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In vitro cytotoxicity evaluation of some substituted isatin derivatives

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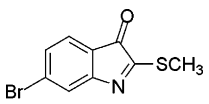
Abstract—A range of substituted 1*H*-indole-2,3-diones (isatins) were synthesized using standard procedures and their cytotoxicity evaluated against the human monocyte-like histiocytic lymphoma (U937) cell line in vitro. SAR studies identified C_5, C_6, and C_7 substitution greatly enhanced activity with some di- and tri-halogenated isatins giving IC_{50} values <10 μM. Of the 23 compounds tested, four were selected for further screening against a panel of five human cancer cell lines. These compounds, in general, showed greater selectivity toward leukemia and lymphoma cells over breast, prostate, and colorectal carcinoma cell lines. The most active compound, 5,6,7-tribromoisatin (**2p**), was found to be antiproliferative at low micromolar concentrations and also activated the effector caspases 3 and 7 in a dose-dependent manner. These results indicate that di- and tri-substituted isatins may be useful leads for anticancer drug development in the future.

1. Introduction

A variety of halogenated marine compounds have been found to exhibit cytotoxic and antineoplastic activity,[1–3] yet a large number still remain uninvestigated. Recently we reported the cytotoxic activity of tyrindoleninone[4] (6-bromo-2-methylthio-3*H*-indol-3-one, **1**), a brominated precursor to tyrian purple, isolated from the egg masses of the Australian mollusk *Dicathais orbita*. Investigations into the cytotoxicity of **1** revealed greater specificity toward cancer cells (IC_{50} 4 μM) than freshly isolated, human, mononuclear cells (IC_{50} 195 μM), in vitro.[5] Although this highlights **1** to be a potentially useful lead for anticancer drug development, our attempts to synthesize this compound have been unsuccessful to date, largely due to the reactivity of the indoleninone nucleus.



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Looking to other less reactive, biologically active compounds in the egg masses of the muricid *D. orbita*, our focus turned to 6-bromoisatin (**2d**, Table 1), a major decomposition product formed through the oxidation of tyriverdin.[6] It was found that 6-bromoisatin also has anticancer activity against a human lymphoma cell line, but was not as active as **1**.[4] This is not surprising as isatin itself, an endogenous compound identified in many organisms, possesses a wide range of biological activities.[7,8] In particular, halogenated derivatives have been reported to exhibit anticancer activity.[9] For example, 5-bromo-3-*o*-nitrophenyl isatin hydrazone was found to be active intramuscularly against Walker carcinoma-256[10] and a series of 5-bromo-(2-oxo-3-indolinyl)thiazolidine-2,4-diones substituted by various Mannich bases were found to exhibit antileukemic activity against P388 lymphocytic leukemia in mice.[9] Recently, SU11248 (Sutent), a 5-fluoro-3-substituted-2-oxoindole, was approved by the US FDA for the treatment of gastrointestinal stromal tumors[11] and advanced renal-cell carcinoma.[12]

In light of this, our study has aimed to optimize the biological activity of 6-bromoisatin to that of **1** by synthesizing a range of stable analogues based on the isatin scaffold. By screening for cytotoxic activity against a

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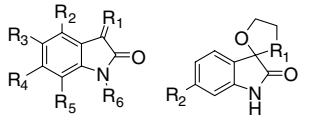
Table 1. Cytotoxicity of isatin derivatives **2a**–**t** on U937[a] cells as calculated from dose–response curves[b]

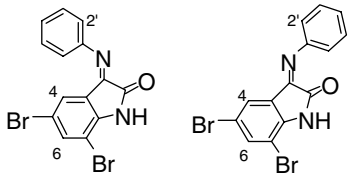


2a-t **3a-c**

Compound	R_1	R_2	R_3	R_4	R_5	R_6	clog P[c]	IC_{50}[d] (μM)
2a	O	H	H	H	H	H	0.828	565
2b	O	Br	H	H	H	H	1.69	214
2c	O	H	Br	H	H	H	1.69	64.5
2d	O	H	H	Br	H	H	1.69	74.8
2e	O	H	H	H	Br	H	1.69	83.0
2f	O	H	F	H	H	H	0.971	98.4
2g	O	H	I	H	H	H	1.95	53.4
2h	O	H	NO_2	H	H	H	0.571	132
2i	O	H	OCH_3	H	H	H	0.747	420
2j	O	H	Br	H	Br	H	2.55	10.5
2k	O	H	Br	Br	H	H	2.35	11.6
2l	O	H	I	H	I	H	3.07	7.85
2m	O	H	Br	H	NO_2	H	1.43	257
2n	O	H	NO_2	Br	H	H	1.23	17.1
2o	O	H	NO_2	H	Br	H	1.43	>369
2p	O	H	Br	Br	Br	H	3.02	6.76
2q	N–C_6H_5	H	H	H	H	H	2.65	76.5
2r	N–C_6H_5	H	Br	H	Br	H	4.38	13.7
2s	N–NHC_6H_5	H	H	H	H	H	2.47	>211
2t	O	H	H	H	H	CH_3	0.564	238
Staurosporine								2.00

[a] U937: human monocyte-like, histiocytic lymphoma.
[b] Sigmoidal dose–response curves (variable slope) were generated using GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[c] clog P values were calculated using ChemDraw Ultra V. 8.0 (CambridgeSoft Corporation).
[d] Values are mean of triplicate of at least two independent experiments.

panel of cancer cell lines, aspects of the structure–activity relationship (SAR) were also determined. Since it is proposed that some isatins inhibit cell proliferation and promote apoptosis,[13] the ability of selected isatins, from those synthesized, to activate effector caspases 3 and 7, and to inhibit cyclin-dependent kinase 2 (CDK2) was also investigated to determine a possible mode of action.



(*E*)-2r **(*Z*)-2r**

2. Results and discussion

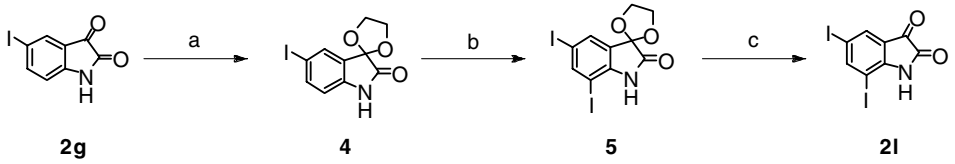
2.1. Chemistry

All compounds tested except **2k** and **2p** (Table 1) were commercially available or prepared using literature procedures. Compounds **2o** and **2r** were synthesized using published methods for analogous compounds. 5,7-Diiodoisatin (**2l**) was made via the ketal intermediate **4** to improve the reactivity of 5-iodoisatin (**2g**) toward further iodination (Scheme 1), using a modification to the procedure previously reported.[14]

Although the six-membered ring of **2a** is activated toward electrophilic attack at position 5 with subsequent attack at position 7 favored, the pattern of substitution was confirmed using gHMBC experiments and/or the magnitude of the coupling constant between H_4 and H_6 (2 Hz *meta* coupling).

The imines **2q** and **2r** were tested as mixtures of *E*- and *Z*-isomers. The ^{1}H NMR spectrum (DMSO-d_6) of the isatin imine **2q** showed only a trace of the minor isomer and assignment of signals for this isomer was not possible. However, the ^{1}H NMR spectrum (DMSO-d_6) of the dibrominated derivative **2r** showed clearly resolved signals for a 4:1 mixture of *E*/*Z*-isomers. The major isomer was assigned the *E* stereochemistry based on the following evidence.

The signal from H_4 of the *E*-isomer (6.32 ppm) was markedly shifted upfield relative to the H_4 signal of both the parent dibromoisatin **2j** (7.63 ppm) and the minor *Z*-isomer (7.68 ppm). In contrast, the chemical shifts of H_4 and H_6 of the *Z*-isomer showed little difference in chemical shift to that for the parent isatin. The signal from H_4 in (*E*)-**2r** is presumably shifted upfield due to interaction with the ring current of the phenyl substituent shielding this proton. The major isomer also exhibited a weak NOE correlation between H_4 and $H_{2'}$. The



Scheme 1. Synthesis of **2l**. Reagents and conditions: (a) ethylene glycol, *p*-TSA (cat.), toluene, reflux (Dean and Stark conditions) 24 h, 43%; (b) ICl, MeOH, reflux, 5 h, 19%; (c) concd HCl/AcOH 4:1, reflux, 40 min, quant.

observed NOE enhancement is only possible for the *E*-isomer. This has recently been described for a range of 3-substituted indolin-2-ones, whereby formation of the *E*-isomer was predominant in all cases, except for one.[15]

2.2. Biological activity and SAR

The in vitro cytotoxic activities of compounds **2a**–**t** were determined initially against the human monocyte-like, histiocytic lymphoma cell line (U937) and are reported as IC_{50} values in Table 1. Results indicate that the toxicity of derivatives varied with structural modification, but only **2p** was as active as **1**. Introduction of electron-withdrawing groups at positions 5, 6, and 7 greatly increased activity from that of isatin (**2a**), with substitution at the 5-position being most favorable. This is not surprising, as C_5 substitution has previously been associated with increased biological activity for a range of indole-based compounds,[13,16] including indirubin, a potent inhibitor of cyclin-dependent kinases (CDKs)[17] and glycogen synthase kinase 3 (GSK3).[18] Halogenation yielded the most active compounds, with di- (**2j**, **2k**, and **2l**) and tri-substitution (**2p**) increasing activity up to >100-fold from the parent molecule **2a**. A comparison of the cytotoxicity (IC_{50}) of compounds **2a**–**t** with their corresponding, calculated log *P* (clog *P*) values (Table 1) found no correlation, indicating that the activity observed is not based on lipophilicity alone.

Preliminary investigation into the biological mode of action of the most active compound **2p** found it to be antiproliferative at low concentrations and cytotoxic at higher concentrations (Fig. 1). This effect was evident within the first 24 h of treatment, and subsequent incubation periods (up to 72 h) did not enhance the activity of **2p** any further. Activation of effector caspases 3 and 7 was dose-dependent in Jurkat cells, with maximum activity detected at 3.1 μg/mL (8 μM) after 5 h (Fig. 2). This was equivalent to the activity observed for staurosporine at 2 μM, a potent, broad-spectrum protein kinase inhibitor. A range of isatin-based molecules have also been reported to display antiproliferative[19,20] and proapoptotic activity in vitro.[21] Mechanistically, isatin itself is proposed to inhibit cell proliferation via interaction with extracellular signal-related protein kinases (ERKs), thereby promoting apoptosis.[13] The oxindoles, for example 3,3-diaryloxindoles, reduce cell growth via Ca^{2+}-mediated inhibition of translation initiation.[20]

Formation of spiroketals **3a** (R_1 = O, R_2 = H) and **3b** (R_1 = O, R_2 = Br) and spirothioketal **3c** (R_1 = S, R_2 = H) at C_3 did not enhance activity relative to that of **2a**. Only mild activity was observed at the highest concentration tested (100 μg/mL) and as a result IC_{50} values were not determined (data not shown). Similarly, imine bond formation at C_3 gave the hydrazone **2s** (R_1 = N–NH–Ph) which showed no activity at the highest concentration tested (50 μg/mL). Formation of the imine **2q** (R_1 = N–Ph) however, increased activity against U937 cells by a factor of 13.5 (Table 1). Di-bromination of **2q** to form **2r** enhanced activity by a further

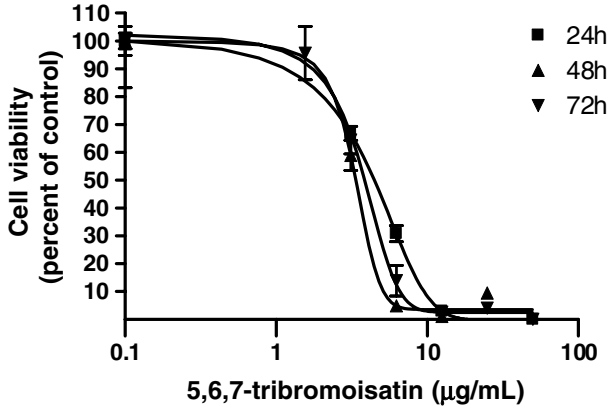


Figure 1. Viability of U937 cells after treatment with various concentrations of 5,6,7-tribromoisatin (**2p**) over time. Briefly, cells were incubated for 24, 48 or 72 h at 37 °C (95% humidity, 5% CO_2) with increasing concentrations of **2p**, then analyzed for a change in metabolic activity and expressed as percent viability in reference to the DMSO control. Each data point is a mean of triplicate ± SE of one representative experiment.

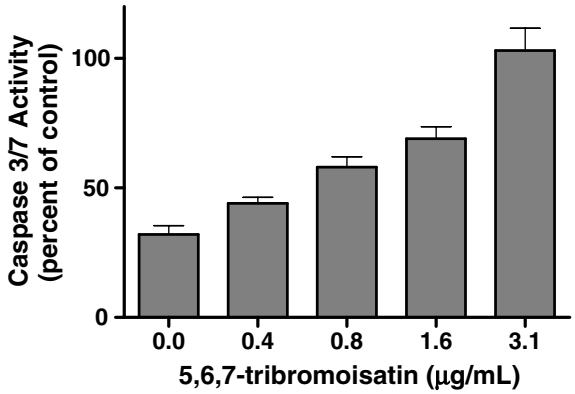


Figure 2. Activation of caspases 3 and 7 in Jurkat cells after treatment with various concentrations of 5,6,7-tribromoisatin (**2p**). Cells were treated with increasing concentrations of **2p** or staurosporine (2 μM) for 5 h. Values are normalized to staurosporine (positive control). Data are means ± SE of one representative experiment performed in triplicate.

18% but **2r** was not as active as the ketone analogue **2j**. This is interesting as many C_3 substituted isatins have been found to selectively target and inhibit PTKs at submicromolar concentrations in cell-based assays.[22,23] SAR studies have shown that the oxindole core occupies a site where the adenine of ATP binds, while substituents at C_3 of various isatins, for example SU5416, contact residues in the hinge region of growth factor receptors.[23] The in vitro activity of isatins **2j**, **2k**, **2l**, and **2p** at low micromolar concentrations and the ability of **2p** to arrest cellular proliferation and promote apoptosis suggests that these compounds may in fact share common interactions with ATP binding pockets associated with receptors of growth factors or even other protein kinases.

In light of this, **2j**, which was a more viable lead compound synthetically, was investigated for its ability to inhibit cyclin-dependent kinase 2 (CDK2), a key regulator of the cell cycle, and was found to cause 44% inhibition at 100 μM (data not shown). Comparison of this data with in vitro cytotoxicity of **2j** in U937 cells (IC_{50} 10.5 μM) suggests that it does not directly interact with CDK2. This does not rule out interactions with other CDKs however, as a number of different inhibitors show potent selectivity based on the structural differences that exist at the proteins' catalytic site.[24] The hydrophobic nature of amino acids lining ATP binding cavities of kinases may also explain the higher cell-based activity observed for di- and tri-halogenated isatins in comparison to unsubstituted compounds (Table 1). Such an effect has similarly been described for 3-substituted-2-oxoindole analogues upon binding to CDK5/p25 and GSK/3β.[15] Furthermore, methylation of **2a** at N_1 to form **2t** increased activity by almost 50% (Table 1). This supports the hypothesis that substituted isatins **2a**–**t** may act on other CDKs or PTKs as the NH amide of the oxindole ring system of various isatins is found to be important for binding to the carbonyl backbone of amino acids in the ATP binding pocket of growth factors PDGF and VEGF[22] as well as CDK2.[25]

Finally, of the 23 compounds screened, the isatins **2j**, **2k**, **2l**, and **2p** were selected for further testing against a panel of human leukemic (Jurkat, T cell), breast (MDA-MB-231 and MCF-7), prostate (PC-3) and colorectal (HCT-116) cell lines (Table 2). Results indicated leukemia and lymphoma cell lines were the most sensitive to treatment, while PC-3 cells, in general, were the least responsive over all (Fig. 3).

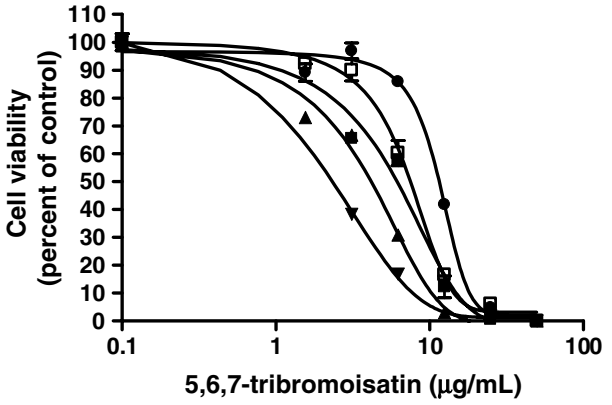


Figure 3. Viability of U937 (▲), Jurkat (▼), HCT-116 (■), PC-3 (●), and MDA-MB-231 (□) cells after treatment with 5,6,7-tribromoisatin (**2p**). Briefly, cells were incubated for 24 h at 37 °C (95% humidity, 5% CO_2) with increasing concentrations of **2p**, then analyzed for a change in metabolic activity and expressed as percent viability in reference to the DMSO control. Each data point is a mean of triplicate ± SE of one representative experiment.

3. Conclusions

In summary, a range of isatin derivatives have been synthesized resulting in the generation of two new compounds (**2o** and **2p**); these were characterized by their MS, ^{1}H NMR, and ^{13}C data. Cytotoxicity screening revealed **2p** to be the most active compound across all cell lines and resulted in the initiation of apoptosis in Jurkat cells after 5 h. A preliminary cyclin-dependent kinase activity assay assessing the inhibitory ability of **2j** suggests CDK2 is not the primary target. However, the structural similarity it shares with other isatins that competitively bind at ATP catalytic sites suggests the molecular mode of action may be via inhibition of growth factors or other protein kinases. These results indicate that structural modification of di- and tri-substituted isatins may lead to new derivatives with enhanced and selective anticancer activity.

Table 2. Cytotoxicity of di- and tri-substituted isatin derivatives (**2j**, **2k**, **2l**, and **2p**) against various cancer cell lines as determined by dose–response curves[a]

Compound	IC_{50}[b] (μM)				
	Jurkat[c]	MDA-MB-231[d]	MCF-7[e]	PC-3[f]	HCT-116[g]
2j	14.3	42.3	31.3	101	24.6
2k	20.2	41.7	20.2	41.0	37.3
2l	20.9	41.8	18.4	71.9	24.0
2p	5.80	21.8	13.3	25.9	15.9

[a] Sigmoidal dose–response curves (variable slope) were generated using GraphPad Prism V. 4.02 (GraphPad Software Inc.).
[b] Values are mean of triplicate of at least two independent experiments.
[c] Jurkat: human leukemic T- cell.
[d] MDA-MB-231: human epithelial, mammary gland adenocarcinoma (metastatic).
[e] MCF-7: human epithelial, mammary gland adenocarcinoma (non-metastatic).
[f] PC-3: human prostate adenocarcinoma.
[g] HCT-116: human colorectal carcinoma.

4. Experimental

4.1. General

4.1.1. Chemistry. All solvents were of AR grade except dichloromethane (DCM) which was of LR grade and distilled before use. The term petroleum spirit refers to petroleum spirit with the boiling range of 40–60 °C. When necessary, the purification of solvents and starting materials was carried out using standard procedures. 4-Bromoisatin (**2b**), 6-bromoisatin (**2d**), and 7-bromoisatin (**2e**) were purchased from Butt Park Ltd., UK. 5-Iodoisatin (**2g**) (98%) and 5-fluoroisatin (**2f**) (98%) were obtained from Alfa Aesar. 5-Methoxyisatin (**2i**) was purchased from the Tokyo Kasei Kogyo Co. Ltd. Isatin (**2a**) (98%) and 5-bromoisatin (**2c**) (90%) were obtained from Aldrich. Compound **2c** was purified by successive crystallizations from absolute ethanol and the purity confirmed using ^{1}H NMR spectroscopy and melting point (mp 258–260 °C, lit.[26] 260–261 °C). Reactions were monitored using thin-layer chromatography (TLC) on aluminum-backed precoated silica gel 60 F_{254} plates (E Merck). In general isatins are highly colored and were visible on a TLC plate; colorless compounds were detected using UV light and/or iodine vapor. Flash chromatography[27] was carried out using silica gel 60 (230–400 mesh) with the solvent system indicated in the individual procedures. All solvent ratios are quoted as vol/vol. Full characterization data are included for known compounds where these data are incompletely reported in the literature. NMR spectra were acquired at 300 (^{1}H NMR) and 75 MHz (^{13}C NMR) on a Varian Unity-300 spectrometer or at 500 (^{1}H NMR) and 126 MHz (^{13}C NMR) on a Varian Inova-500 spectrometer with a probe temperature of 298 K. The NMR spectra are referenced to the residual solvent peak of the solvent stated in the individual procedures. Hydrogen and carbon assignments were made using standard NOE, APT, gCOSY, gHSQC, and gHMBC spectroscopic techniques. Low-resolution EI mass spectra (LR EIMS) were determined on a Shimadzu QP5050 spectrometer. High-resolution EI mass spectra (HR EIMS) were determined on VG Autospec spectrometer operating at 70 eV with a source temperature of 250 °C and were referenced with PFK. Melting points were determined on a Reichert melting point apparatus and are uncorrected.

4.1.2. Cell culture reagents. RPMI-1640 was purchased from Gibco™ (Invitrogen Life Technologies, NSW, Australia). Fetal calf serum (FCS) was obtained from MultiSer™ (ThermoTrace, Vic., Australia). Sodium bicarbonate was purchased from Univar Analytical Reagents (Ajax Chemicals, Australia). Trypsin–EDTA solution was purchased from Sigma–Aldrich (MO, USA). The CellTiter 96® AQueous One Solution Cell Proliferation Assay and Apo-ONE Homogeneous Caspase-3/7 Assay were purchased from Promega Co. (Madison, WI, USA).

4.1.3. Cell lines and culture. The human leukemic (U937, monocyte and Jurkat, T cell), breast (MDA-MB-231 and MCF-7), prostate (PC-3), and colorectal (HCT-116) cell lines were all obtained from American Type Culture Collection (ATCC, VA, USA) distributed by Cryosite, NSW, Australia. Cell lines were routinely cultured at 37 °C in a Heracell incubator (Kendro Laboratory Products, Langenselbold, Germany) in 95% humidified atmosphere, containing 5% CO_2. Cells were grown in culture media (10.4 g/L RPMI-1640 with 2 mM L-glutamine and 2 g/L $NaHCO_3$) supplemented with 5% (v/v) fetal calf serum (FCS), and routinely passaged at confluency for up to 20 passages. Adherent cell lines were detached using sterile trypsin–EDTA, washed with culture media, and centrifuged at 1500 rpm (514*g*) for 5 min at room temperature (RT) before reseeding. All cell experiments were performed using cells in exponential growth, cultured 48 h before without change of media. Adherent cells were detached prior to experiments with sterile 1 × phosphate-buffered saline (PBS) containing 5 mM EDTA (pH 7.4), cells were then centrifuged at 1500 rpm (514*g*) for 5 min at RT. Cell viability and cell number were assessed by the Trypan blue exclusion method and viable cells counted with the aid of a hemocytometer.

4.2. Chemistry

4.2.1. 5,7-Dibromoisatin (2j). The synthesis of **2j** was based on the method of Lindwall.[28] Isatin (**2a**) (5.0 g, 34 mmol, 1 equiv) was warmed in ethanol (95%, 100 mL) with stirring until it dissolved. Bromine (16.3 g, 102 mmol, 5.2 mL, 3.0 equiv) was added dropwise to the stirred isatin solution whilst maintaining the temperature of the reaction mixture between 70 and 75 °C. The solution was cooled to room temperature and placed on ice for 30 min. The resulting precipitate was washed with water and cold ethanol and then crystallized from ethanol to yield bright orange-red crystals of 5,7-dibromoisatin (**2j**) (6.6 g, 63%), mp 253–255 °C (lit.[28] 248–250 °C), R_f 0.58 (silica, DCM–MeOH, 9:1). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.63 (d, 1H, J = 2 Hz, H4), 7.98 (d, 1H, J = 2 Hz, H6), 11.42 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 105.7, 114.6, 121.2, 125.9 (C4), 141.1 (C6), 148.6, 159.3 (C2), 182.5 (C3). LREI-MS m/z 303/305/307 ([M]$^+$/[M+2]$^+$/[M+4]$^+$).

4.2.2. Bromination of 6-bromoisatin. 6-Bromoisatin (**2d**, 200 mg, 0.88 mmol, 1 equiv) was stirred and warmed in glacial acetic acid (8 mL) until it dissolved. A solution of bromine (312 mg, 100 μL, 1.94 mmol, 2.2 equiv) in acetic acid (2 mL) was added dropwise with stirring to the 6-bromoisatin solution and the reaction mixture was stirred at reflux for 16 h and then cooled and poured into ice water. The resulting orange precipitate was filtered, washed with cold water, and oven-dried at 80 °C for 6 h. The LR EIMS and ^{1}H NMR spectrum of the solid showed it was a 2:1 mixture of 5,6-dibromo (**2k**) and 5,6,7-tribromoisatin (**2p**). The crude mixture of products was purified using flash column chromatography, with the crude product adsorbed onto silica to load and then eluted with DCM to separate the isatins:

5,6-Dibromoisatin (**2k**) was a bright red-orange solid (135 mg, 50%), mp > 265 °C (lit.[29] 287–290 °C), R_f 0.49

(silica, DCM–MeOH, 9:1). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.24 (s, 1H, H7), 7.82 (s, 1H, H4), 11.24 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 116.7, 116.8 (C7), 118.8, 128.6 (C4), 133.1, 149.8, 159.0 (C2), 182.3 (C3). HREI-MS *m/z* calcd for $[M+2]^+$ $C_8H_3{}^{79}Br^{81}BrNO_2$: 304.8510; found: 304.8514.

5,6,7-Tribromoisatin (**2p**) was a bright red-orange solid (73 mg, 22%), mp 261–262 °C, R_f 0.56 (silica, DCM–MeOH, 9:1). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.83 (s, 1H, H4), 11.48 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 109.0, 117.6, 119.8, 126.9 (C4), 135.6, 149.5, 159.7 (C2), 182.2 (C3). HREI-MS *m/z* calcd for $[M+2]^+$ $C_8H_2{}^{79}Br_2{}^{81}BrNO_2$: 382.7616; found: 382.7615.

4.2.3. 5-Nitroisatin (2h). A general method for the nitration of the isatins was based on the method of Calvery.[30] A solution of isatin (500 mg, 3.4 mmol) in concd H_2SO_4 (3.2 mL) was added dropwise to a solution of KNO_3 (344 mg, 3.4 mmol) in concd sulfuric acid (3.8 mL) over a period of 1 h whilst maintaining the temperature between 0 and 4 °C. The reaction mixture was poured into 25 mL of ice water and the precipitate collected and washed with water. The crude 5-nitroisatin was purified by flash chromatography eluting with DCM–MeOH (98:2). The product was a bright yellow/orange solid (350 mg, 47%), mp 252–254 °C (lit.[30] 254–255 °C), R_f 0.49 (silica, DCM–MeOH, 98:2). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.07 (d, J = 9 Hz, 1H, H7), 8.18 (d, J = 2 Hz, 1H, H4), 8.78 (dd, J = 2, 9 Hz, 1H, H6), 10.92 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 113.2, 118.8, 120.3, 133.8, 143.3, 156.0, 160.6, 183.1. LREI-MS *m/z* 192 ($[M]^+$).

4.2.4. 5-Bromo-7-nitroisatin (2m). This compound was prepared from 5-bromoisatin (**2c**, 1.0 g, 4.4 mmol) using the method for the preparation of (**2h**). The crude product was purified using flash chromatography. The crude mixture was adsorbed onto silica to load and eluted with DCM to yield a bright yellow solid (703 mg, 59%), mp 246–248 °C (lit.[31] 244–245 °C), R_f 0.25 (DCM–MeOH, 98:2). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.11 (s, 1H, H4), 8.42 (s, 1H, H6), 11.82 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 113.9, 124.0, 133.1, 133.2 (C4), 133.3 (C6), 144.8, 160.3 (C2), 180.9 (C3). LREI-MS *m/z* 270/272 ($[M]^+$/$[M+2]^+$).

4.2.5. 6-Bromo-5-nitroisatin[32] (2n). This compound was synthesized from 6-bromoisatin (**2d**, 100 mg, 0.4 mmol) using the method for the preparation of **2h**. Pure **2n** was isolated from the crude mixture by flash chromatography by adsorbing the crude product onto silica to load the sample and then eluting with DCM. The product was a bright yellow solid (80 mg, 67%), mp 251–253 °C, R_f 0.33 (DCM–MeOH, 9:1). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.29 (s, 1H, H7), 8.15 (s, 1H, H4), 11.57 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz) δ 117.2 (C7), 117.5, 121.8 (C4), 123.6, 144.4, 153.0, 159.7 (C2), 181.7 (C3). HREI-MS *m/z* calcd for $[M+2]^+$ $C_8H_3{}^{81}BrN_2O_4$: 271.9256; found 271.9262.

4.2.6. 7-Bromo-5-nitroisatin (2o). This compound was synthesized using the method for the preparation of **2h** using 7-bromoisatin (100 mg, 0.4 mmol) as the starting material. Pure **2o** was isolated from the crude mixture by flash chromatography. The crude product was adsorbed on to silica to load and eluted with DCM. The product was a bright yellow solid (65 mg, 54%), mp 215–217 °C, R_f 0.19 (DCM–MeOH, 98:2). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.18 (d, J = 2 Hz, 1H, H4), 8.62 (d, J = 2 Hz, 1H, H6), 11.94 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 105.1, 117.8 (C4), 118.9, 120.5, 135.5 (C6), 143.6, 161.2 (C2), 182.9 (C3). HREI-MS *m/z* calcd for $[M+2]^+$ $C_8H_3{}^{81}BrN_2O_4$: 271.9256; found 271.9253.

4.2.7. 5′-Iodo-spiro[1,3-dioxolane-2,3′-[3H]indol]-2′(1′H)-one (4). This compound was prepared from 5-iodoisatin in accordance with the literature method.[33] The crude product was partially purified by flash chromatography (9:1–7:3 pet. spirit/ethyl acetate) and the resulting yellow solid was crystallized from ethanol to afford the desired colorless product (0.68 g, 43%), mp 172–174 °C (lit.[33] 173–175 °C) R_f 0.35 (DCM–MeOH, 95:5). ^{1}H NMR (CD_3OD, 500 MHz) δ 4.28 (AA′BB′ m, B signal, 2H, $H4_B$ and $H5_B$), 4.43 (AA′BB′ m, A signal, 2H, $H4_A$ and $H5_A$), 4.86 (br s, 1H, NH), 6.66 (d, J = 8 Hz, 1H, H7′), 7.59 (d, J = 8, 2 Hz, 1H, H4′), 7.64 (dd, 1H, J = 2, 8 Hz, H6′). ^{13}C NMR (CD_3OD, 126 MHz) δ 66.0 (C4, C5), 84.4, 102.0, 112.7, 127.6, 133.8, 140.3, 142.6, 175.3. LREI-MS *m/z* 317 ($[M]^+$).

4.2.8. 5,7-Diiodoisatin (2l). This compound was prepared using the method of Bass.[14] 5′-Iodo-spiro[1,3-dioxolane-2,3′-[3*H*]indol]-2′(1′*H*)-one (**4**) (200 mg, 0.63 mmol) was heated at reflux in methanol (20 ml) with ICl (213 mg, 1.31 mmol) for 5 h. The reaction mixture was poured into water and extracted with ether. The combined ether layers were dried over $MgSO_4$, filtered and the ether removed. The crude product was purified using flash chromatography (DCM). The identity of the colorless product (53 mg, 19%), R_f 0.22 (DCM) was confirmed using LREI-MS *m/z* 443 ($[M]^+$) and the compound used without additional characterization. The spiroketal (**5**) (11 mg, 0.02 mmol) was heated at reflux in a mixture of HCl–AcOH (3:1, 4 mL) for 40 min. The resulting bright orange solution was poured onto ice water (5 mL) and the precipitate collected and washed with water. Compound **2l** was obtained as a bright red solid (8 mg, quant.), mp 259–260 °C (lit.[34] 261–263 °C), R_f 0.57 (DCM). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.70 (d, J = 2 Hz, 1H,), 8.21 (d, J = 2 Hz, 1H), 11.10 (br s, 1H, NH). LREI-MS *m/z* 399 ($[M]^+$).

4.2.9. Spiro[1,3-dioxolane-2,3′-[3H]indol]-2′(1′H)-one (3a). This compound was prepared in accordance with the literature method.[33] The crude product was partially purified by flash chromatography (7:3 pet. spirit–ethyl acetate) and the resulting yellow solid was crystallized from benzene to afford the desired product as colorless needles (0.47 g, 36%), mp 129–130 °C (lit.[33] 131–132 °C, lit.[35] 134 °C), R_f 0.59 (9:1 DCM–methanol). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.32 (AA′BB′ m, B signal, 2H, $H4_B$ and $H5_B$), 4.56 (AA′BB′ m, A signal, 2H, $H4_A$ and $H5_A$), 6.81 (d, J = 8 Hz, 1H, H7′), 7.04 (t, J = 8 Hz, 1H, H5′), 7.28 (t, J = 8 Hz, 1H, H6′), 7.34

(d, J = 8 Hz, 1H, H4′), 8.74 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 126 MHz) δ 65.8 (C4, C5), 102.4 (C3a′), 110.8 (C7′), 123.2 (C5′), 124.3 (C3′), 125.0 (C4′), 131.6 (C6′), 141.8 (C7a′), 175.8 (C2′). LREI-MS m/z 191 ($[M]^+$).

4.2.10. 6′-Bromo-spiro[1,3-dioxolane-2,3′-[3H]indol]-2′(1′H)-one (3b). This compound was prepared using the method for **3a**.[33] The crude product was purified by flash chromatography (7:3 pet. spirit–ethyl acetate–6:4 spirit–ethyl acetate) to afford the desired product as a colorless solid (0.11 g, 96%), mp 218–220 °C, R_f 0.55 (9:1 DCM–methanol). ^{1}H NMR (DMSO-d_6, 500 MHz) 4.23 (AA′BB′ m, B signal, 2 H, $H4_B$ and $H5_B$), 4.32 (AA′BB′ m, A signal, 2H, $H4_A$ and $H5_A$), 6.98 (d, J = 2 Hz, 1H, H7′), 7.19 (dd, J = 2, 8 Hz, 1H, H5′), 7.27 (d, J = 8 Hz, 1H, H4′), 10.60 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) δ 65.6 (C4, C5), 101.2, 113.5, 123.9, 124.3, 125.1, 126.8, 144.4, 174.1. LREI-MS m/z 269/271 ($[M]^+$/$[M+2]^+$).

4.2.11. Spiro[3H-indole-3,2′[1,3]oxathiolan]-2(1H)-one (3c). This compound was prepared in accordance with the literature method.[33] The crude product was partially purified by flash chromatography (95:5 DCM–methanol) and the resulting pale yellow solid was crystallized from benzene (0.21 g, 15%), mp 159–160 °C (lit.[33] 155–156 °C), R_f 0.54 (9:1 DCM–methanol). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.45 (dt, J = 6, 10 Hz, 1H, $H4'_B$), 3.65 (ddd, J = 6, 6, 10 Hz, 1H, $H4'_A$), 4.54 (dt, J = 6, 9 Hz, 1H, $H5'_B$), 4.77 (dt, J = 6, 9 Hz, 1H, $H5'_A$), 6.85 (dd, J = 1, 8 Hz, 1H, H4 or H7), 7.07 (dt, J = 1, 8 Hz, 1H, H5 or H6), 7.27 (dt, J = 2, 8 Hz, 1H, H6 or H5), 7.43 (ddd, J = 1, 2, 8 Hz, 1H, H4 or H7), 8.55 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 126 MHz) δ 34.0, 72.6, 110.4, 123.3, 125.7, 126.7, 130.8, 140.5, 177.8. LREI-MS m/z 270 ($[M]^+$).

4.2.12. 3-(Phenylimino)-2-indolinone (2q). Synthesis of this compound was based on the method of Kumar.[36] The product was isolated as bright yellow needles (98 mg, 63%), mp 232–234 °C (lit.[36] 200 °C), R_f 0.56 (9:1 DCM–methanol). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.65 (d, J = 8 Hz, 1H, ArH), 6.74 (t, J = 8 Hz, 1H, ArH), 6.95 (d, J = 8 Hz, 1H, ArH), 7.04 (d, J = 8 Hz, 2H, H2′), 7.26 (t, J = 8 Hz, 1H, ArH), 7.30 (t, J = 8 Hz, 1H, ArH), 7.44 (t, J = 8 Hz, 2H, H3′), 9.78 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 112.1, 116.4, 118.1 (2C), 123.0, 125.7, 126.6, 129.7 (2C), 134.6, 146.0, 150.3, 155.0, 165.8. Spectral data showed that the product contained a trace of a minor isomer however these signals were not sufficiently resolved to make assignments. LREI-MS m/z 222 ($[M]^+$).

4.2.13. 5,7-Dibromo-3-(phenylimino)-2-indolinone (2r). This compound was made using the method for **2q**. The crude product was recrystallized from ethanol to yield a mixture of the E and Z isomers (4:1 E:Z) as bright orange needles (127 mg, 48%), mp 223–224 °C, R_f 0.57 (9:1 DCM–methanol). ^{1}H NMR (DMSO-d_6, 500 MHz) major (E) isomer δ 6.32 (d, J = 1 Hz, 1H, H4), 6.99 (d, J = 8 Hz, 2H, H2′), 7.29 (t, J = 8 Hz, 1H, H4′), 7.49 (t, J = 8 Hz, 2H, H3′), 7.81 (d, J = 1 Hz, 1H, H6), 11.40 (br s, 1H, NH), minor (Z) isomer δ 7.06 (d, J = 8 Hz, 2H, H2′), 7.14 (t, J = 8 Hz, 1H, H4′), 7.32 (t, J = 8 Hz, 2H, H3′), 7.68 (d, J = 1 Hz, 1H, H4), 7.88 (d, J = 1 Hz, 1H, H6), 11.40 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 126 MHz) major isomer δ 105.8, 113.9, 117.8 (C2′), 119.3, 126.2 (C3′), 127.2 (C4), 130.4, 138.7 (C6), 146.2, 150.5, 154.4 (C3), 163.9 (C2); minor isomer δ 104.9, 114.9, 120.2, 124.8, 125.5, 126.0, 129.0, 138.3, 144.8, 148.8, 152.3 (C3), 158.8 (C2). HREI-MS m/z calcd for $[M+2]^+$ $C_{14}H_8{}^{79}Br^{81}BrN_2O$: 379.8983; found: 379.8978.

4.2.14. Isatin-3-phenylhydrazone (2s). A mixture of isatin (100 mg, 0.67 mmol), phenylhydrazine hydrochloride (108 mg, 0.75 mmol), and sodium acetate (112 mg, 1.36 mmol) in absolute ethanol (100 mL) was heated at reflux for 16 h. The reaction mixture was allowed to cool and the resulting precipitate filtered from the solution. The crude product was purified using flash chromatography eluting with DCM. The product was a bright yellow solid (159 mg, 75%), mp 214–216 °C (lit.[37] 213–214°C), R_f 0.50 (silica, 3:7 ethyl acetate–pet. spirit). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.91 (d, J = 8 Hz, 1H, H4 or H7), 7.03 and 7.04 (2 overlapping t, 2× 1H, H4 and H5 or H6), 7.24 (dt, J = 1, 8 Hz, 1H, H5 or H6), 7.36 (t, J = 8 Hz, 2H, H3′), 7.42 (d, J = 8 Hz, 2H, H2′), 7.54 (d, J = 7 Hz, 1H, H4 or H7), 11.00 (br s, 1H, NH), 12.74 (s, 1H, NH). ^{13}C NMR ($CDCl_3$, 126 MHz) δ 110.5, 114.0, 118.6, 121.1, 121.8, 122.9, 127.7, 128.5, 129.4, 139.8, 142.5, 163.1 (C2). LREI-MS m/z 237 ($[M]^+$).

4.3. Biological assays

4.3.1. Cell viability assay. The toxicity of isatin and its derivatives on various cell lines was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS), in 96-well microplates, as described previously.[38]

4.3.2. Apoptosis assay. The activation of effector caspases 3 and 7 was determined in Jurkat cells after incubation with test compound or 2 μM staurosporine (positive control) using the Apo-ONE Homogeneous Caspase-3/7 Assay kit. Briefly, cells (2.0×10^4 in a total volume of 90 μL, complete media) were seeded into 96-well microtiter plates and increasing concentrations of test compound added. After 5 h incubation (37 °C, 95% humidity, 5% CO_2), 50 μL of supernatant was taken from each well and transferred to a black plate and an equivalent volume of caspase reagent added. The reagent was made up fresh by diluting the substrate 1:100 with the buffer. Cells were then mixed at 300–500 rpm on a plate shaker for 1 h at room temperature. The fluorescence was measured using the FLUOstar fluorescence plate reader (BMG Labtech) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

4.3.3. CDK2 inhibitory assay. Inhibition studies were performed using an assay established by Vesely et al.[39] Briefly, radiolabeled [^{32}P] ATP was added to buffered **2j** (dissolved in 1% DMSO) and incubated at 30 °C for 10 min. After incubation, the assay mixture was spotted onto phosphocellulose filter paper, allowed to dry for

20–30 s, and then transferred to stirring 1% phosphoric acid to dilute the phosphate substrate, thereby terminating the reaction. The filters were washed a further 5–6 times, and dried, before being placed on a scintillation counter. The amount of [^{32}P] incorporated into histone was measured by counting the number of disintegrations per minute. This gave the value of pmol enzyme activity. Control assays were also carried out in 1% DMSO, allowing the percentage inhibition to be calculated, the control was classed as having a 100% activity. The IC_{50} value for **2j** was determined by plotting % inhibition values against inhibitor concentration.

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