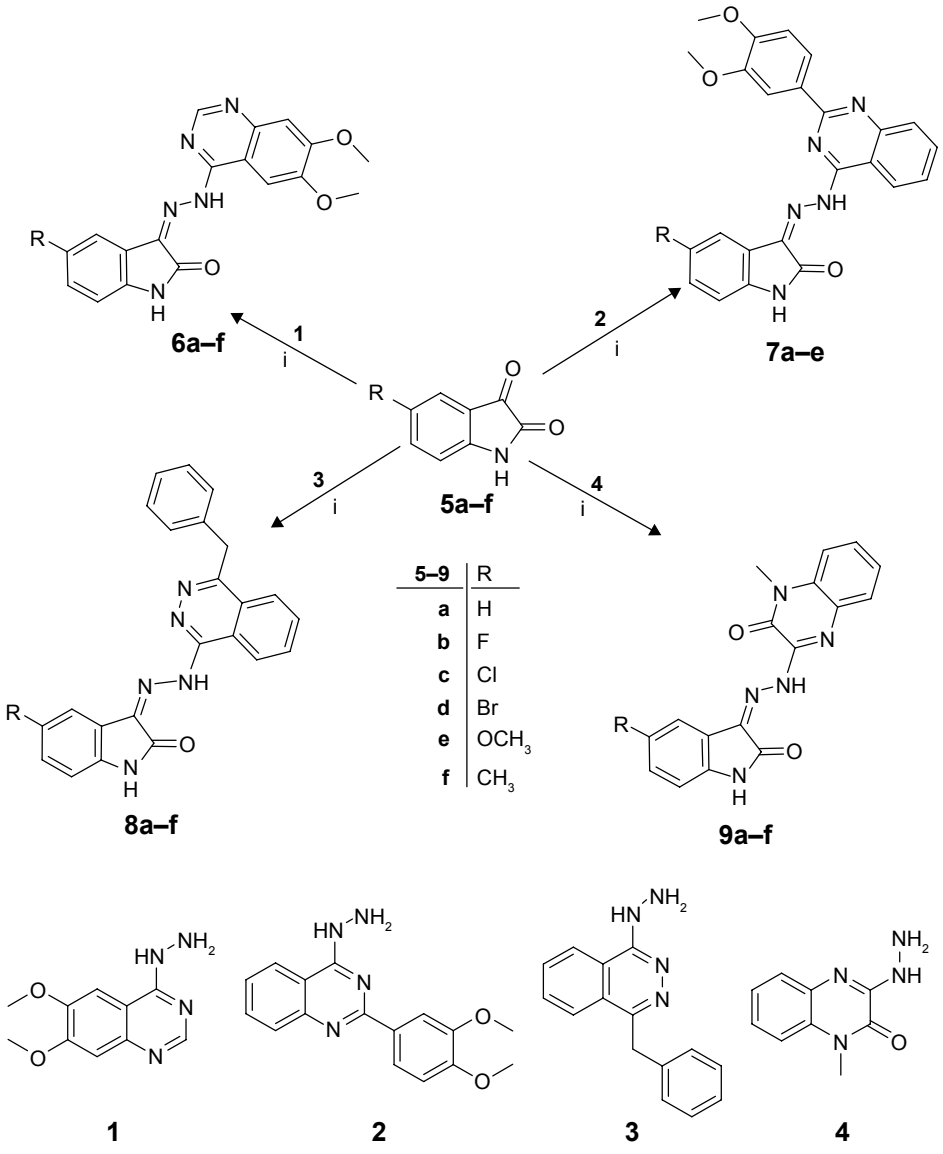
The study protocol was approved by the Research Ethics Committee at College of Pharmacy, King Saud University. Animals were maintained according to the guidelines of Animal Care Center, College of Pharmacy, King Saud University, and approved by the Local Animal Care and Use Committee of King Saud University. The details of the experimental protocols[40–42] are provided in Supplementary materials.

Results and discussion

Chemistry

The synthetic pathway employed to prepare the target isatin derivatives is outlined in Scheme 1. The target compounds **6a–f**, **7a–e**, **8a–f** and **9a–f** were obtained by the reaction of the appropriate indoline-2,3-diones **5a–f** with the hydrazinyl intermediates **1–4** in refluxed ethanol in the presence of a catalytic amount of glacial acetic acid with 70%–86% yields (Scheme 1).

IR spectra of the target compounds **6a–f**, **7a–e**, **8a–f** and **9a–f** showed absorption bands due to the NH groups in the region 3,347–3,421 cm^{-1}, in addition to carbonyl bands in the region 1,697–1,718 cm^{-1}. Their ^1H NMR spectra showed two singlet signals attributable to NH protons of the isatin and the hydrazine function (=N–NH–) in the region



Scheme 1 Synthesis of targets hydrazino-isatins **6a–f**, **7a–e**, **8a–f** and **9a–f** and the chemical structures of the intermediates **1–4**. Reagents and conditions: (i) Ethanol/glacial acetic acid (catalytic)/reflux for 1 h.

δ 10.40–11.44 and 11.24–13.81 ppm. Also, the methoxy ($-OCH_3$) protons of compounds **6a–f** appeared as singlet signals around δ 4.00 ppm, while the methoxy protons of derivatives **7a–e** appeared around δ3.80 ppm in the ^{1}H NMR spectra. Furthermore, the ($-CH_2$) protons of benzylic moiety of **8a–f** appeared as a singlet signal in the range δ4.36–4.37 ppm, while in case of **9a–f** the signals of the aliphatic protons ($N-CH_3$) were observed as singlets near to δ3.70 ppm.

Pharmacological evaluation

Antiproliferative activity

A total of 23 compounds were analyzed for cancer cell growth inhibitory activity. These studies were carried out using cells derived from human lung, colon and breast tumors (A-549, HT-29 and ZR-75 cells, respectively). This initial assessment of activity tested each compound in quadruplicate at a single concentration of 30 μM, if solubility permitted. As indicated in Table 1, compounds **8b–d** are the most potent congeners, inhibiting growth of all three cell lines with average growth inhibition values of 93.8, 96.5 and 96.4%, respectively, at a test concentration of 30 μM. The rest of the compounds showed an average growth inhibition values from 6.1%–81.2% at the tested concentration levels.

Table 1 Antiproliferative (cell growth inhibitory activity at 30 μM concentration) activity of the target compounds **6a–f**, **7a–e**, **8a–f** and **9a–f** against HT-29, ZR-75 and A-549 cell lines

Compound	HT-29	ZR-75	A-549	Average growth inhibition %
6a	38.7±9.1	50.4±19.4	32.9±7.0	40.7
6b*	13.2±10.5	19.2±8.6	7.1±8.2	13.2
6c	5.5±3.2	16.4±13.2	10.9±11.1	11.0
6d*	18.6±4.6	4.2±12.7	−4.3±16.4	6.1
6e*	20.9±8.1	32.0±18.6	−10.4±8.5	14.2
6f	49.3±5.5	40.4±11.3	20.5±15.0	36.7
7a	38.1±6.8	54.9±14.3	58.6±10.5	50.6
7b	9.6±9.2	26.5±6.6	4.9±8.5	13.7
7c	5.3±7.9	14.2±14.4	10.9±12.6	10.2
7d*	70.8±7.6	78.8±7.6	77.1±6.3	75.6
7e	6.2±6.4	23.1±8.2	17.0±9.0	15.4
8a	87.6±8.3	54.3±9.4	81.1±7.4	74.4
8b	98.5±1.0	86.3±5.4	96.5±2.2	93.8
8c	95.7±2.8	96.1±2.1	97.8±3.0	96.5
8d	96.8±3.0	97.2±4.2	95.2±3.5	96.4
8e	9.3±10.2	30.9±18.1	13.5±14.5	17.9
8f	89.1±8.6	69.6±6.6	85.0±15.0	81.2
9a	73.0±14.2	52.1±11.9	95.2±2.6	73.4
9b	48.1±7.3	22.6±14.2	53.2±10.0	41.3
9c	16.1±14.5	29.8±14.3	27.1±14.7	24.3
9d	49.4±16.2	62.1±18.5	84.9±4.7	65.5
9e	48.5±13.7	76.0±2.8	85.9±6.2	70.2
9f	47.6±7.5	71.0±10.8	68.8±10.3	62.5
Sunitinib	59.5±2.3	90.7±4.5	85.7±2.7	78.7

Note: *Tested concentration was 10 μM.

Table 2 IC_{50} of antiproliferative activity of the selected compounds **8b–d** and sunitinib against HT-29, ZR-75 and A-549 cell lines

Compound	IC_{50} (μM)			Average IC_{50} (μM)
	HT-29	ZR-75	A-549	
8b	6.69±0.4	13.25±2.0	7.19±1.3	9.04
8c	5.31±1.2	5.90±0.3	5.39±0.6	5.53
8d	6.23±0.7	7.77±2.7	7.02±1.0	7.01
Sunitinib	10.14±0.8	8.31±2.4	5.87±0.3	8.11

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

The most active promising compounds **8b–d** in the preliminary antiproliferative screening were subjected to quantitative inhibitory concentration 50% (IC_{50}) determination for their cell growth inhibitory activity towards A-549, HT-29 and ZR-75 cancer cell lines and the results are presented in Table 2.

Compound **8c** bearing 4-benzylphthalazine moiety exhibited the best average IC_{50} value of 5.53 μM, as compared with the positive control, sunitinib, which showed an average IC_{50} =8.11 μM. Therefore, compound **8c** was subjected to deeper pharmacological investigations in order to gain insight into its pharmacological profile.

Apoptosis and caspase 3/7 activity

Compound **8c** was analyzed for apoptosis-inducing activity in cancer cells. These studies were carried out using cells derived from human lung (A-549). This further assessment of activity tested compounds in quadruplicate at concentrations equivalent to IC_{50} value to inhibit growth and a concentration 3-fold above the IC_{50} concentrations over a time course ranging from 2–48 h. As indicated in Figure 3, compound **8c** at 5 μM increased caspase activity by 3-fold after 16 h

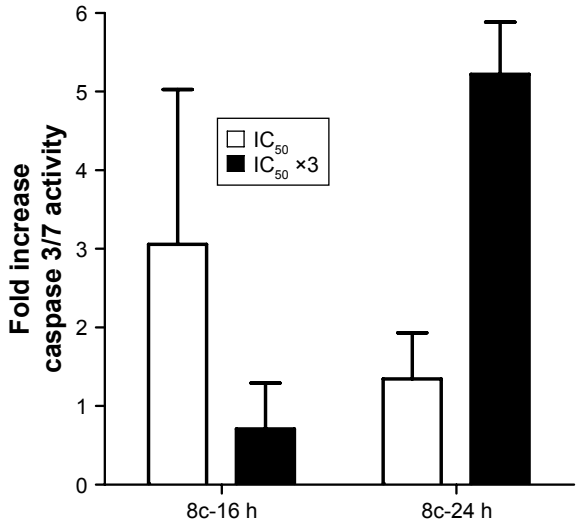


Figure 3 Caspase 3/7 activity of compound **8c**.
Abbreviation: IC_{50}, inhibitory concentration 50%.

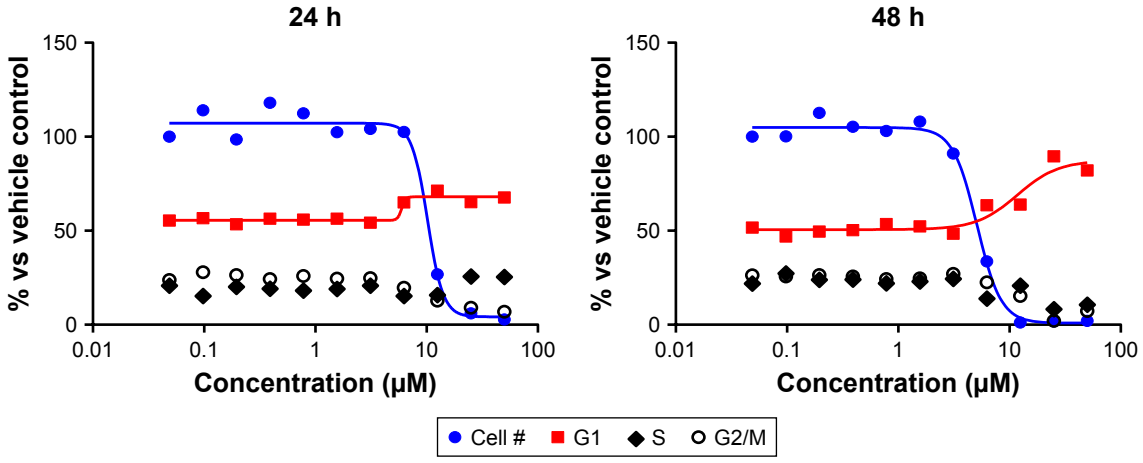


Figure 4 Cell cycle effects of compound **8c** after 24 and 48 h of incubation.

of treatment and to over 5-fold after 24 h of treatment at a concentration of 15 μM.

Cell cycle effects

Compound **8c** was analyzed for effects on various aspects of the cell cycle progression in human cancer cells. These studies were carried out using cells derived from lung adenocarcinoma (A-549). This follow-up assessment of activity tested compounds using immunofluorescent imaging of phosphorylated Rb protein and total DNA content of each cell to assess phase of cell cycle. The ability of test compounds to affect cell cycle distribution and Rb phosphorylation was tested over a range of concentrations less than 100 nM to 50 μM. As shown in Figure 4, compound **8c** produced dose-dependent effects on the tested parameters; however, compound **7d** displayed no effects on the tested parameters (not shown). Compound **8c** caused a significant reduction in the total cell number after 24 h of treatment with IC_{50} value =10.19 μM and with IC_{50} value =5.11 μM after 48 h (Table 3).

In addition, compound **8c** caused an increase in the percentage of cells in the G1 phase of the cell cycle with corresponding decrease in S and G2/M phases. This suggests that part of the compound effects on growth may be attributable to the decreased rate of progression through the cell cycle and corresponding decrease in proliferation. By contrast, sunitinib caused a reduction in the percentage of cells in G1, with corresponding increases in S or G2/M phases. Arrest in G2 may represent a checkpoint blockade, whereas mitotic arrest may, in some cases, lead to mitotic catastrophe and subsequent programmed death of cells with multiple or aberrant nuclei.

As with other cell cycle parameters, levels of phosphorylated Rb protein were substantially reduced in a dose-dependent manner by the control and the test compound **8c**. After 24 h of treatment, the IC_{50} value was lower than the IC_{50} value for reductions in the cell number caused by compound **8c** (Table 3). This may support the hypothesis that inhibition of cyclin-dependent kinases by isatin compounds plays a role in their growth inhibitory activity. However, the correlation is less apparent at the 48-h time point. Furthermore, compound **8c** was analyzed for effects on total cellular levels of phosphorylated tyrosine residues in human cancer cells. These studies were carried out using cells derived from lung adenocarcinoma (A-549) and immunofluorescent imaging. The ability of the test compounds to affect acute serum stimulation of tyrosine phosphorylation was tested over a range of concentrations less than 100 to 50 μM. Compound **8c** had no significant effect on P-Tyr labeling.

Selectivity

As an indicator of the selectivity for tumor cells, compound **8c** was analyzed for its cell growth inhibitory activity in

Table 3 IC_{50} for reductions in the total cell number and cell cycle effects of compound **8c** and sunitinib

Compound	IC_{50} (μM) for reductions in the total cell number		IC_{50} (μM) for reduction in Rb phosphorylation		Cell cycle effects
	24 h	48 h	24 h	48 h	
8c	10.19±1.3	5.11±1.1	6.55±0.3	6.18±0.1	G1 increased, S and G2/M phase decreased
Sunitinib	12.54±2.9	3.48±1.6	3.16±0.1	7.99±4.1	G1 decreased and G2/M phase increased

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

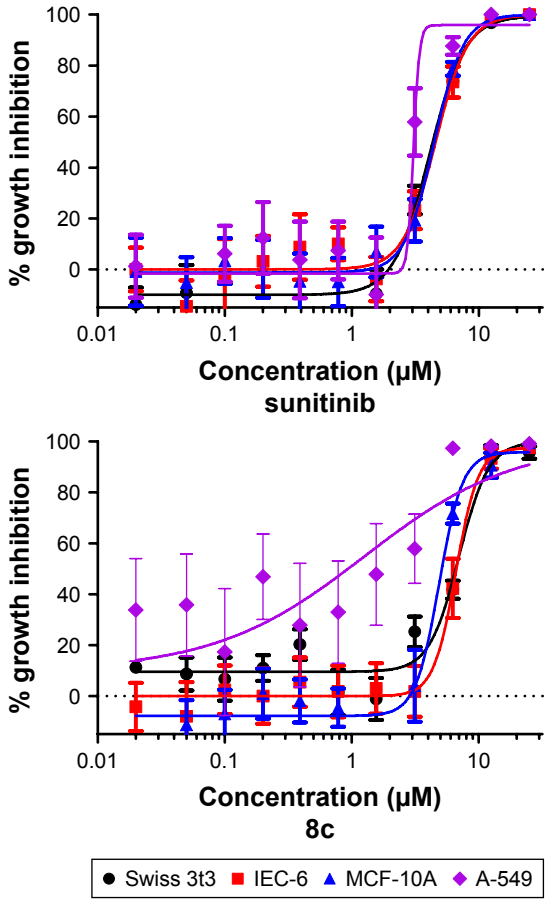


Figure 5 Selectivity profile of sunitinib and compound **8c**.

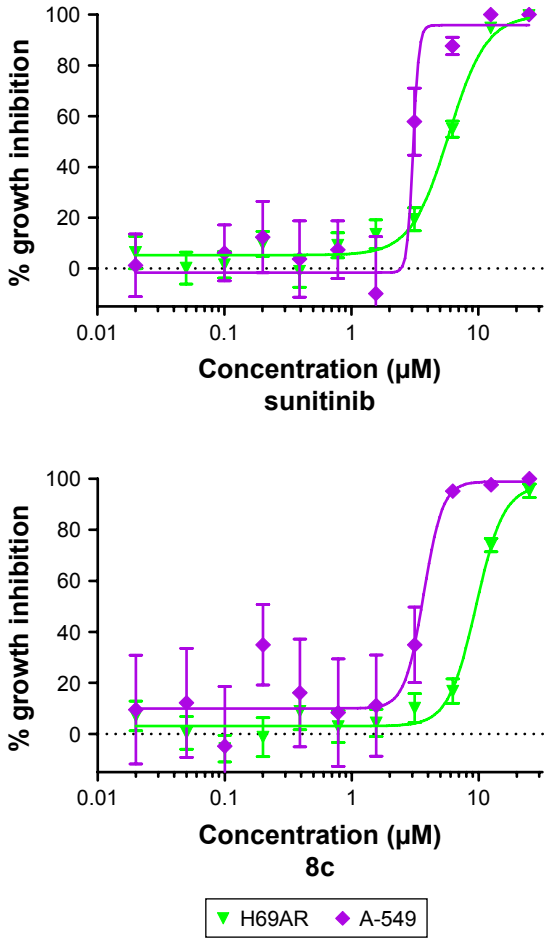


Figure 6 Activity of sunitinib and compound **8c** against sensitive and resistant cancer cell lines.

three non-tumorigenic cell lines. IEC-6 cells derived from rat intestine exhibit morphologic and karyotypic features of normal intestinal epithelial cells.[43] Cultures derived from human fibrocystic mammary tissue (MCF-10A) are non-tumorigenic and exhibit features of primary cultures of breast tissue including dome formation.[44] Fibroblasts derived from embryonic tissue from mice (Swiss 3t3 fibroblasts) are both non-tumorigenic and contact inhibited.[45] For comparison, A-549 human non-small cell lung cancer (NSCLC) cell line was included. This assessment of growth inhibitory activity tested compounds in quadruplicate at maximum concentrations of 25 μM, followed by 10 serially diluted concentrations. As demonstrated in Figure 5 and Table 4, compound **8c** inhibited growth in both normal and tumor cell lines by >50%. Compound **8c** not only inhibited NSCLC with IC_{50} value =1.27 μM but also inhibited non-tumor cells less potently with 4.8-fold selectivity value. For the control compound, sunitinib, there was a modest degree of selectivity (1.4-fold difference between mean IC_{50} in non-tumor cell lines versus the NSCLC cells).

Table 4 Selectivity for compound **8c** and sunitinib toward tumor and non-tumorigenic cell lines

Compound	IC_{50} (μM)				Mean tumor selectivity
	Intestine IEC-6	Breast MCF-10A	Fibroblast Swiss 3t3	NSCLC A-549	
8c	6.60±1.1	4.87±1.1	6.98±0.6	1.27±1.5	4.8
Sunitinib	4.56±0.9	4.43±0.8	4.07±0.5	3.06±0.9	1.4

Abbreviations: IC_{50}, inhibitory concentration 50%; NSCLC, non-small cell lung cancer.

Multidrug-resistant lung cancer cell line

Compound **8c** was analyzed for cancer cell growth inhibitory activity in a sensitive NSCLC cell line (A-549) and a multidrug-resistant lung cancer cell line (NCI-H69AR) that expresses the ABCC1 efflux pump protein. This assessment of activity tested compound **8c** in quadruplicate at maximum concentrations of 25 μM, followed by 10 serially diluted concentrations. As illustrated in Figure 6 and summarized in Table 5, compound **8c** inhibited growth in both sensitive and resistant cancer cell lines with IC_{50} values =1.3 and 9.5 μM, respectively, being 7.5-fold less sensitive toward the resistant

Table 5 Cancer cell growth inhibitory activity of compound **8c** and sunitinib toward sensitive (A-549) and resistant NCI-H69AR cancer cell lines

Compound	IC_{50} (μM)		Fold resistance
	Sensitive A-549	Resistant NCI-H69AR	
8c	1.3±1.5	9.5±0.6	7.5
Sunitinib	3.1±0.9	5.8±0.4	1.9

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

NCI-H69AR cell line, indicating that this compound may be subjected to efflux by ABCC1. Sunitinib showed a lesser degree of fold resistance being 1.9-fold less sensitive toward the resistant NCI-H69AR cell line.

Metabolic investigations

The study of drug metabolism is a core part of the process of drug discovery and development; it has evolved from being a complementary step to that process to becoming crucial to it.[46] Nowadays, metabolic profiles of new drugs have to be investigated prior to any clinical use of such drugs. This approach has been prejudiced by data accumulation assuming that poor pharmacokinetics is the main reason for failure of drug substances, in which the metabolic liability of a drug molecule is the primary determinant.[47,48] In this study, comparison of the extracted ion chromatograms between incubations with or without RLMs as well as comparison of the product ion mass spectra of the postulated metabolites of **8c** allowed the detection of ten metabolites. Such metabolites resulted from the incubation of **7d** and **8c** with RLMs that involved various metabolic reaction types, namely, demethylation for **7d** and isomerization, reduction, hydroxylation and oxidation for **8c** (Figure 7 and Scheme 2). Table 6 summarizes the product ions, retention times and metabolic reactions for the in vitro phase I **8c** metabolites.

Conclusion

Quinazoline-isatin hybrids **6a–f** and **7a–e**, phthalazine-isatin hybrids **8a–f** and 1-methylquinoxaline-isatin hybrids **9a–f** were synthesized and characterized with different spectroscopic techniques. The preliminary in vitro antiproliferative activity of the synthesized compounds against various human cancer cell lines revealed that compounds **8b–d** were the most active candidates. Therefore, they were subjected to quantitative IC_{50} determination. Detailed pharmacological investigations were carried out on the most promising compound **8c** in order to gain insight into its pharmacological profile. Compound **8c** induced apoptosis through increasing caspase 3/7 activity by about 5-fold at 15 μM concentration using human cancer A-549 cell line. In addition, it displayed an increase in the G1 phase and a decrease in the S and G2/M phases in the cell cycle effects assay and it showed IC_{50} value of 9.5 μM against resistant NCI-H69AR cancer cell lines. In vitro metabolic profiling of compound **8c** predicted its possible metabolites. Overall, the current study demonstrated that the new chemical entity **8c** might be harnessed for cancer therapy after integration of the required preclinical studies.

Supporting materials

The details of the experimental methods that were adopted for the pharmacological investigations of the prepared compounds, the protocols that were used for metabolic studies and representative NMR (^{1}H and ^{13}C) spectra of the target compounds are provided as Supplementary materials.

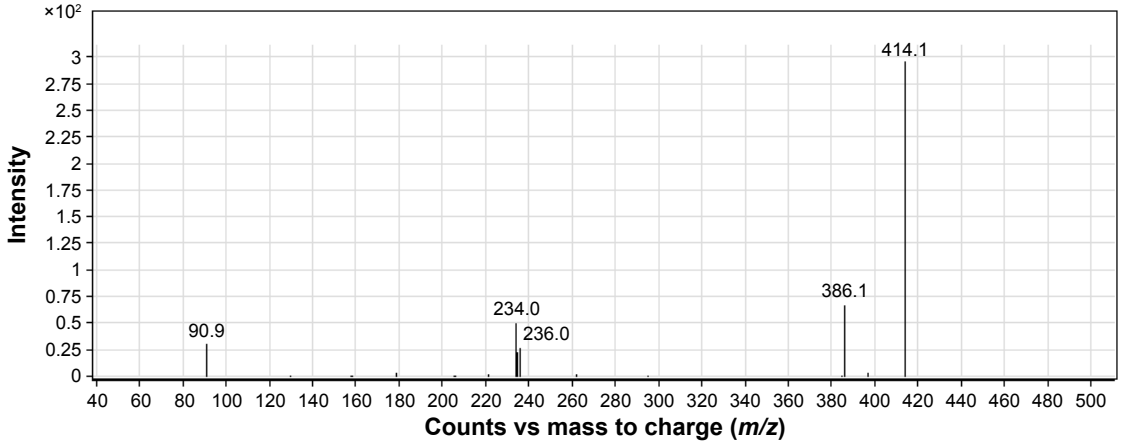
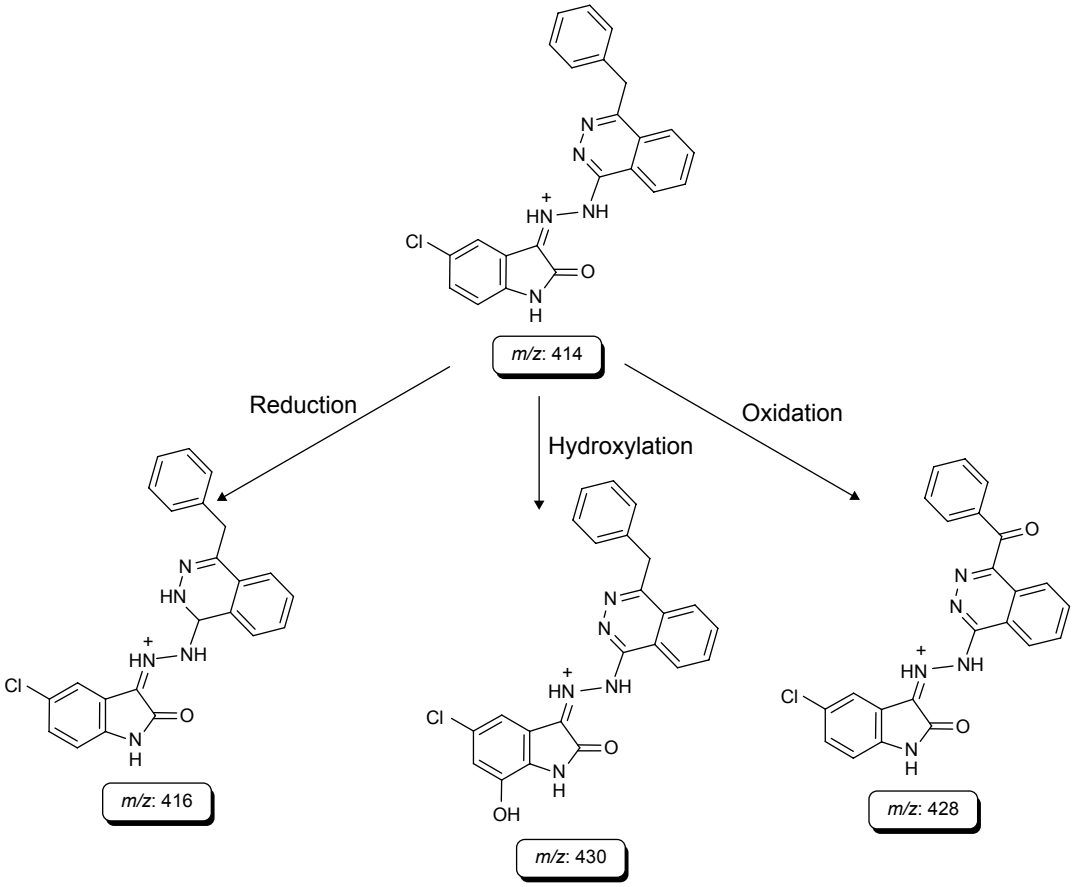


Figure 7 A representative product ion spectrum of compound **8c** (retention time =36.40 min).



Scheme 2 Postulated in vitro metabolic pathway of compound **8c**.

Table 6 In vitro RLMs metabolites of compound **8c**

Compound	Compound/metabolite	Metabolic reaction	Monoisotopic mass (*m*/*z*)	Product ion (*m*/*z*)	Retention time (min)
8c	**8c**	–	414	386, 236, 234, 91	36.40
	8c Reduced	Reduction	416	388, 234, 91	36.35
	8c Reduced isomer	Reduction and isomerization	416	388, 234, 91	43.82
	8c Hydroxylated	Arom hydroxylation	430	234, 181	31.62
	8c Hydroxylated isomer 1	Arom hydroxylation and isomerization	430	412, 233, 107	32.30
	8c Hydroxylated isomer 2	Arom hydroxylation and isomerization	430	412, 250, 233, 107	38.96
	8c Hydroxylated isomer 3	Arom hydroxylation and isomerization	430	412, 233, 107	40.34
	8c Oxygenated	oxidation	428	322, 107, 79	27.25
	8c Oxygenated isomer 1	oxidation and isomerization	428	322, 107, 79	25.70
	8c Oxygenated isomer 2	oxidation and isomerization	428	105, 77	40.35
	8c Oxygenated isomer 3	oxidation and isomerization	428	179, 105, 77	44.24

Abbreviations: RLM, rat liver microsome; Arom, aromatic.

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Disclosure

The authors report no conflicts of interest in this work.

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