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Recent highlights in the development of isatin-based anticancer agents

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Keywords
highlights, recent, anticancer, isatin, development, agents

Disciplines
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Recent Highlights in the Development of Isatin-Based Anticancer Agents

Running Title: *Isatin-based Anticancer Agents*

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Abstract:

Isatin (1*H*-indole-2,3-dione) and its derivatives are responsible for a broad spectrum of biological activities. Among these the cytotoxic and antineoplastic properties have been the most widely reported. The synthetic versatility of the isatin, due to its privileged scaffold, has led to the generation of a large number of structurally diverse derivatives which include analogues derived from either mono-, di-, and tri-substitution of the aryl ring A, and/or those obtained by derivatisation of the isatin nitrogen and C2/C3 carbonyl moieties. These compounds inhibit cancer cell proliferation and tumour growth via interaction with a variety of intracellular targets such as DNA, telomerase, tubulin, P-glycoprotein, protein kinases and phosphatases. Herein we review recent highlights in the development of isatin-based compounds as anticancer agents with a particular focus on the cytotoxicity and structure activity relationships.

Key words: isatin, 1*H*-indole-2,3-dione, 2,3-dioxindole, indolinone, cytotoxicity, anticancer, enzyme inhibitor, kinase, tubulin.

1. INTRODUCTION

Isatin (1*H*-indole-2,3-dione) (**1**), an oxidised derivative of indole, was first discovered by Erdmann and Laurent in 1840 as a product arising from the oxidation of indigo (**2**) using nitric and chromic acids [1-2] (Fig. (**1**)). The compound was considered synthetic for almost 140 years until it was found to be present in plants from the *Isatis* genus [3], in fruits of the cannon ball tree, *Couroupita guianensis* Aubl. [4] and in secretions from the parotid gland of the *Bufo* frog [5]. Various substituted isatins have also been identified in plants [6-8], fungi [9], symbiotic bacteria [10] and marine molluscs [11-14], where they are postulated to play a defensive role against pathogenic organisms (Fig. (**1**)).

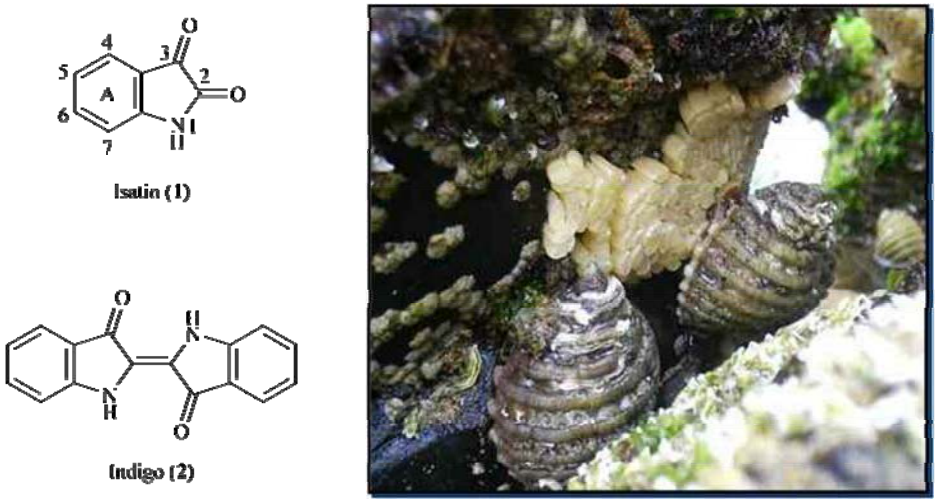


Fig. (1). The chemical structures of isatin (**1**) and indigo (**2**). The photograph shows adult Australian Muricid molluscs (*Dicathais orbita*) amongst freshly laid egg capsules, a rich source of the cytotoxic isatin derivative tyrindoleninone (6-bromo-2-methylthio-3*H*-indol-3-one) [15].

In humans and other mammals, isatin (**1**) is found as an endogenous molecule. Although the metabolic pathways of isatin (**1**) have not yet been fully elucidated, it has been proposed that it is

synthesised *in vivo* from tryptophan-rich foods such as meat, dairy and whole grains. In this pathway, tryptophan is converted to indole by bacteria from the gastrointestinal tract and then transported to the liver where it is oxidized [16-17]. Isatin (**1**) also plays a role in many physiological pathways, which are beyond the scope of this chapter (for further information see refs. [18-20]).

Isatin (**1**) contains a relatively large number of functionalisable groups and this together with its long term use in the synthetic dye industry has led to its widespread exploitation as a substrate/intermediate for organic synthesis. A number of review articles, including a comprehensive survey by da Silva *et al.* [21] describing the synthesis and chemistry of isatin have been published [22-24]. Reviews that discuss the utility of isatin as a precursor for the synthesis of other heterocyclic compounds are also available [25-27]. More recently, reviews have focused on the biological role of endogenous isatin [19-20, 28] and the diverse range of biological activities displayed by assorted isatins and isatin derivatives [18, 29-34] including oxindoles and their copper complexes [35-36]. Most recently, isatin derivatives have received considerable attention due to their potent anticancer activities [31]. Herein, we review the current literature describing the cytotoxic and anticancer activities of isatin analogues derived from either mono-, di-, and tri-substitution of the aryl ring A, and/or those obtained by derivatisation of the isatin nitrogen and C2/C3 carbonyl moieties (Fig. (**2**)). Advances in the development of more sophisticated isatin-based chemotherapeutics, including dual action drugs and selectively deliverable conjugates, and the potential utility of isatin anticancer drugs in combination therapies is also discussed.

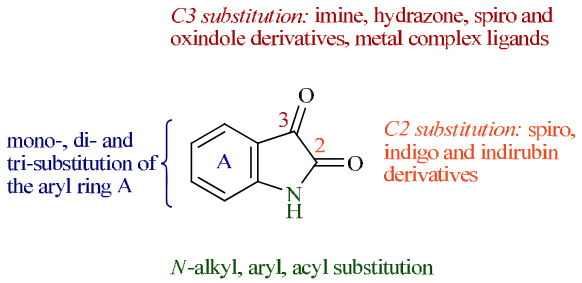


Fig. (2). The various substitution types and patterns possible for the isatin scaffold.

Finally, the cancer cell killing ability, or cytotoxicity, of a particular compound is typically defined as the concentration required to inhibit the growth of (and ultimately kill) 50% of a cell population *in vitro*, and is reported as an IC_{50}, EC_{50}, LD_{50} or GI_{50} value. It is important to note, however, that whilst this standard nomenclature allows for greater comparison of compounds across different structural classes, the use of diverse cell lines, assays (e.g. MTS, MTT, lactate dehydrogenase, Sulforhodamine B/ Kiton Red, WST-1 and clonogenic), incubation times and concentration units (i.e. moles *vs.* grams per litre) always need to be taken into consideration when comparing the potency of the compounds discussed in this chapter.

2. CYTOTOXIC AND ANTICANCER ACTIVITIES OF ISATIN DERIVATIVES

2.1 Mono-, di- and tri-substituted aromatic isatin derivatives

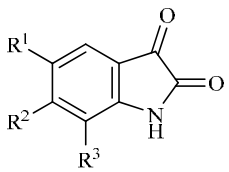
The isatin scaffold is a versatile moiety and its success as a new class of antineoplastic agents is supported by the approval of the oxindole, sunitinib maleate (Sutent®) by the Food and Drug Administration for the treatment of advanced renal carcinoma [37], gastrointestinal stromal tumours [38] and, more recently for neuroendocrine tumours of the pancreas (see section 2.4.1). Of importance

to its activity is the fluorine atom at C5, which is not surprising, as substitution at the 5-position has previously been associated with increased biological activity for a range of isatin-based compounds. For example, in 2007 Vine *et al.* reported the *in vitro* cytotoxicity of a range of mono-substituted isatins (**3a-g,** Fig. (**3**)) on a human monocyte-like, histiocytic lymphoma (U937) cell line [39]. Structure activity relationship (SAR) studies revealed that substitution at position 5 was favoured over positions 4, 6 or 7, leading to greater cancer cell killing ability. Nitration at C5 (**3f**) improved the anticancer activity by a factor of 4, while the addition of a methoxy group (**3g**) only mildly improved cytotoxicity (i.e. decreasing the IC_{50} by 145 μM) over a 24 h period. Furthermore, halogenation yielded the most active compounds, with 5-bromo-, 5-iodo-, and 5-fluoroisatin (**3a**, **3e** and **3d** respectively) being 5-10 times more active than the unsubstituted parent compound [39]. Increasing the number of electron-withdrawing groups on the ring to make combinations of dibromo-, tribromo-, iodo- and nitroisatin derivatives (**3h-n**) also enhanced the overall activity against a panel of human cancer cell lines, by up to 100-fold from that of the parent molecule (**1**). Despite this trend, 6,7-dimethylisatin was deemed inactive in a brine shrimp lethality assay (LD_{50} 96 ppm) compared to other substituted heterocyclic isatins [40], suggesting that together with substitution on the aromatic ring A, substitution at N1, C2 and/or C3, may also significantly enhance the molecule's cytotoxic effect (see sections 2.2 - 2.4).

In terms of its mode of action, isatin itself is proposed to inhibit cancer cell proliferation via interaction with extracellular signal-related protein kinases (ERKs), thereby promoting apoptosis. In 2000, Cane *et al*. [41] reported that isatin inhibited the proliferation of a human promyelocytic leukemia (HL60) cancer cell line by 80% at a concentration of 0.1 mM, and consequently induced DNA fragmentation and chromatin condensation. Moreover, treatment of N1E-115 neuroblastoma

cells with isatin at the same concentration inhibited the phosphorylation of ERK-2, but not ERK-1, by 35% compared to untreated control cells. A subsequent study using human neuroblastoma SH-SY5Y cells confirmed that the effect of isatin on cell proliferation was dose- and time-dependent [42]. For example, after 24 h of exposure, isatin caused 35% detachment (or cell death) of SH-SY5Y cells treated with high concentrations (400 μM) of the compound. Following a 48 h exposure, the percentage of detached cells significantly increased to 82%. Apoptosis was observed in cells exposed to lower concentrations of isatin (50 μM), as indicated by morphological changes (including cell shrinkage and nuclear condensation), internucleosomal DNA fragmentation and externalisation of plasma membrane phosphatidylserine. At higher concentrations, (greater than 200 μM) cell death increased even further, however evaluation of membrane permeability by fluorescence-activated cell sorting revealed that isatin had significantly damaged the plasma membrane, consistent with necrotic cell death [42]. Such a time- and dose-dependent switch from apoptosis to necrosis was also observed by Vine *et al.* whereby 5,6,7-tribromoisatin (**3n**) was found to be anti-proliferative (and induce apoptosis) at low concentrations (4 μM), but cytotoxic (and induce necrosis) at high concentrations (130 μM) in U937 cells [39]. ERK activation was not tested in this study.

	R^1	R^2	R^3
3a	Br	H	H
3b	H	Br	H
3c	H	H	Br
3d	F	H	H
3e	I	H	H
3f	NO_2	H	H
3g	OMe	H	H



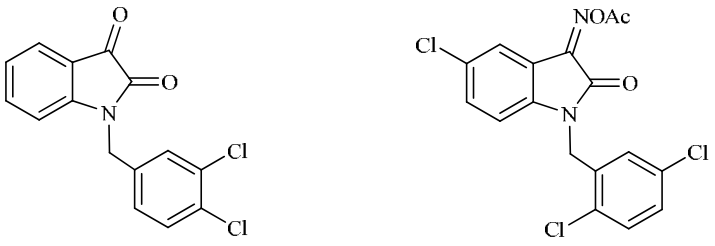
3

	R^1	R^2	R^3
3h	Br	H	Br
3i	Br	Br	H
3j	I	H	I
3k	Br	H	NO_2
3l	NO_2	Br	H
3m	NO_2	H	Br
3n	Br	Br	Br

Fig. (3). Selected isatin derivatives with mono-, di- and tri- substitution on ring A.

2.2 *N*-Alkyl substituted isatin derivatives

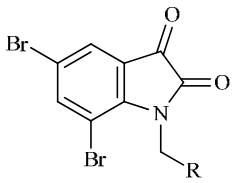
N-Alkylated indoles are well known to exhibit anticancer activity [43-45]. However, until recently, little had been reported on the antineoplastic activity of *N*-alkyl and *N*-aryl isatins. In 2003, compound **4** (Fig. (**4**)), was the first *N*-alkylisatin described to induce apoptosis in a panel of human cancer cell lines, but not normal cells, at micromolar concentrations [43]. Generally it was found that normal cell lines such as peripheral blood lymphocytes and human mammary epithelial cells were resistant to *N*-(3,4-dichlorobenzyl)-1*H*-indole-2,3-dione (**4**) induced apoptosis, while cancer cell lines of lymphoid origin were the most sensitive. Further screening by the National Cancer Institute (NCI) found that the dichlorinated compound exerted a cytostatic effect (inhibiting cell growth by 50-100%) on 40 out of the 48 cell lines tested, at a concentration of 10 μM. At 10 times this concentration however, the compound exhibited 100% cytotoxicity on virtually all cell lines, suggesting that this effect may be due to non-specific toxicity [43]. Later in 2003, Liu *et al.* [44], identified a class of isatin *O*-acyl oximes (e.g. compound **5**) that selectivity inhibited neuronal ubiquitin *C*-terminal hydrolase (UCH-L1) at sub-micromolar concentrations in the H1299 lung cancer cell line (Fig. (**4**)). Despite being linked to Parkinson's disease, UCH-L1 is also expressed in many primary lung tumours and lung cancer cell lines, but not normal lung tissue [46-47], where it is proposed to be linked to tumour progression upon upregulation [44]. Expression of UCH-L1 also correlates with tumour progression in colorectal cancer [48]. Hence, its inhibition by small molecules like compound **5**, may be of great therapeutic benefit to lung and colorectal cancer patients in the future.

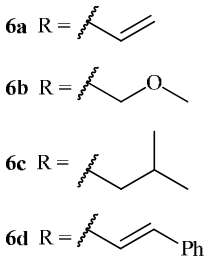


4 5

Fig. (4). Examples of the first synthetic cytotoxic *N*-substituted isatin-based molecules.

In 2007, Vine *et al.* [49] synthesised a series of dibrominated *N*-substituted isatins (e.g. **6a-d** and **7a-n**, Fig. (**5**)). Given the increased potency of the halogenated isatins (see section 2.1) and the fact that *N*-methylation significantly enhanced the cytotoxicity of the parent compound (**1**) [39], these compounds were evaluated for their cytotoxicity against a panel cancer cell lines *in vitro* [49]. SAR studies indicated that the introduction of an aromatic ring with a one or three carbon atom linker at N1 increased the activity relative to that of the allyl (**6a**), 2′-methoxyethyl (**6b**) and 3′-methylbutyl (**6c**) *N*-substituted isatins. Furthermore, electron-withdrawing groups substituted at the *meta* or *para* position of the substituent phenyl ring were favoured over the *ortho* orientation. Of the 24 compounds screened, nine displayed sub-micromolar IC_{50} values and in general demonstrated greater selectivity toward leukemia and lymphoma cell lines over other carcinoma cell lines tested. 5,7-Dibromo-*N*-(*p*-methylbenzyl)isatin (**7f**) was the most active compound, inhibiting the metabolic activity of two haematopoietic cancer cell lines by 50% at 0.49 μM. This effect was further enhanced by at least a factor of two when the incubation time was increased from 24 h to 72 h, making this class of compounds >10 times more active than the conventional chemotherapeutic agents 5-fluorouracil (5-FU), paclitaxel and vinblastine against U937 cells [50].



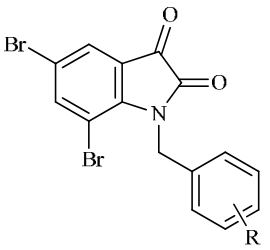


6a R =

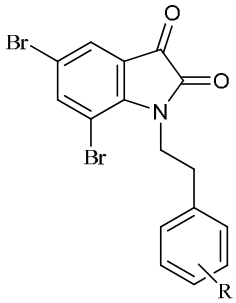
6b R =

6c R =

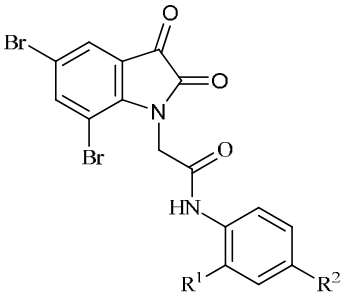
6d R =



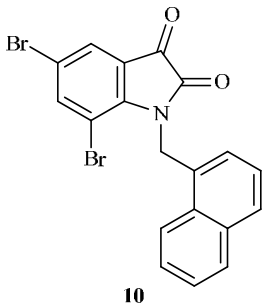
7a R = H
7b R = *p*-I
7c R = *p*-Br
7d R = *p*-Cl
7e R = *p*-CF_3
7f R = *p*-Me
7g R = *p*-CO_2H
7h R = *p*-CO_2Me
7i R = *p*-(*t*-Bu)
7j R = *p*-Ph
7k R = *p*-OMe
7l R = *m*-OMe
7m R = *p*-NO_2
7n R = *o*-NO_2



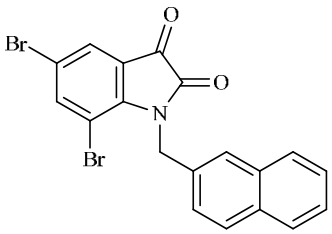
8a R = H
8b R = *m*-Br
8c R = *p*-Br
8d R = *m*-OMe
8e R = *p*-OMe



	R^1	R^2
9a	H	H
9b	NO_2	H
9c	CH_3	H
9d	H	NO_2
9e	H	CH_3



10



11

Fig. (5). Examples of cytotoxic *N*-substituted 5,7-dibromoisatins including the *N*-naphthyl isatin derivatives (**10** and **11**).

Continuing this work, in 2008 Matesic *et al.* synthesised a family of *N*-phenethyl derivatives (**8a-e**, Fig. (**5**)) [51]. All five *N*-phenethyl derivatives (**8a-e**) exhibited low to sub-micromolar cytotoxic activity against a panel of human leukemic, lymphoma and carcinoma cell lines where introduction of a hydrophobic bromo substituent in the *meta* (**8b**) or *para* (**8c**) position yielded the most active compounds [51]. Interestingly, the corresponding phenacyl derivatives (not shown) were at least 3-5 times less active against the U937 cells [51]. Related *N*-phenylacetamide derivatives (**9a-e**) prepared by Modi *et al.* [52] were also active against a MCF-7 human mammary adenocarcinoma cell line with IC_{50} values ranging from 1.09 - 3.91 μM and exhibiting up to 20-fold selectivity for cancer cells over non-cancerous cells.

In 2009, further examples of new *N*-alkylisatin derivatives were synthesised and evaluated for their cytotoxic activity, including Singh *et al*. [53], who described five *N*-alkylbromo and five *N*-alkylphthalimido isatin derivatives of which the most active compound gave IC_{50} values of 4.57, 10.90, 11.75, 12.40 and 54.20 μM after 48 hour incubation against HeLa, PC-3, HCT-15, THP-1 and Colo-205 cell lines respectively. Solomon *et al*. [54] prepared a series of 15 dialkyl/diaryl amino methyl, morpholinyl and piperidinyl *N*-alkylisatins bearing either an unsubstituted aromatic ring or halogenation (Cl or Br) solely at position 4 of the aromatic ring. All 15 compounds, along with their analogous 3-substituted benzothiazole derivatives (see section 2.5), were screened against three breast cancer cell lines (MDA-MB-231, MDA-MB-468 and MCF-7) and two non-cancer cell lines (184B5 and MCF10A). The derivatives were found to be active in the mid-micromolar range (>10-100 μM) after a 48 h incubation, with the most active compound, 4-bromo-1-diethylaminomethyl-1*H*-indole-2,3-dione (IC_{50} 11.68 μM; MDA-MB-468), displaying 10-15 fold greater selectivity for cancer over non-cancer cells. It is interesting to note that the derivatives in the above two studies lacked

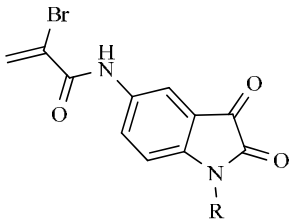
substitution at the 5- and/or 7-position of the aromatic moiety known to be important for cytotoxic activity, which may be reflected in their activities being in the mid-micromolar rather than sub-micromolar range.

Other examples of *N*-substituted isatins that display potent cytotoxic activity include the dibrominated 1- and 2-naphthyl derivatives **10** and **11** (Fig. (**5**)). The *N*-naphthyl isatins were found to display sub-micromolar cytotoxicity against U937 and human leukemic T–cell (Jurkat) cell lines (e.g. IC_{50} for the 1-naphthyl derivative (**10**) of 0.19 and 0.91 μM, respectively), along with low micromolar cytotoxicity towards a panel of human carcinoma cell lines, including those derived from breast (MDA-MB-231 and MCF-7), colon (HCT-116) and prostate (PC-3) [51]. Interestingly, compound **10** represents one of the most cytotoxic dibrominated *N*-alkylisatin reported to date, when compared with other *N*-alkylisatins tested over a 24 h time period. This suggests that increasing the hydrophobicity of the *N*-substituent through site specific placement of an additional aromatic ring is important for activity.

In 2010, Sabet *et al*. [55] reported a QSAR study that utilised biological data from 47 of the isatin derivatives reported previously by Vine [39, 49] and Matesic [51] to reveal that positive effects to anticancer activity were related to the number of halogen atoms and secondary carbons, while negative effects were related to the number of secondary amides and ketones. Their study also further confirmed the importance of lipophilic substituents at position 5 and 7 on the aromatic ring to the anticancer activity of isatin derivatives. These findings were further supported by another QSAR study in 2011, where Modi *et al*. [52] found that electron-withdrawing substituents at the *para*-position of the phenyl ring, substitution at the 5,7-positions of the isatin ring and increasing lipophilicity of the compound enhanced the cytotoxic activity.

In addition to their potent cell killing ability, various *N*-alkylisatins were also found to induce G2/M cell cycle arrest and dramatically alter lymphocyte morphology [49, 51, 54]. As these morphological alterations were also seen in vinblastine and paclitaxel-treated cells, Vine *et al.* suggested that *N*-alkylisatins may either disrupt or stabilise microtubules in a similar fashion [49]. Various *N*-alkylbenzylisatins including representative phenethyl and naphthyl derivatives were therefore screened for their ability to interfere with microtubule dynamics and were found to strongly inhibit the rate of microtubule polymerisation. This effect was shown to be dose-dependent, and appeared to be independent of the nature of the *p*-substituent and size of the benzene carbon linker, as compounds **6d**, **7e**, **7f**, and **7j** all inhibited microtubule polymerisation at a similar rate [49]. All *N*-alkylbenzylisatins tested, including the phenethyl and naphthyl compounds were found to be potent microtubule destabilisers, which is consistent with reports that structurally similar indole [45, 56] and indolinone [57-58] compounds inhibit tubulin polymerisation.

In 2009, Romagnoli *et al*. [59] combined the α-bromoacrylamido found in a number of natural cytotoxic alkaloids such as discorhabdin A and G with the *N*-alkylisatin type structures described earlier [49] to generate a new series of 20 α-bromoacrylamido *N*-substituted isatin derivatives (**12a-t,** Fig. (**6**)). The majority of the compounds exhibited sub-micromolar cytotoxicity when incubated for 72 h with IC_{50} values ranging from 0.15 - 8.53 μM against human myeloid leukemia HL60, U937, and human lymphoid leukemia MOLT-3 cells. In addition, the authors used morphological changes, flow cytometry and western blot analyses to show that the antiproliferative activity of the compounds in human leukemia cells is likely to be mediated by apoptosis caused by cytochrome c release and caspase activation.



12

12a R = H
12b R = CH_3
12c R = CH_2CH_3
12d R = $(CH_2)_2CH_3$
12e R = $CH_2CH{=}CH_2$
12f R = CH_2CCH
12g R = $CH_2CH(CH_2)_2$
12h R = $(CH_2)_3CH_3$
12i R = $(CH_2)_4CH_3$
12j R = $(CH_2)_5CH_3$
12k R = C_6H_5
12l R = 4-Me-C_6H_4
12m R = 4-OMe-C_6H_4
12n R = 4-F-C_6H_4
12o R = $C_6H_5CH_2$
12p R = 4-Me-C_6H_4
12q R = 4-*t*-Bu-C_6H_4
12r R = 4-F-C_6H_4
12s R = 4-Cl-C_6H_4
12t R = 4-CF_3-C_6H_4

Fig. (6). Cytotoxic α-bromoacrylamido *N*-substituted isatin derivatives.

2.3 C2-Substituted isatin derivatives

The most famous of the C2-substituted isatins are the widely employed indigo pigments used since ancient times. This class includes indigo carmine (5,5'-indigodisulfonic acid sodium salt), an extensively used non-toxic, inexpensive stain for chromo-colonoscopic diagnosis and management of early colorectal cancer [60-63]. The best known cytotoxic C2-substituted isatin derivative is indirubin (**13**, Fig. (**7**)), the red component of indigo pigments. This compound is found in plants belonging to the genii *Indigofera*, *Isastis* and *Polygonum*, and in marine molluscs of the *Murex* genus [64]. Indirubin is the active component in the traditional Chinese antileukemia medicine Danggui Longhui Wan, and the synthesis and biological activities of indirubin and its derivatives have been well studied and reviewed [65-68]. The indirubins are thought to have great potential for pharmaceutical use however, they are plagued by extremely poor aqueous solubility. As a consequence, investigations focus on developing indirubins with improved water solubility and recent studies have often explored the more soluble 3'-oxime substituted derivatives and/or side chains designed to enhance aqueous solubility. A number of these derivatives with various substitution patterns exhibit sub-micromolar

and/or high-nanomolar IC_{50} values against human cancer cell lines *in vitro* (Fig. (**7**), compounds **14-16**) [69-73].

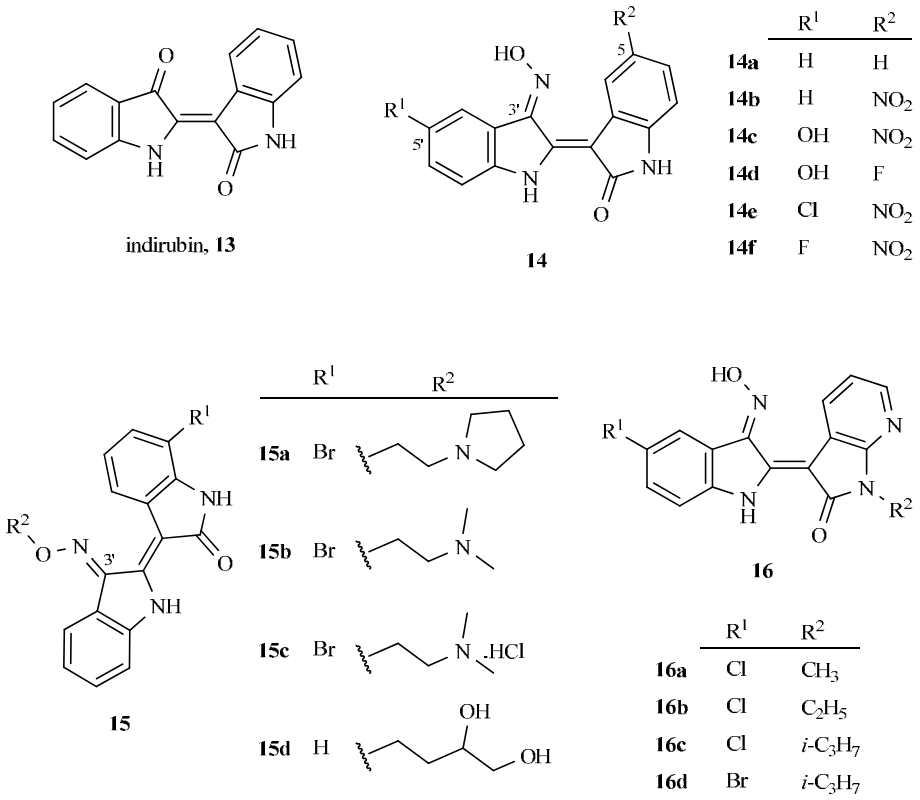


Fig. (7). The structure of indirubin (**13**) and some indirubin-3'-oxime derivatives (**14a-f**), E804 (**15d**) and a number of recently disclosed soluble indirubin-3'-oximes (**15a-c**) [69-70] and azaindirubins (**16a-d**) [71] that exhibit low micromolar/high nanomolar IC_{50} values (≤ 3.8 μM) against a variety of different human cancer cell lines *in vitro*.

Indirubins exert their cytotoxic action *via* a number of modes [74] and they are recognised for their ATP-competitive inhibition of both CDK1 and CDK2 [75-77] and induction of apoptosis through cell cycle arrest at G2/M, as a result of inhibition of GSK3 [78], c-Src kinase and NF-κB activation and

expression [79-80] as well as the activation of the aryl hydrocarbon receptor [81-82]. Based on a range of mechanistic and crystallographic studies of indirubins with GSK3 and CDK2, a vast number of indirubin derivatives have been synthesised and their biological activity and specificity evaluated [76, 83-84]. A number of recent studies have identified numerous alternative modes for the cytotoxic and antiangiogenic actions displayed by indirubin and some of its derivatives. Other actions of the indirubins include suppression of the VEGFR-2-mediated JAK/STAT3 signalling pathway by indirubin (**13**) [85]; inhibition of alternative kinases such as Fms-like tyrosine kinase 3 by indirubin-3'-oxime (**14a**) and its 6-bromo analogue [86]; and inhibition of polo-like kinase 1 [87] as well as downregulation of Notch-1 and inhibition of Integrin β1/FAK/Akt signalling by 5-nitroindirubin-3'-oxime (**14b**) [86, 88-90].

In addition to increased solubility relative to 3'-keto compounds, the 3'-oxime substituted derivatives often exhibit either equivalent or greater cytotoxicity against cancer cell lines *in vitro* [71, 86, 91-92]. The 3'-oxime indirubin derivative 3'-(3,4-dihydroxybutoxy)iminoindirubin or E804, (**15d**, Fig. (**7**)) has also recently shown potent antiangiogenic activity [93]. E804 is known to block the Src-STAT3 signalling pathway [94] and demonstrated strong inhibition of STAT5 and subsequently induced apoptosis in K562 chronic myelogenous leukemia (CML), imatinib resistant KCL-22M and primary CML cells [95]. A recent study that combined the indirubin moiety with amide-linked side chains terminating in assorted amine groups (**17a-d**, Fig. (**8**)) to produce derivatives with structural similarities to sunitinib (see section 2.4.1). A series of these compounds were tested against a panel of cell lines and were particularly active against SK-BR-3 breast carcinoma cells with compound **17a** showing sub-nanomolar activity (IC_{50} = 0.8 nM), significantly higher potency than that observed for the sunitinib control (3.55 μM) in this cell line [96].

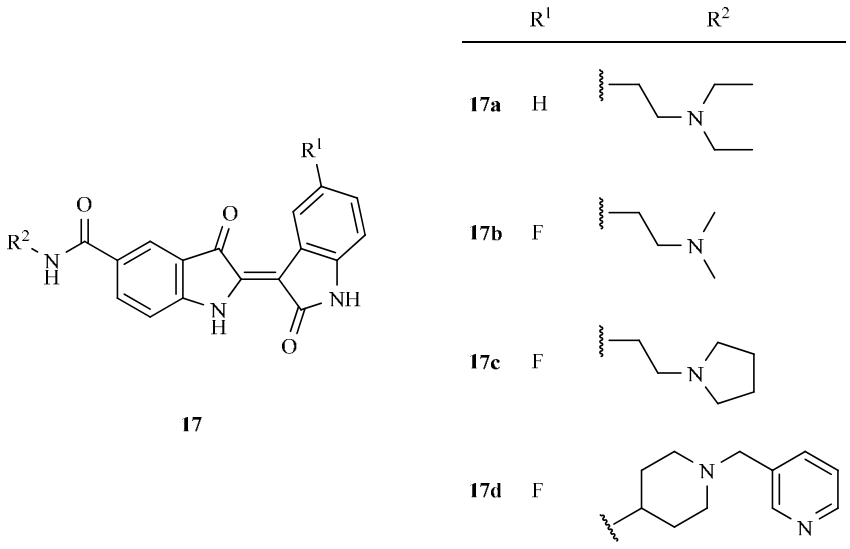


Fig. (8). Structure of indirubin derivatives whose design was based on the structure of sunitinib.

In addition to indirubin derivatives, the marine environment has yielded an array of other novel C2-substituted indolinones including the alkaloid matemone (**18**, Fig. (**9**)) [97]. Isolated from the Indian Ocean sponge *Iotrochota purpurea*, matemone (**18**) was found to intercalate DNA, to inhibit the division of sea-urchin eggs and exhibited mild cytotoxicity towards a range of human carcinoma cell lines. More recent examples of marine-based isatins have been provided by the sea cucumber-associated fungus *Aspergillus fumigatus,* which was found to produce several novel prenylated indole diketopiperazine alkaloids, including compound **19** and the three spirotryprostatins C-E (**20a-c**, Fig. (**9**)). The prenylated indoles were evaluated for their cytotoxicity with derivative **20c** exhibiting the most potent activity with IC_{50} values of 2-3 μM against the human non-small cell lung carcinoma (A549), the human acute lymphoblastic leukemic (MOLT-4) and HL60 cell lines [98].

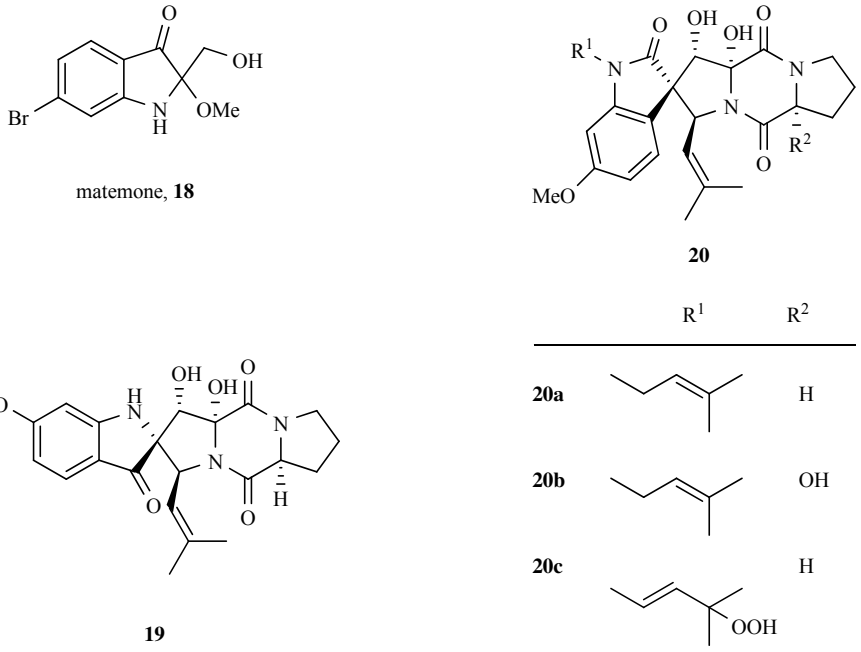


Fig. (9). Cytotoxic, marine-derived C2-substituted isatin derivatives.

Based on previous studies of the aurones, a range of 2-arylidenedihydroindole-3-ones (**21a-f**, Fig. (**10**)), or azaaurones, have been synthesised and evaluated for their antiproliferative and apoptotic abilities against bladder tumours. Azaaurone (**21c**), the most active compound, inhibited proliferation and induced apoptosis *via* a fibroblast growth factor receptor-3 (FGFR) dependent pathway in DAG-1 and RT112 bladder tumour cell lines [99].

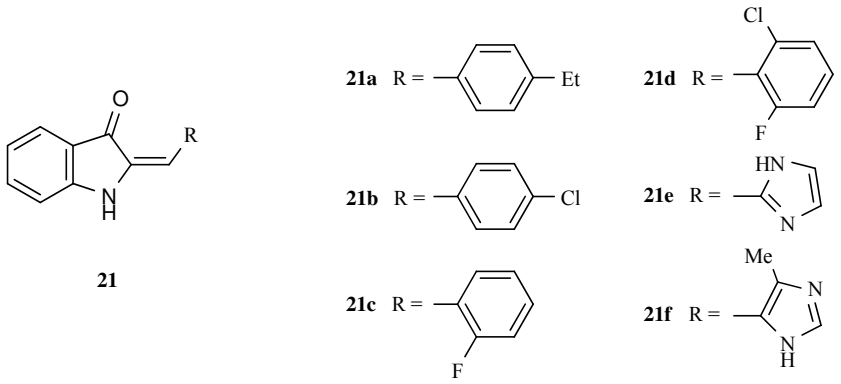


Fig. (10). Structures of some bioactive azaaurones reported by Gerby *et al* [99].

2.4 C3-Substituted isatin derivatives

A plethora of C3-substituted isatins (also known as indolinones or oxindole derivatives) have been generated due to the susceptibility of isatin to be attacked by nucleophiles at the C3 position. This is reflected by the vastly greater proportion of biologically active 3-substituted isatins reported in the literature relative to the other substitution patterns. This section will highlight the recent progress of C3-substituted isatins containing arylidene, hydrazone/imine and metal functionalities, together with isoindigo and other miscellaneous isatin analogues.

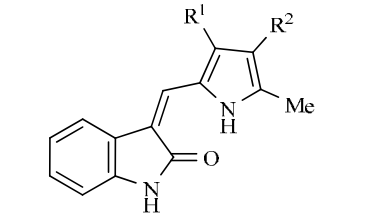
2.4.1 3-Arylidene derivatives

Indolinones such as SU5416 (semaxanib, **22**) have been discontinued in clinical trials due to dangerous side effects [100], although the compound is extremely potent with GI_{50} values of < 10 nM against 46 out of 53 NCI cell lines [101]. In contrast, SU11248, (sunitinib, Sutent®, **23**), is now the standard first-line treatment for the treatment of gastrointestinal stromal cancers and renal cell

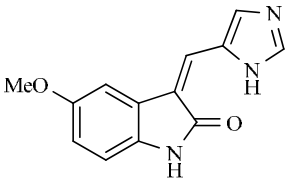
carcinoma [102]. The drug is a multiple receptor tyrosine kinase (RTK) inhibitor, targeting VEGFR-2, platelet-derived growth receptor-β (PDGFR-β), stem cell factor receptor, fetal liver tyrosine kinase receptor-3 [100], colony-stimulating factor type 1 and glial cell-line derived neurotrophic factor receptor [103]. 3-Substituted indolinones possessing the *Z* configuration are potent inhibitors of the FGFR, while compounds adopting the *E* configuration inhibit epidermal growth factor receptor (EGFR) [56].

SU9516 (**24**) is capable of causing mitochondrial damage, caspase activation and inducing apoptosis in a dose- and time-dependent manner in U937 cells [104]. Pyrrolyllactone and pyrrolyllactam derivatives of **24** have been synthesised and described as potent CDK2 inhibitors, particularly compounds exhibiting a polar substituent in the 4-position [105].

The indolinone SU5402 (**25**), encompasses a *Z* configuration about the alkenyl bond. Twenty analogues of **25** bearing the *E* configuration were synthesised and subsequently assessed for cytotoxicity [106]. The lead compound, containing a methyl ester on the pyrrole group displayed IC_{50} values of 98 nM and 65 nM against A549 lung and MDA-MB-468 breast carcinoma cell lines after a 72 h exposure.



	R^1	R^2
22	Me	H
25	$(CH_2)_2COOH$	Me
26	Me	$(CH_2)_2COOH$



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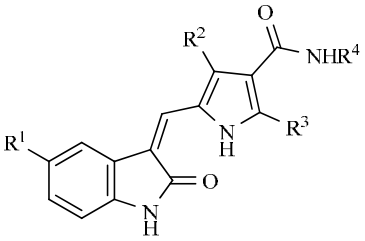


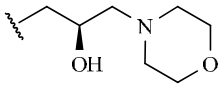
	R^1	R^2	R^3	R^4
23	F	Me	Me	$N(Et)_2$
27	F	Me	Me	
28	F	Cl	Me	NHEt
29	Cl	Cl	Me	$N(Et)_2$
30	F	C_2H_5	Cl	NHEt

Fig. (11). Examples of 3-pyrroloarylidene isatin derivatives including the clinically used sunitinib (**23**).

SU6668 (**26**) significantly suppresses tumour angiogenesis [107], and is capable of inducing substantial tumour regression, regardless of initial tumour size. The drug was evaluated in four separate Phase I trials, but was discontinued due to the superior activity of sunitinib (**23**). SU14813 (**27**) has also emerged as a multiple RTK inhibitor with strong antiangiogenic and antitumour activity. It expresses dose-dependent antitumour efficacy and assists in the regression of human renal (786-O), human acute myelogenous leukemia (MV4;11), human colon (Colo-205 and MV522) and rat glioma (C6) tumours [108]. Additionally, the compound displays moderate systemic clearance and inhibition

of tumour growth can be improved when **27** is combined with the anti-mitotic chemotherapeutic, docetaxel.

An extension of this scaffold included 18 novel 3-substituted indolinones incorporating chloropyrroles [109]. The most bioactive compounds in the series, as determined by evaluating the analogues against four tumour cell lines, were **28-30** (Fig. (**11**)). Indolinone **28** displayed 3-4 fold greater cytotoxicity than sunitinib (**23**) against KB oral epithelial and K111 melanoma cells, while analogues **29** and **30** were highly cytotoxic against A549 cells, with compound **29** being up to 10-fold more cytotoxic than **30** in this cell line. Furthermore, a derivative of sunitinib (**23**) containing a *N*-(dimethylamino)propyl group has been found to be up to twice as cytotoxic as **23** against HL60, K562 leukemia and MDA-MB-231 breast cancer cells [110].

Luo *et al.* have also prepared a series of 3-pyrrolo-substituted indolinones and compared their cytotoxicity to sunitinib (**23**) [111]. 5-Vinylsulfonyl indolinones such as **31a-c** (Fig. (**12**)) were found to exhibit IC_{50} values < 10 μM against A549, SQC-7901, ECA-109 and HCT-116 cells. Compound **31b** was more active than sunitinib (**23**) against all four cell lines. Additionally, 5-sulfonyl indolinones **32a** and **32b** were also found to possess potent cytotoxicity and compound **32b** was also more active than **23** against the four cell lines.

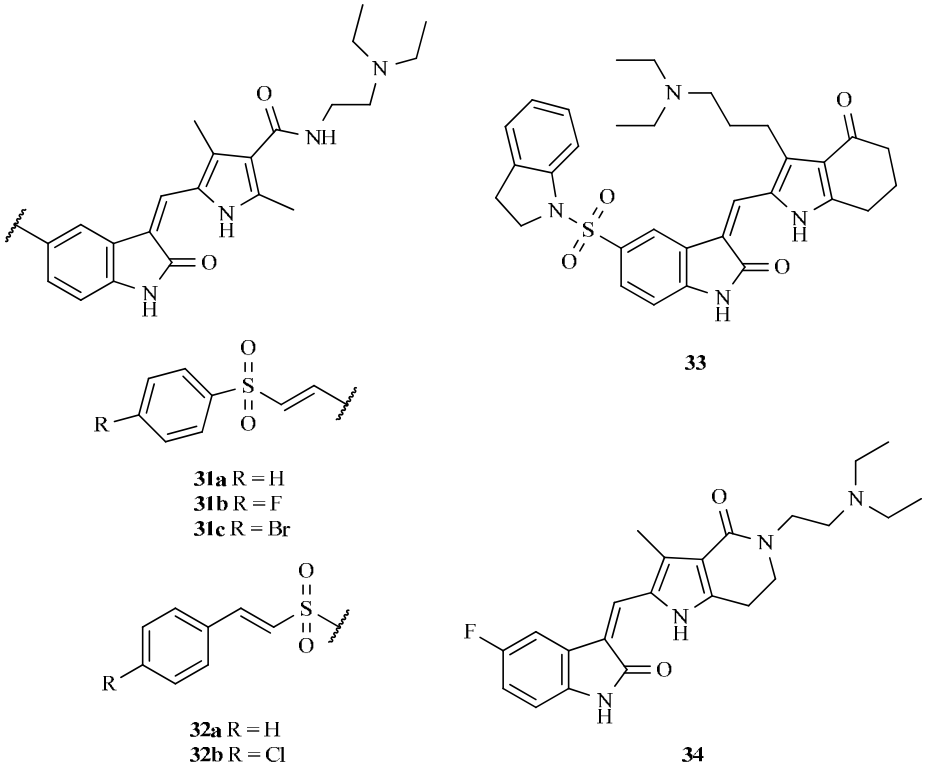
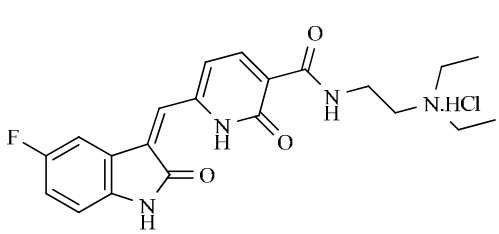


Fig. (12). Examples of 3-pyrroloarylidene isatins containing a sulfonyl functionality at C5 of isatin (**31** and **32**) and 3-pyrrolo-fused heterocyclic isatins (**33** and **34**).

Indolinones incorporating a cycloalkanone fused onto the pyrrole ring have also been prepared [112]. The most cytotoxic analogue (**33**, Fig. (**15**)) displayed an IC_{50} value of 1.62 μM against HeLa cells after a 72 h exposure and sub-micromolar IC_{50} values against colonic HCT-116 and HT-29 cells. Additionally, the compound was efficacious as an Aurora kinase inhibitor, particularly towards Aurora kinase B. Pyrrolo-fused heterocyclic analogues have been investigated and compound **34** has been evaluated in human HT-29 colon tumour xenografts assay in BALB/cA-nude mice [113]. After 14 days of once daily administration, the compound led to antitumour efficacy in a dose-dependent manner and the tumour growth was inhibited by 74.2% at the maximum dose of 30 mg/kg.

Indolinones possessing a pyrrole ring, similar to the above mentioned compounds, have additionally been identified as inhibitors of the cAkt2 protein kinase, which is activated by phosphoinositide-dependent kinase-1 through the support of phosphoinositide 3-kinase activity in cells [114]. Activity towards the cAkt2 kinase was increased almost 400-fold through the addition of a urea group at C5 on the isatin core and a methyl group on the alkene bond at C3. The novel compound named BX-517, was optimised by exploring the C4′ position on the pyrrole ring and led to potent phosphoinositide-dependent kinase-1 inhibitors with superior pharmacokinetic properties compared to BX-517 [115].



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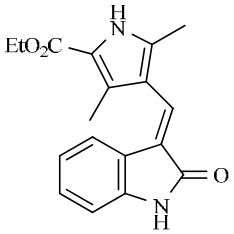
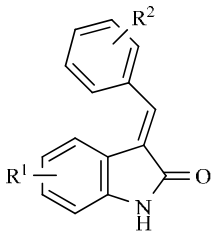
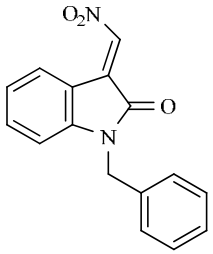


B5, **36**



37a R^1 = 6-OCH_3, R^2 = 3-OCH_3
37b R^1 = H, R^2 = 2,5-$(OCH_3)_2$



38

Fig. (13). Examples of (*E*)-3-arylidene indolinone derivatives (**36-38**) and an indolinone containing a dihydripyridine at the C3 position of isatin (**35**).

Indolinones containing a dihydropyridine instead of a pyrrole ring such as **35** (Fig. (**13**)), have been found to be cytotoxic and exhibit 3-fold greater activity than sunitinib (**23**) against HCT-116 colon, A549 lung and HepG2 liver cells [116]. When **35** was modified to include a 4- or 7-azaindolinone core, the biological activity diminished and the analogues were less cytotoxic than sunitinib (**23**) in most of the cell lines evaluated.

B5 (**36**, Fig. (**13**)), a pyrrole-substituted indolinone possessing an *E*-isomeric configuration, has been identified as an extremely active cytotoxin against a panel of lung, breast and gastric cancer cell lines [117]. Other potent *E*-isomeric indolinones include the methoxy-substituted indolinones (**37a**) and (**37b**), which display IC_{50} values < 10 μM against MCF-7 and HCT-116 cells, together with selectivity over normal human diploid embryonic lung fibroblast (CCL-186) cells [118]. Moreover, (*E*)-3-substituted indolinones incorporating alkyl-nitromethylene units such as **38** have shown excellent inhibitory activity against P388 leukemia and A549 lung cancer cells [119].

The indolinones BIBF1000 (**39**) and BIBF1120 (**40**) induce apoptosis and both compounds have an IC_{50} value of 8 nM against the leukemic Kasumi-1 cell line, along with similar activity against the leukemic Mono-Mac-1 cell line [120]. BIBF1120 (**40**, Fig. (**14**)), inhibits VEGF, FGF and PDGF receptors, displays efficacy *in vivo* amongst multiple animal models and is currently in Phase III trials for the treatment of a variety of cancers [121].

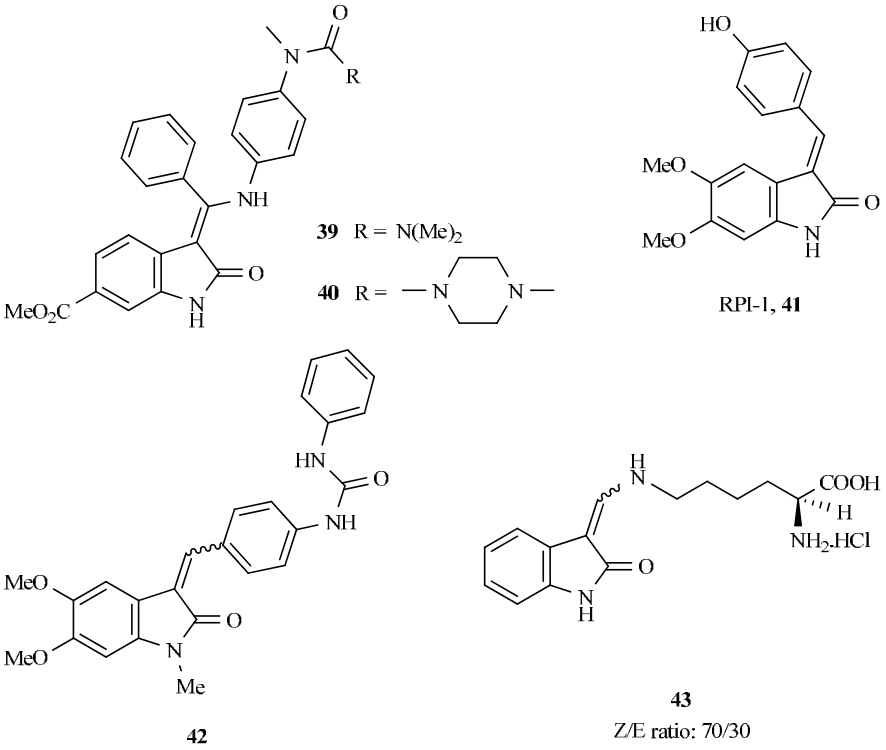


Fig. (14). Structures of the indolinones BIBF1000 (**39**), BIBF1120 (**40**), RPI-1 (**41**) and a potent derivative (**42**) as well as an example of an aminomethylene-lysine indolinone (**43**).

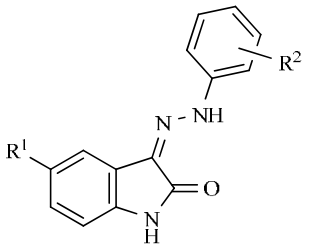
RPI-1 (**41**, Fig. (**14**)), a 3-benzylidene-indolinone, has been shown to exhibit antiproliferative activity particularly towards a modified mouse embryonic fibroblast cell line NIH3T3 (NIH3T3^{MEN2A}) (IC_{50} 3.6 μM after 72 h) [122]. Derivatives containing a diphenyl urea functionality were introduced to the 5,6-dimethoxyindolinone core of RPI-1. This led to *N*-methyl-3-arylureidobenzylidene-indolin-2-one (**42**) being identified as the lead compound due to its increased activity against 3 modified NIH3T3 cell lines [122].

A C3-substituted aminomethylene-lysine indolinone (**43**, Fig. (**14**)) was found to exhibit cytotoxicity after 48 h towards human colon (DLD-1) and ovarian (PA-1) carcinoma cell lines (IC_{50}

values ranged from 10-17 μM) [123]. Compounds containing more rigid side chains derived from D-phenylalanine and L-phenylglycine were found to be inactive.

2.4.2 Hydrazones and Imines

Isatin-based hydrazones (**44a-c**, Fig. (**15**)) have been identified as inhibitors of the protein tyrosine phosphatase Shp2, which plays an important role in cell signalling, cell proliferation, differentiation and migration [124]. Compounds containing a carboxylic acid group on the hydrazone aromatic ring and a *p*-halosulfonamide at the C5 position (i.e. **44a** and **44b**) selectively inhibit Shp2 in the low micromolar region. Analogue **44c** was the most active in the series, exhibiting an IC_{50} value of 0.8 μM against Shp2.



44

	R^1	R^2
44a	$SO_2NHCH_2(4\text{-}ClC_6H_4)$	3-CO_2H
44b	$SO_2NHCH_2(4\text{-}FC_6H_4)$	3-CO_2H
44c	CO_2H	2-CO_2H

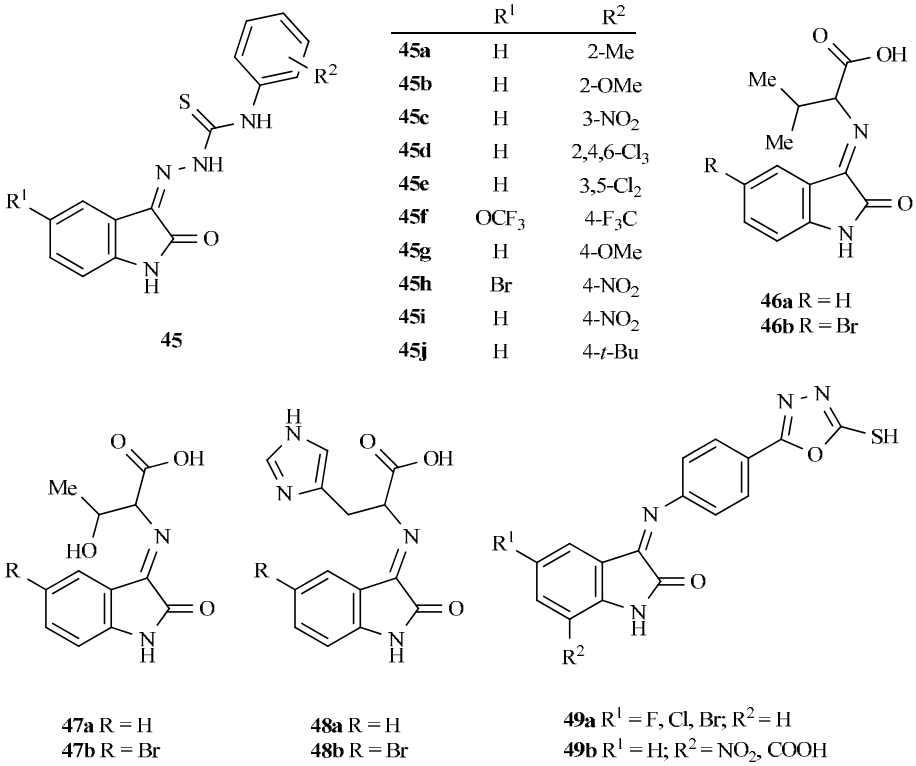
Fig. (15). Isatin based inhibitors of Shp2.

In recent years, Pervez and co-workers have reported the cytotoxic activity of isatin derivatives containing a thiosemicarbazone moiety (a hydrazone derivative containing a sulfur atom) [125-128]. Initially, 15 N^4-substituted isatin-3-thiosemicarbazones were screened for cytotoxicity against brine shrimp (*Artemia salina)* [125]. Only the 2-methylphenyl (**45a**), 2-methoxyphenyl (**45b**) and

3-nitrophenyl (**45c**) derivatives displayed significant cytotoxic activity (Fig. (**16**)). The most active analogue was **45b** with a LD_{50} value of 11 μM against *Artemia salina.* Subsequent studies assessed N^4-substituted isatin-3-thiosemicarbazones including one, two or three phenyl substituents [126]. Eleven of the 12 derivatives contained at least one fluorine atom although intriguingly, the most active compound was the trichloro-substituted analogue **45d** with a LD_{50} value of 11 μM against *Artemia salina.* Other novel N^4-substituted isatin-3-thiosemicarbazones have been synthesised and it was determined that the 3,5-dichloro-substituted derivative **45e** possessed a LD_{50} value of 17.5 μM in the brine shrimp assay [127]. Other chlorinated analogues with substituents at the 2,3-, 2,4- and 2,5-positions were far less potent, with LD_{50} values > 100 μM. To further expand the SAR of N^4-substituted isatin-3-thiosemicarbazones, the authors have also investigated the effects of an electron-withdrawing group (in this case, the trifluoromethoxy group) at the C5 position of isatin, together with mainly halogenated phenyl substituents on the thiosemicarbazone functionality [128]. Ten of the 21 compounds tested were considered to exhibit promising cytotoxicity with LD_{50} values between 11.1 and 180 μM. The 4-trifluoromethyl derivative **45f** was the most potent analogue (LD_{50} = 11.1 μM) in the series and was more active than the clinically used anticancer agent etoposide (LD_{50} = 12.7 μM) against *Artemia salina* [128].

Isatin thiosemicarbazones **45g** and **45h** (Fig. (**16**)) have been found to display cytotoxicity against the KB-3-1 cell line (a HeLa derivative) after a 72 h exposure (IC_{50} = 14.2 and 28.4 μM, respectively) [129]. More importantly, these compounds exhibit MDR1 selectivity (multidrug resistance that includes the P-glycoprotein (P-gp) family), which is calculated as a ratio between a compound's IC_{50} value against P-gp negative KB-3-1 cells divided by its IC_{50} value against P-gp positive KB-V1 cervical adenocarcinoma cells. A value of > 1 denotes that the compound kills P-gp-expressing cells

more effectively than parental cells. Further research by this group has led to the discovery of thiosemicarbazones **45i** and **45j** which display similar cytotoxicity to **45g** and **45h** against KB-3-1 cells (IC_{50} values of 17.15 and 15.90 μM respectively), however, the MDR1 selectivity increased to 8.3 and 14.8 respectively [130].



	R^1	R^2
45a	H	2-Me
45b	H	2-OMe
45c	H	3-NO_2
45d	H	2,4,6-Cl_3
45e	H	3,5-Cl_2
45f	OCF_3	4-F_3C
45g	H	4-OMe
45h	Br	4-NO_2
45i	H	4-NO_2
45j	H	4-*t*-Bu

Fig. (16). Examples of cytotoxic isatin-3-thiosemicarbazones, iminoisatins incorporating the amino acids valine (**46**), threonine (**47**) and histidine (**48**), and examples of 3-iminoisatins monosubstituted on the isatin ring (**49**).

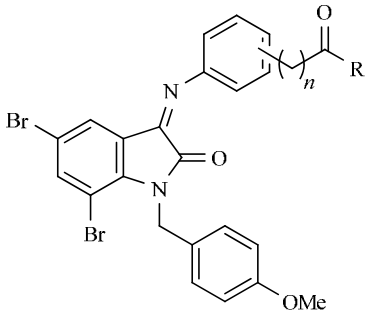
3-Imino-substituted indolinones comprising amino acids have been assessed as kinase inhibitors and cytotoxins. Imino groups incorporating valine (**46a** and **46b**, Fig. (**16**)) and threonine (**47a** and

47b) residues proved to be inactive against the CDK1/cyclin B, CDK5/p25 and GSK3α/β protein kinases [131]. Conversely, histidine imino groups (**48a** and **48b**) increased kinase activity and subsequent halogenation at C5 on the isatin nucleus yielded highly potent kinase inhibitors, with the lead compound, a 5-bromo derivative (**48b**), displaying an IC_{50} value of 0.37 μM against the CDK5/p25 kinase. Interestingly, none of the analogues synthesised displayed cytotoxicity towards human breast, lung or glioblastoma cell lines, although this may be attributed to the presence of the carboxylic acid functionality which may inhibit the entry of the compound into tumour cells.

Gudipati *et al.* have also investigated twelve 3-iminoisatins monosubstituted at the C5 or C7 positions of the isatin ring system [132]. The compounds were tested for cytotoxic activity against HeLa, IMR-32 (neuroblastoma) and MCF-7 cells and all compounds exhibited IC_{50} values between 10.6-33.6 μM against the three cell lines, which were comparable to the IC_{50} values of cisplatin. Once again, halogen atoms at the C5 position yielded the most potent compounds (e.g. **49a**, Fig. (**16**)), while analogues bearing a C7 substituent were less active (e.g. **49b**).

Previously reported *N*-alkylated 3-iminoisatins [133] (**50a-e**, Fig. (**17**)) have been found to be extremely cytotoxic, with two derivatives (**50c** and **50e**) displaying nanomolar activity towards U937 lymphoma cells after a 24 h exposure [134]. Lysine derivatives of these 3-iminoisatins (**51a-e**) also exhibited potent cytotoxic effects, with all five analogues possessing nanomolar activity and being more cytotoxic than their corresponding imino derivatives (a 19-fold increase in the case of **50d** *vs.* **51d**). Within the 3-iminoisatin series, the increase in chain length correlated with an increase in cytotoxicity (i.e. **50a** < **50c** and **50d** < **50e**), however, this trend was not observed within the isatin-lysine series (**51a-e**). Generally, *meta*-substituted compounds were more cytotoxic than their corresponding *para*-substituted analogues. The enhanced cytotoxicity of the isatin-lysine series (**51a-e**)

when compared to the 3-iminoisatins (**50a-e**), suggests that increasing the alkyl chain length of 3-iminoisatins leads to a change in intracellular target.



Imine	*n*	Substitution	IC_{50} (uM)
R = OH			
50a	0	*para-*	1.95 (± 0.69)
50b	1	*para-*	2.13 (± 0.51)
50c	2	*para-*	0.422 (± 0.09)
50d	0	*meta-*	3.14 (± 0.39)
50e	1	*meta-*	0.463 (± 0.08)
R = $(CH_2)_4CH(NHAc)CO_2Me$			
51a	0	*para-*	0.575 (± 0.37)
51b	1	*para-*	0.642 (± 0.03)
51c	2	*para-*	0.399 (± 0.11)
51d	0	*meta-*	0.165 (± 0.06)
51e	1	*meta-*	0.391 (± 0.13)

Fig. (17). Cytotoxic 5,7-dibrominated-*N*-alkylated 3-iminoisatins.

Furthermore, bis-Schiff bases of 5-fluoroisatin (**3d**) such as 3,3'-[oxybis(4,1-phenylenenitrilo)]bis(1,3-dihydro)-5-fluoro-2*H*-indol-2-one have been identified to be cytotoxic towards the human embryonic cell line (HEL) while a dichlorinated derivative, 3,3'-[methylenebis(2-chloro-3,5-diethyl-4,1-phenylenenitrilo)]bis(1,3-dihydro)-2*H*-indol-2-one, displayed cytotoxicity at 16 μg/mL against African green monkey kidney (Vero) and HeLa cell lines [135].

2.4.3 Metal complexes

Isatin–diimine copper complexes, Cu(isapn) (**52**, Fig. (**18**)) and $Cu(isaepy)_2$ (**53**), have been evaluated against SH-SY5Y, M14 (melanoma) and U937 cell lines and were found to induce apoptosis

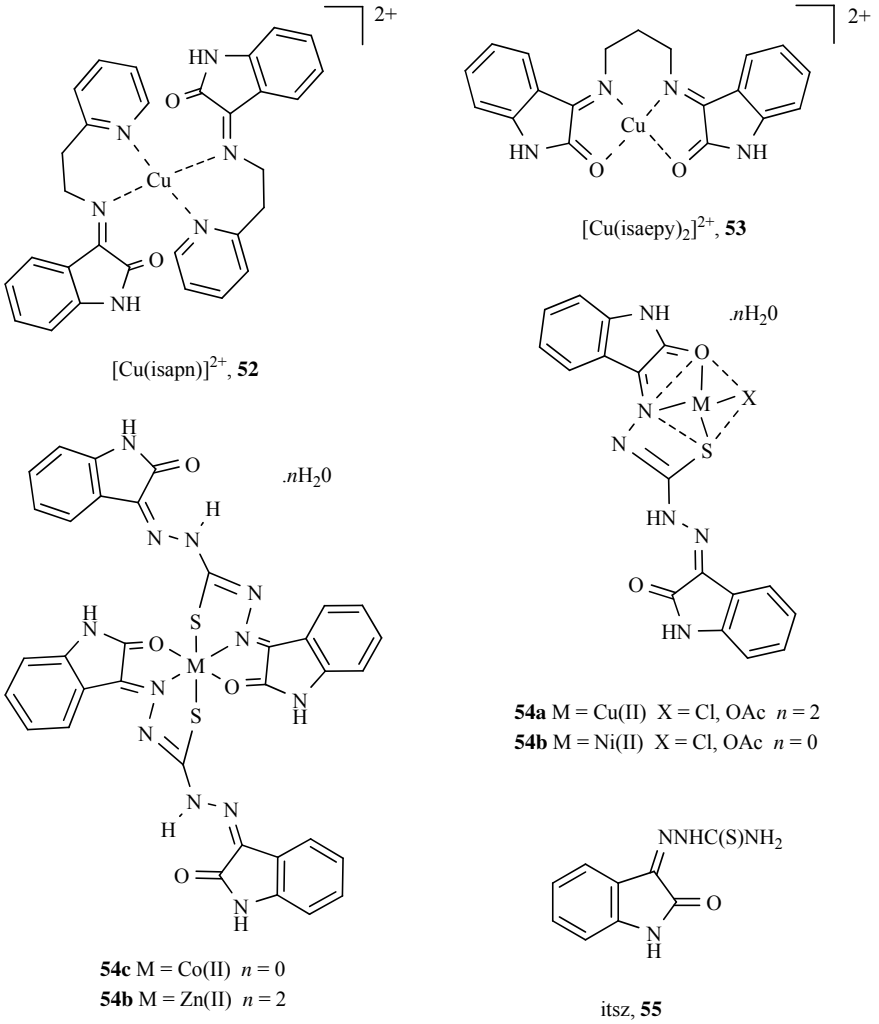


Fig. (18). The structures of some Isatin diimine complexes (**52, 53**), bis-isatin thiocarbohydrazone metal complexes (**54a-d**) and an isatin thiosemicarbazone ligand (**55**).

through the mitochondrial pathway and/or copper-dependent oxidative stress [136]. Apoptosis of these copper complexes was also caspase-dependent, which appears to be the principal mechanism of cell death.

Bis-isatin thiocarbohydrazones have been coordinated to Cu(II), Ni(II), Co(II) and Zn(II) ions (**54a-d**, Fig. (**18**)) [137]. All of the compounds displayed cytotoxic activity after 24 h in the brine shrimp assay and produced 50% cell death at or below a concentration of 19.1 μg/mL against Ehrlich ascitic carcinoma (EAC) cells. *In vivo* studies involving female Swiss Albino mice inoculated with EAC cells revealed that the compounds reduced weight gain, prolonged the mean survival times and reversed the tumour-induced increase in white blood cell counts compared to the control [137].

Karki *et al.* synthesised the isatin thiosemicarbazone ligand, itsz, (**55**, Fig. (**18**)) and subsequently chelated it to ruthenium-1,10-phenathroline and 2,2'-bipyridine derivatives [138]. The compounds were tested *in vitro* against leukemic human (MOLT 4/C8 and CEM) and murine (L1210) cell lines. The ruthenium complexes displayed greater activity than their ligands and the 1,10-phenathroline derivative was the more active of the two with an IC_{50} value of 3.9 μM against the CEM cell line. These compounds were once again evaluated *in vivo* against EAC bearing mice and found to increase life span and mean survival time compared to the control.

Isatins containing sulfonamides through an imine or hydrazone linkage have been complexed with Co(II), Ni(II), Zn(II) and Cu(II) ions (**56a-d**, Fig. (**19**)) [139]. Evaluation of the complexes in the brine shrimp bioassay revealed one Ni(II) and two Cu(II) complexes displayed potent cytotoxicity. The maximum activity observed was a LD_{50} value of 156 nM. The authors extended this work to include derivatised compounds of type **57** [140]. The four heterocyclic ligands were attached to each of the

four metal complexes. Only the copper complexes (**57d**) displayed potent cytotoxicity against *Artemis salina* ($LD_{50} = 4.5 \times 10^{-4} – 6.4 \times 10^{-4}$ µg/mL); the other metal complexes were deemed to be inactive.

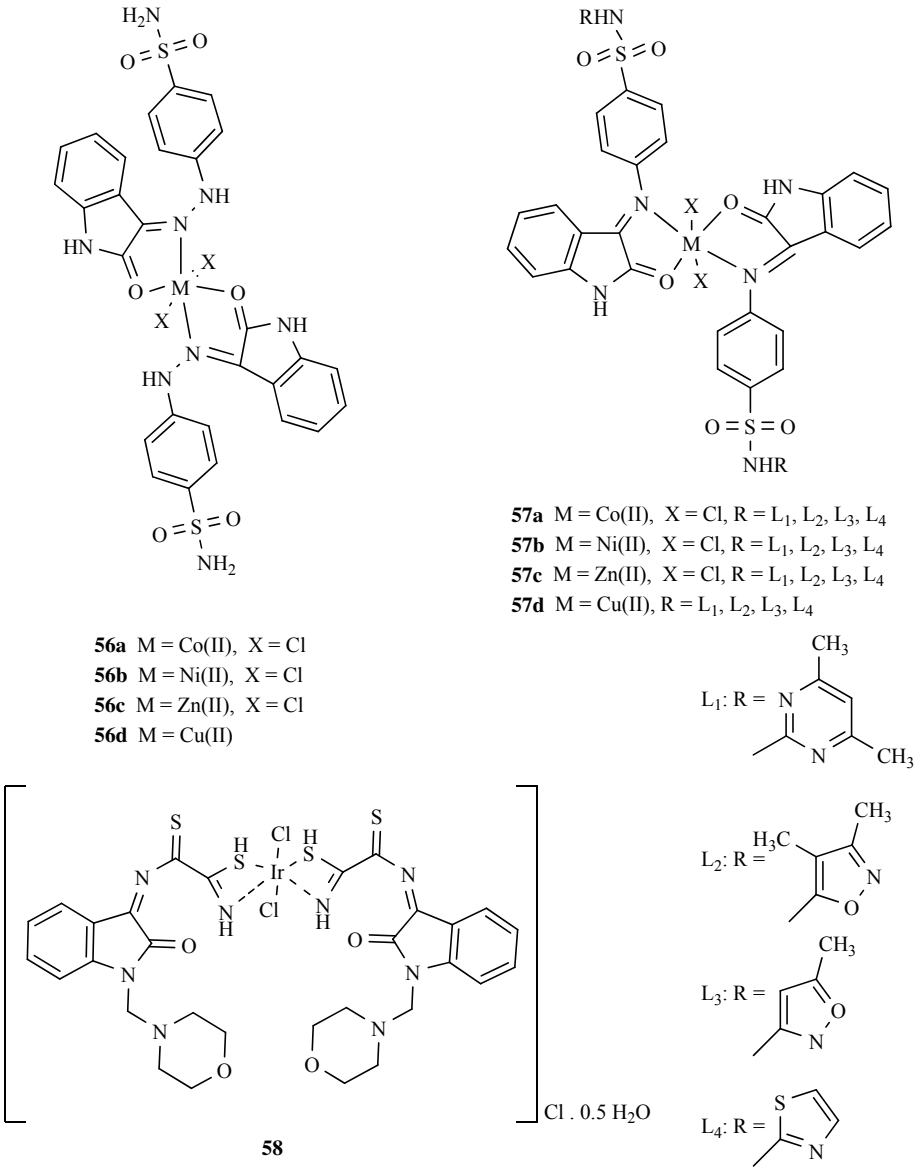


Fig. (19). Examples of isatin sulfonamide (**56** and **57**) metal complexes and an isatin-metal complex containing a Schiff base (**58**).

Other new isatin-metal complexes include Schiff and Mannich base ligands coordinated to metal salts such as Co(II), Ni(II), Mn(II) and Ir(III) [141]. The complexes were evaluated against the Hep-2 larynx carcinoma cell line and the Ir(III) complex **58** (Fig. (**19**)) was found to be the most active complex, with cytotoxic activity increasing in a dose-dependent manner.

2.4.4 Isoindigo and derivatives

Isoindigo (**59**, Fig. (**20**)) is the isomer of indirubin (**13**) containing two indolinone moieties. Glycosyl-isoindigo derivatives (**60**) have been prepared bearing benzyl or acetyl protecting groups on the sugar residue [142]. Protected analogues are speculated to enhance cellular penetration and therefore, display superior biological activity compared to the corresponding non-protected compounds. Acetylated glycosyl-isoindigo derivatives prepared by Sassatelli *et al.* displayed greater cytotoxicity against a panel of human solid tumour cell lines than the analogous benzylated compounds [142].

N-Methylisoindigo, more commonly known as meisoindigo (**61**), is approved for the treatment of CML [143] and induces apoptosis in the transformed human vein endothelial cell line, ECV304 [144]. Lee *et al.* have also investigated the potential of meisoindigo (**61**) against acute myeloid leukemia (AML) [145]. The compound is capable of inducing apoptosis in HL60 and other leukemic cells in a dose- and time-dependent manner. Further studies revealed **61** was also active in primary cells derived from eight AML patients and appears to exhibit moderate anti-AML activity in NOD/SCID mice.

Structural modifications to meisoindigo (**61**) have been prepared in an attempt to increase cytotoxicity. The *N*-substituent in **61** was replaced by arylalkyl substituents of varying chain lengths

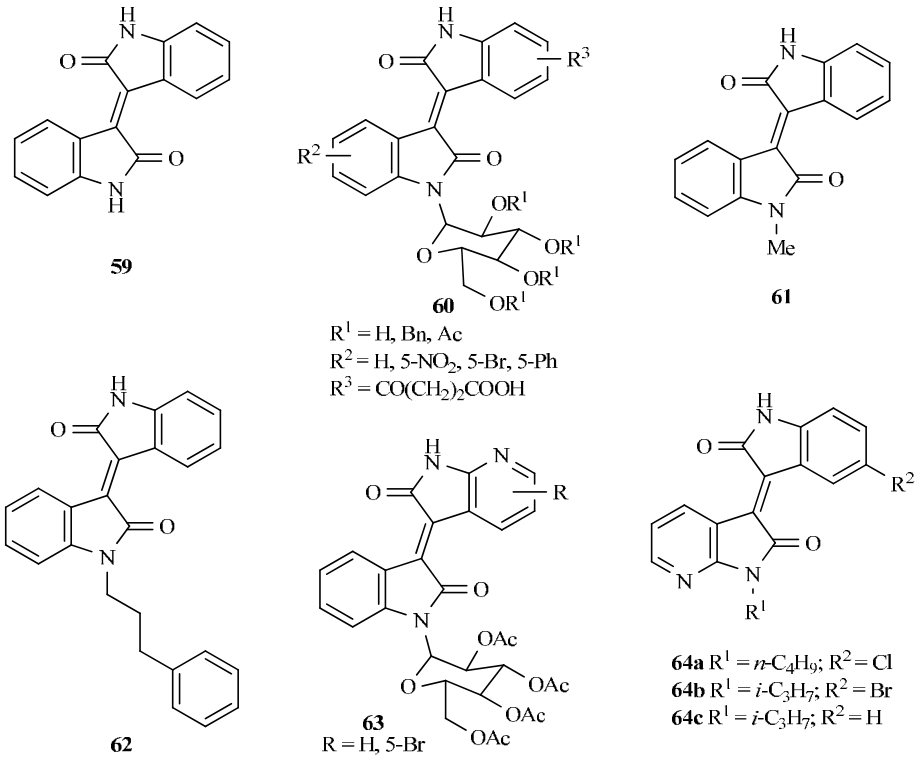


Fig. (20). Cytotoxic isoindigo and 7'-azaisoindigo derivatives.

and alternative substituents being introduced to the phenyl ring of the phenethyl side chain [146]. *In vitro* results revealed the test compounds exhibited specificity for leukemic cells (HL60 and K562) rather than HCT-116 colon or HuH7 hepatoma cells. Additionally, the analogues displayed selectivity towards one leukemic cell line than the other after a 72 h exposure (K562: IC_{50} 1.4-8.5 μM; HL60: 3.8-16.6 μM). The introduction of a second *N*-methyl group to meisoindigo (**61**) significantly decreased cytotoxicity amongst all cell lines tested. It appears the presence of one lactam NH is imperative for activity as is an intact exocyclic double bond on the isoindigo core [146]. *N*-(Phenpropyl)-isoindigo (**62**) was determined to be the most cytotoxic compound of the series, with consistently greater potency than meisoindigo (**61**) amongst the four tumour cell lines.

Compounds integrating a 7'-azaisoindigo scaffold (**63**, Fig. (**20**)) have been recently synthesised [147-148]. These compounds were discovered to exhibit micromolar IC_{50} values against the human buccal carcinoma cell line KB, and K562 and HL60 leukemic cell lines after 72 h, suggesting that the presence of an additional nitrogen atom to the isoindigo nucleus improves cytotoxicity. *N*-Alkylated analogues of 7'-azaisoindigo also display cytotoxic properties. Wang *et al*. reported that compounds possessing an isopropyl or butyl substituent at *N*1' (**64a-c**) were twice as cytotoxic (i.e. 8.3-9.1 μM) as meisoindigo (**61**) against DU-145 hormone-independent prostate carcinoma cells after a 24 h exposure [149]. The non-chlorinated analogue of **64a** has also exhibited micromolar activity against HeLa, A549, ECA-109 and HepG2 cell lines after a 48 h exposure [150].

2.4.5 Miscellaneous

Compounds incorporating a spirocycle at C3 on the isatin nucleus (also known as spiro-oxindoles) possess biological activities and the scaffold is common within the *Gelsemium* alkaloids such as 14-acetoxygelsenicine (**65**, Fig. (**21**)), which has an EC_{50} value of 0.25 μM against the A431 human epidermoid carcinoma after a 48 h exposure [151]. Spiro-oxindoles integrating silyl functionalities have been synthesised recently [152]. Two of these compounds, one including a tricyclic isatin core (**66**) and the other a *N*-benzylisatin analogue (**67**), were evaluated against A549 and HepG2 cell lines. Both compounds displayed comparable cytotoxicity towards the A549 cells after 24 h (EC_{50} = 42.8 μM and 42.6 μM respectively). However, when assessed against the HepG2 cell line, **67** exhibited twice the activity of the compound **66** (EC_{50} = 16.8 μM and 32.5 μM, respectively).

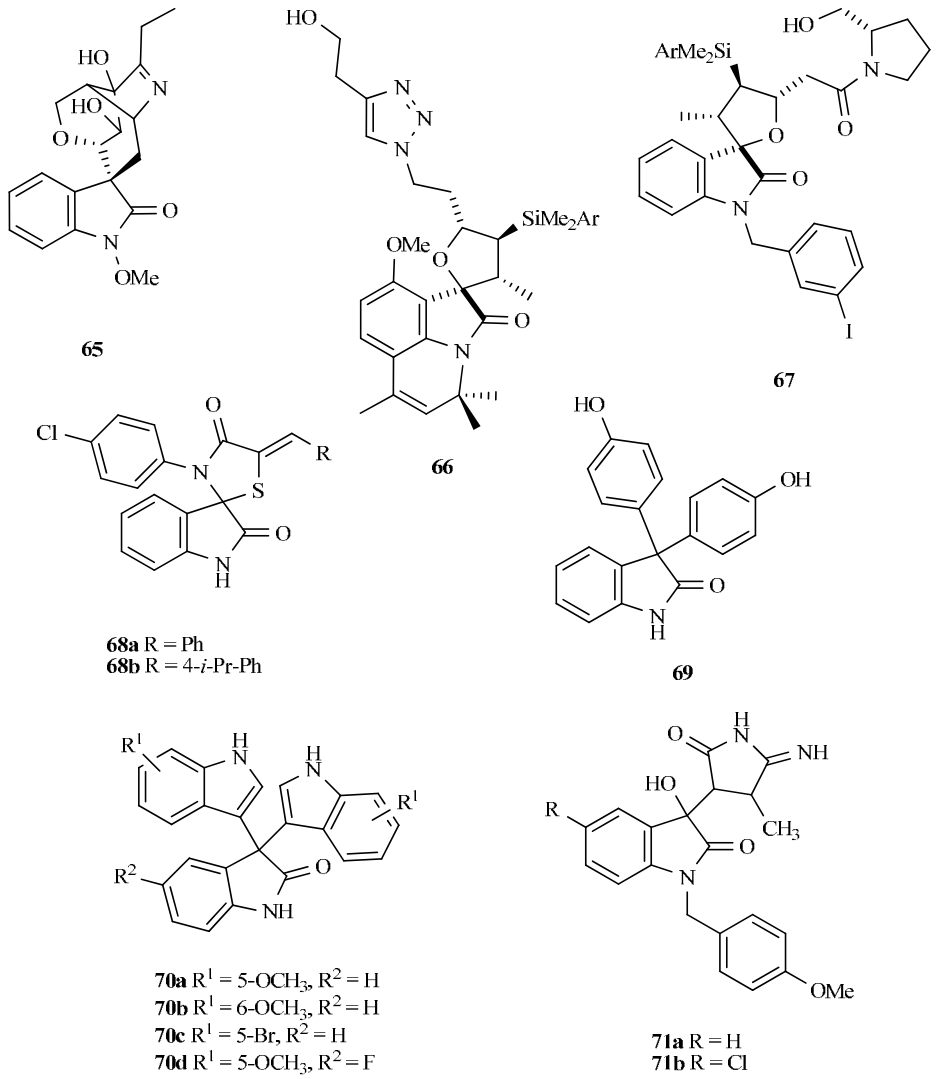


Fig. (21). Examples of biologically active spiro-oxindoles and 3,3-disubstituted oxindoles.

Kaminskyy *et al.* have screened a variety of oxindoles incorporating a spiro-thiazolidinone moiety against 60 cancer cell lines from the NCI [153]. Derivatives **68a** and **68b** were identified as the most potent and displayed LC_{50} values < 4 μM against a range of cancer cell types in the panel.

Another class of oxindoles which have been evaluated for their anti-proliferative activity are the 3,3-diaryloxindoles which include oxyphenisatin (**69**, Fig. (**21**)). Uddin *et al.* demonstrated that the compound and its derivatives were potent antiproliferatives, particularly against the MDA-MB-468 cell line [154] (IC_{50} value of oxyphenisatin (**69**) is 112 nM). The two hydroxyl groups on **69** are essential for activity and cytotoxicity can be increased (to less than 10 nM) by replacing small and lipophilic substituents in the 6- and/or 7-position of the isatin moiety. The lead analogue was the 6,7-difluorooxyphenisatin which displayed an IC_{50} of 3 nM against MDA-MB-468 cells [154]**.**

An extension of the 3,3-diaryloxindoles are the 3,3-diindolyloxindoles. This class of compounds (e.g. **70a-d**, Fig. (**21**)) were examined for cytotoxicity against five human cancer cell lines by Kamal *et al*. [155]. The 3,3-diindolyloxindoles were most susceptible towards DU-145 prostate cancer cells, with **70a-d** exhibiting IC_{50} values of < 5 μM after a 48 h exposure. Generally, introduction of methoxy groups onto the indole ring systems led to increased potency towards all cell lines, while a fluoro substituent at the 5-position on the oxindole core resulted in decreased potency, particularly against HepG2 liver cancer cells. Analogues containing nitro substituents on the indole ring systems displayed IC_{50} values of ~ 8 μM against DU-145 cells but were inactive against MCF-7 breast cancer cells. More recently, Subba Reddy *et al*. condensed isatin and 5-chloroisatin with 2- and/or 5-substituted indoles to prepare a new series of 3,3-diindolyloxindoles which exhibited sub-micromolar cytotoxicity against A549 lung cancer and SK-N-SH neuroblastoma cells, but were completely inactive against the MRC5 normal human lung cell line [156].

A series of *N*-alkylisatins containing a hydroxy substituent and a functionalised imidazole at the C3 position on isatin were evaluated for their cytotoxicity against 57 NCI cell lines [157]. Compounds **71a** and **71b** (Fig. (**21**)) exhibited growth inhibitory properties against 89% and 93% of all cancer cell lines

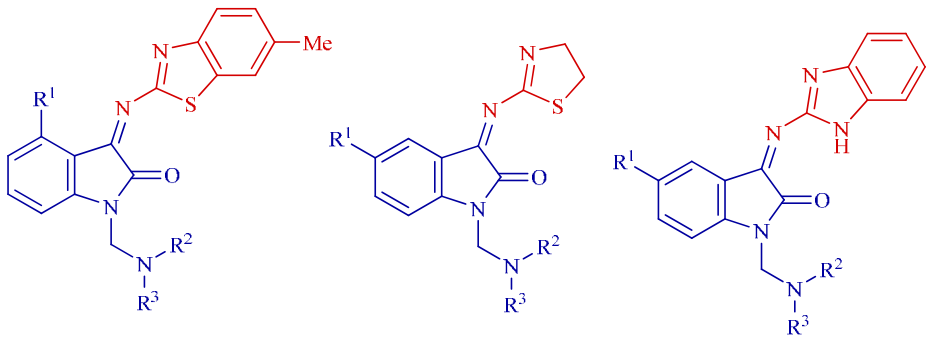
in the panel respectively, with GI_{50} values in the range of 2-60 μM for **71a** and 0.19-30 μM for **71b**. Both compounds were capable of inhibiting the growth of the five leukemic cell lines in the panel with GI_{50} values in the range of 2-5 μM.

2.5 Isatin-based conjugates

The synthetic versatility of isatin has lead to its further use in drug conjugate strategies whereby isatin or its analogues are chemically coupled to other bioactive and/or target specific molecules to improve drug efficacy and versatility of action. Two key approaches have been taken and are discussed further in sections 2.5.1 and 2.5.2.

2.5.1 Dual action/ hybrid agents

The hybridisation of two or more bioactive drug fragments with complementary functions or different mechanisms of action into a single molecule is a novel approach that often results in synergistic activity and enhanced drug efficacy. Solomon *et al*. was the first to report this dual action/hybrid pharmacophore strategy using isatin (**1**) by linking benzothiazole to isatin through its C3 carbonyl by Schiff formation [158] to yield compounds of type **72** (Fig. (**22**)). It was postulated that the combined anticancer activities of the isatin Mannich base derivatives and benzothiazole would yield highly potent targeted dual drugs for the treatment of breast cancer. Among the 30 isatin-benzothiazole derivatives screened, 4-chloro-1-dimethylaminomethyl-3-(6-methyl-benzothiazol-2-ylimino)-1,3-dihydroindol-2-one emerged as the most active against a panel of human breast cancer cell lines, with GI_{50} values in the range of 10.92 - 28.09 μM. Addition of the benzothiazole moiety increased activity by up to a factor of 12 over that of the unsubstituted isatin Mannich base.



72
R^1 = H, Cl, Br
R^2R^3 = methyl, ethyl, phenyl, piperidinyl, morpholinyl

73
R^1 = H, Br
R^2R^3 = phenyl, piperazinyl, piperidinyl, morpholinyl

74
R^1 = H, Br
R^2R^3 = phenyl, piperazinyl, piperidinyl, morpholinyl

Fig. (22). Examples of isatin-benzothiazole (**72**), isatin-thiazoline (**73**) and isatin-benzimidazole (**74**) hybridised dual action derivatives.

Taher *at al*. further contributed to this work in the search for new anti-breast cancer agents through the synthesis of novel isatin-thiazoline and isatin-benzimidazole derivatives (compounds of type **73** and **74**, Fig. (**22**)) via condensation of isatin Mannich bases with either 2-aminothiazoline or 2-aminobenzimidazole, respectively [159]. Benzimidazole-based compounds have been reported to demonstrate cytotoxic activity against hormone-dependant breast cancer cells such as MCF-7 [160], while thiazoline derivatives possess both cytotoxic and cytostatic activity against a broad spectrum of cancer cell lines [161]. Of the 16 hybrid compounds prepared, 11 were screened for cytotoxic activity against a human breast adenocarcinoma cell line and all were found to be active in the nanomolar range. The most lethal compound, 3-(1*H*-benzimidazol-2-ylimino)-1-{[*N*,*N*-diphenyl-amino-1-yl]-methyl}indol-2(3*H*)-one, derived from the benzimidazole series **74**, showed potent activity with an IC_{50} of 22.59 nM against MCF-7 breast cancer cells [159].

Diazoles, such as pyrazoles and pyrazolines are small molecules that have also been found to exhibit remarkable anticancer activities and have been identified as inhibitors of CDKs [162], VEGF [163] and P-gp [164]. These observations led Lesyk and colleagues to synthesise new isatin-pyrazoline hybrids (**75a-d**, Fig. (**23**)) with the aim of discovering new active and selective compounds that elicit synergistic anticancer activity [165]. The further conjugation of isatin-pyrazoline hybrids with 4-thiazolidinones to from isatin-based hybrids with three distinct bioactive drug components (**76a-d**) builds on the work of Taher *et al*., above. The synthesised hybrids were tested for anticancer activity in the NCI's 60 cell line screen and found the isatin-pyrazoline derivative 5-bromo-1-{2-[5-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydropyrazol-1-yl]-2-oxothyl}-1*H*-indole-2,3-dione (**75d**) to demonstrate the highest activity, particularly towards a leukemia subpanel with GI_{50} values in the range of 0.69 - 3.35 μM. SAR studies revealed that hybridisation of isatin with the pyrazoline ring system (**75a-d**) leads to enhanced anticancer activity and introduction of a bromine atom at the 5-position of the isatin fragment further enhances potency. However, condensation of the active isatin-pyrazoline hybrids with 4-thiazolidinones (**76a-d**) results in complete loss of activity [165].

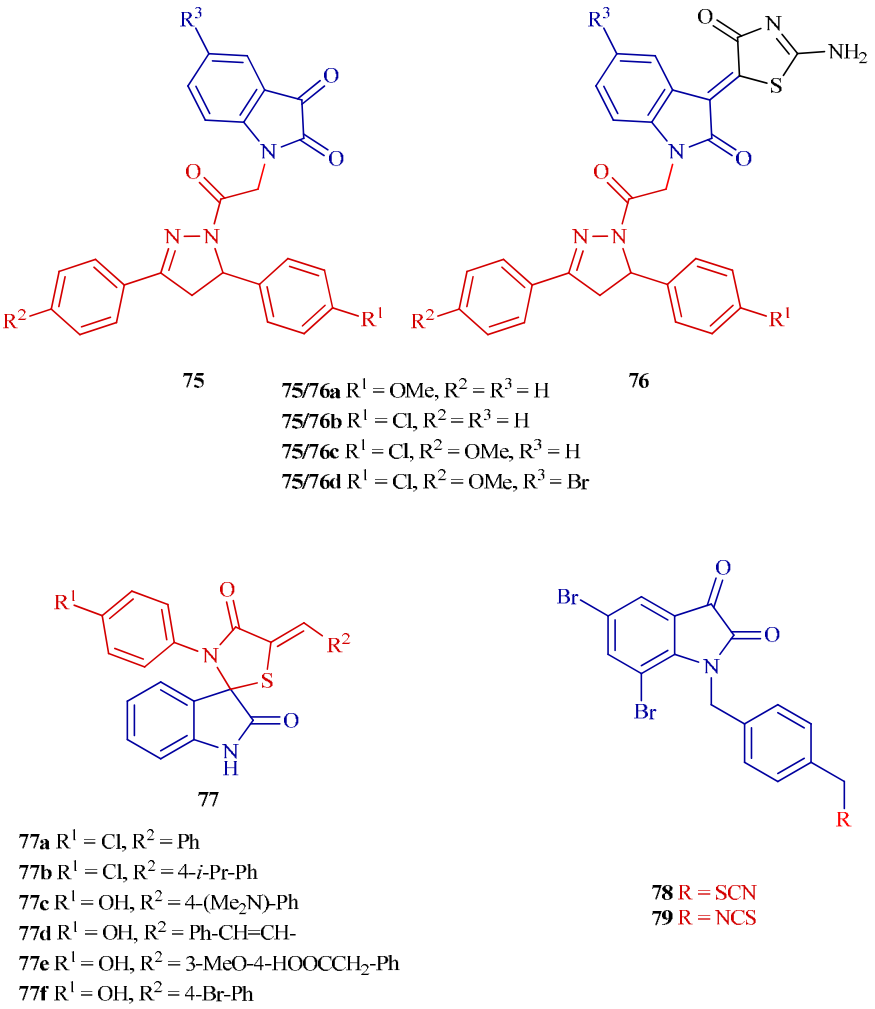


Fig. (23). Examples of pyrazoline-isatin hybridised dual action derivatives (**75** and **76**), spiro-thiazolidinone-isatin hybridised derivatives (**77**) and cytotoxic 5,7-dibromo-*N*-alkylisatins containing cyano-based substituents (**78** and **79**).

Spiro thiazolidinone-isatin derivatives (**77a-f**, Fig.(**23**)) have also been explored and were found to exhibit modest activity *in vitro* (GI_{50} values in the range of 4.13 - 6.55 μM) against the NCI's full panel of 60 human cancer cell lines [166]. Despite the rationale behind this hybrid pharmacophore approach, all of the compounds described above were found to display novel modes of action and did not retain

the mechanism of the individual fragments alone, as determined by the NCI's COMPARE Analysis Program [167-168].

Conversely, hybridisation of thiocyanate, isothiocyanate or selenocyanate moieties (potent inhibitors of the PI3K/Akt pathway) to 5,7-dibromoisatin (**3h**) afforded 12 novel 5,7-dibromo-*N*-alkylisatin derivatives that concomitantly inhibit tubulin polymerisation and phosphorylation of Akt [169]. *In vitro* biological evaluation and SAR studies against a panel of human cancer cell lines found three of the isatin-selenocyanate analogues (containing a selenocyanate group in the alkyl chain) to be most active against the MCF-7 breast cancer cell line (IC_{50} 1.45 – 1.65 μM), while the isatin-thiocyanate and isatin-isothiocyanate derivatives (containing a thiocyanate or isothiocyanate group in the alkyl chain) were most active against the HT-29 colon (IC_{50} 1.09 – 3.24 μM), A549 lung (IC_{50} 2.13 – 5.53 μM) and UACC903 melanoma (IC_{50} 2.06 – 4.37 μM) cell lines. The two most active compounds, 5,7-dibromo-*N*-(*p*-thiocyanomethylbenzyl)isatin (**78**) and 5,7-dibromo-*N*-(*p*-isothiocyanatomethylbenzyl)isatin (**79**, Fig. (**23**)) emerged as promising dual inhibitors of microtubule assembly and Akt and are being further evaluated *in vivo* as antitumour agents for the treatment of colon cancer.

2.5.2 Selectively deliverable conjugates

Chemical conjugation of a potent cytotoxin to a tumour targeting moiety such as a protein, peptide or aptamer to increase the therapeutic index of the drug is another relatively new strategy used to confer high tumour selectivity and reduced toxicity to cytotoxic drugs. Vine *et al*. was the first to publish this approach using isatin whereby two potent *N*-alkylisatin-based microtubule destabilisers

were coupled to the delivery agents, plasminogen activator inhibitor type-2 (PAI-2) or transferrin (Tf) [170-171], to achieve site-specific drug delivery. Tf is an iron ferrying glycoprotein that has commonly been exploited to deliver potent chemotherapeutics to the intracellular space of cancer cells via the Tf receptor (TfR/CD71) [172], while PAI-2 is an irreversible, specific inhibitor of the proven metastatic marker urokinase plasminogen activator (uPA) [173]. Both ligands, upon interaction with appropriate cell surface receptors, are specifically endocytosed by the low density lipoprotein receptor family [172, 174] and can thus selectively deliver attached toxins to the intracellular space of targeted cells [175]. Exposure of the conjugate to high concentrations of lytic enzymes or the acidic conditions of the endosome or lysosome results in the selective release the cytotoxin and localised cell death (Fig. (**24**)).

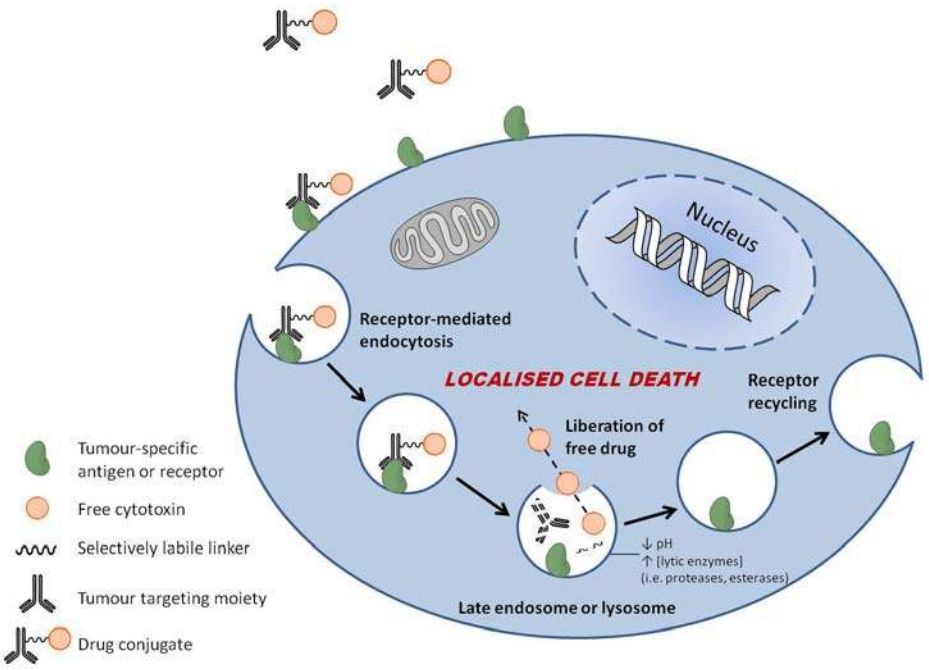


Fig. (24). Schematic representation of selectively deliverable drug conjugates. The drug conjugate is internalised by receptor-mediated endocytosis and then cleaved in the acidic environments of the late endosomes or lysosomes to release the free cytotoxin into the cytosol and cause cell death.

In one report, 5,7-dibromo-*N*-(*p*-hydroxymethylbenzyl)isatin (**80**) was functionalised for attachment to Tf and PAI-2 via an esterase-labile succinate linker to form Tf- and PAI-2-*N*-alkylisatin conjugates (Tf-*N*-AIE and PAI-2-*N*-AIE, respectively) (**83**, Fig. (**25**)) [171]. Conjugation of the *N*-hydroxysuccinimide-derived active ester (**82**) to the targeting ligands Tf and PAI-2 was achieved by forming stable amide bonds between the ε-amino group of available surface lysine residues on the protein and the carboxylic acid group of the succinate linker on the functionalised *N*-alkylisatin (**81**). Tf-*N*-AIE was up to 24 times more active than the free drug (**80**) (IC_{50} 0.38 μM *vs.* IC_{50} 9.01 μM) against the high TfR expressing MCF-7 breast cancer cell line and showed clear cytotoxicity patterns based on TfR levels. On the other hand, PAI-2-*N*-AIE showed equivalent activity compared to the

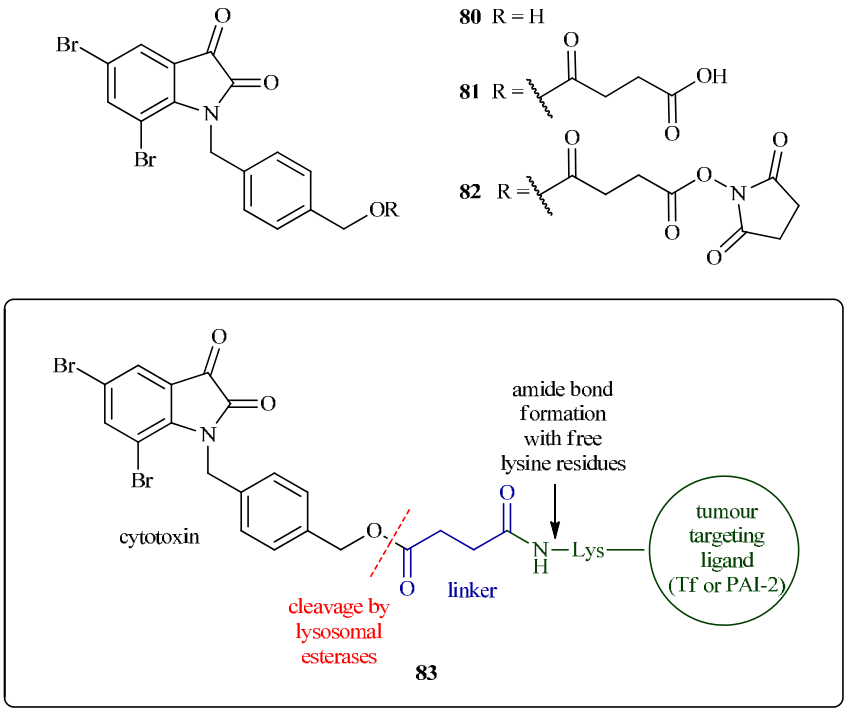


Fig. (25). The structures of Tf- and PAI-2-*N*-alkylisatin conjugates (**83**) and their precursor molecules (**80-82**).

parent drug (IC_{50} 1.02 μM *vs.* IC_{50} 1.38 μM) against the high uPA/uPAR expressing MDA-MB-231 breast cancer cell line and strong selectivity patterns for uPA levels. Both Tf- and PAI-2-*N*-AIE conjugates exhibited impressive tumour growth delay in a metastatic, orthotopic human breast tumour xenograft mouse model and were efficacious at 1/20th and 1/10th of the dose of the free drug respectively, with no observable signs of toxicity [171].

In another report, 5,7-dibromo-*N*-(*p*-methoxybenzyl)isatin (**7k**) was conjugated to Tf via an acid-labile imine-functionalised (*para*-phenylpropionic acid) linker to form a novel *N*-alkylisatin–imine–Tf (NAI-imine-Tf) conjugate (**84**, Fig. (**26**)) [170]. The bifunctional linker was selected on the basis of hydrolytic studies led by Matesic *et al*. on a series of isatin-based imino acid derivatives [176]. The derivatives were functionalised at the C3 carbonyl group of 5,7-dibromo-*N*-(*p*-methoxybenzyl)isatin (**7f**) and were stable at physiological pH but readily cleaved at pH 4.5. Observed rates of hydrolysis for the embedded imine-acid moiety were in the order *para*-phenylpropionic acid > phenylacetic acid (*para* > *meta*) > benzoic acid (*meta* > *para*). The half-life of the *para*-phenylpropionic acid derivative under acidic conditions was 17 min. The NAI-imine-Tf conjugate (**84**) was equipotent to the free drug (**7k**) against MCF-7 breast cancer cells (IC_{50} 2.00 μM *vs.* IC_{50} 1.67 μM) and demonstrated clear receptor-dependent cytotoxicity [170].

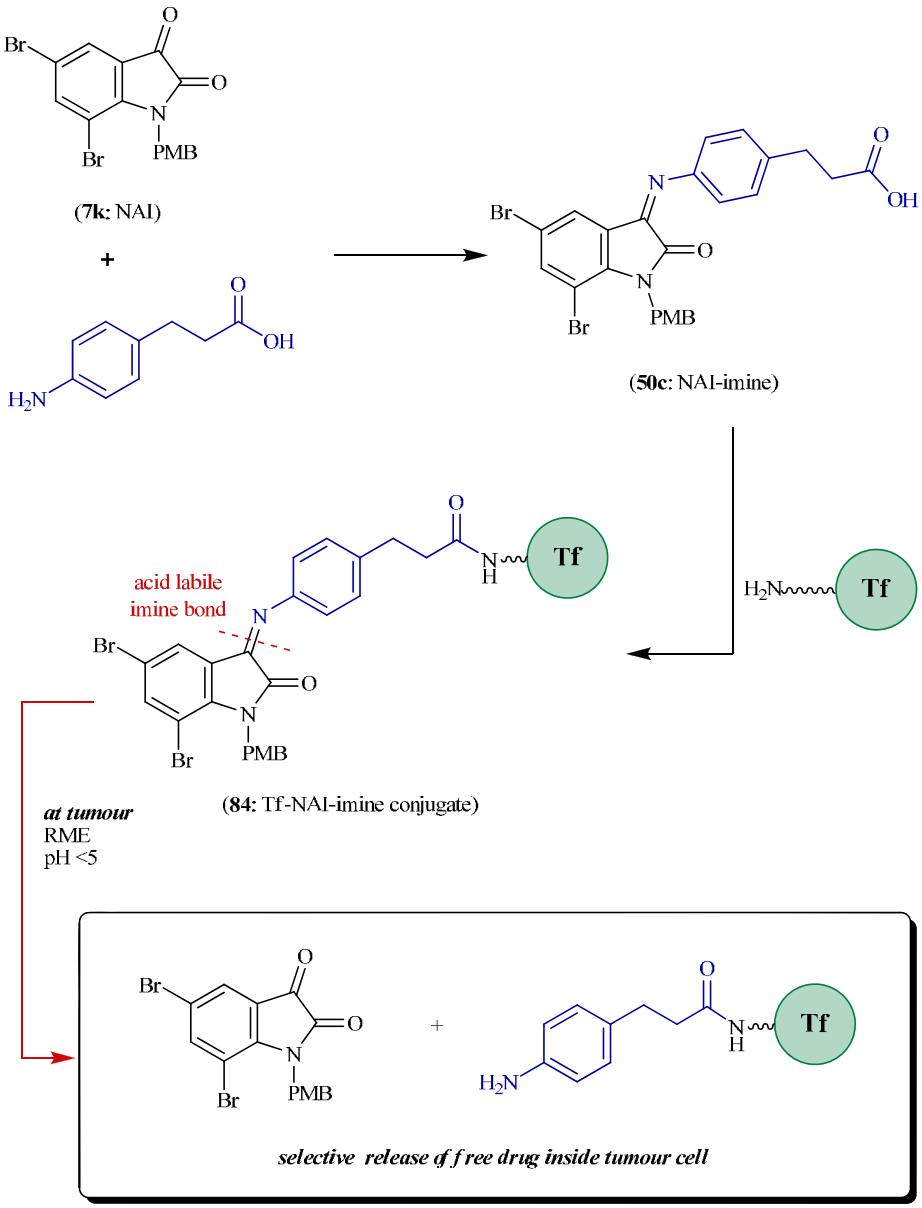


Fig. (26). *N*-Alkylisatin **7k** bound to the lysine residues of transferrin (Tf) via an acid-labile, imine linker (**84**). Upon receptor mediated endocytosis of the conjugate (**84**) at the target tumour, the free cytotoxin (**7k**) is selectively released inside the tumour cell along with modified protein.

2.6 Isatins in combination therapy

Combination chemotherapy is now the standard of care for the adjuvant (post-surgery) treatment of most solid and haematological cancers. The rationale behind this approach is two-fold: i) to minimise the dosage of each individual drug and therefore reduce non-specific toxicity and ii) through limited drug exposure, reduce the emergence of multi-drug resistance (MDR). Recently, the effect of combined administration of isatin (**1**) and the omega-3 fatty acid eicosapentaenoic acid (EPA), on a human breast cancer cell line was described [177]. In this study, EPA was found to significantly increase the cytotoxicity of isatin towards MDA-MB-231 cells *in vitro* via modulation of lipid peroxidation. Omega-3 fatty acids have previously been shown to increase the efficacy of various cancer chemotherapy drugs such as doxorubicin [178-179], tamoxifen [180], and mitomycin C [181], as well as radiation therapy [182].

More recently, Vine *et al.* has demonstrated additivity and synergy with combinations of *N*-alkylisatin-based microtubule destabilisers and the commercial anticancer agents vinblastine, paclitaxel, 5-FU and colchicine. When combined at a molar ratio of 1:1 and concomitantly administered to either a MDR human uterine sarcoma cell line (MES-SA/Dx5) or a human histiocytic lymphoma cell line (U937), combinations were found to exhibit a significant increase in cytotoxicity over and above that of the individual drugs alone (Table **1**).

Table 1. Cytotoxicity (IC_{50}) of single-agent *vs.* combination treatments in the human U937 and MES-SA/Dx5 cancer cell lines.

	IC*$_{50}$ *(μM)*	
Individual Drugs and Drug Combinations	**MES-SA/Dx5[†]**	**U937[§]**
5,7-dibromo-*N*-(*p*-methoxybenzyl)isatin (**7k**)	4.8	-
5,7-dibromo-*N*-(*p*-trifluoromethylbenzyl)isatin (**7e**)	-	1.24
Vinblastine	5.5	5
Colchicine	>10	-
Paclitaxel	-	>5
5-fluorouracil (5-FU)	-	>5
(**7k**) + Vinblastine	1.9	-
(**7k**) + Colchicine	2.9	-
(**7e**) + Vinblastine	-	0.42
(**7e**) + Paclitaxel	-	1.02
(**7e**) + 5-FU	-	0.98

*IC_{50} values were calculated from sigmoidal dose response curves (variable slope), generated using GraphPad Prism V.5 software. Briefly, cells were incubated for 24 h in the presence of increasing concentrations of single agent or combinations (1:1) of drug and cell viability determined using the CellTiter 96 Aqueous One Cell Proliferation (MTS) Assay. Values are the means of triplicate determinations.
[†] MES-SA/Dx5 = MDR human uterine sarcoma
[§] U937 = human monocyte-like histiocytic lymphoma
(-) = Not tested.

3. CONCLUSIONS

Isatin is the core nucleus of an array of cytotoxic and antineoplastic compounds. A SAR summary for the isatin derivatives discussed in this review is shown in Fig. (**27**). Derivatisation at X to form the mono- di- and tri-aryl ring substituted isatin series (see section 2.1) are generally found to induce cancer cell death via apoptosis in the mid-low micromolar range and necrosis in the high micromolar range. This is proposed to be linked to a reduction in ERK activity. Small electron-withdrawing groups at positions 5, 6 and/or 7 enhance, but are not essential for anti-tubulin or anti-kinase activity when found as part of a larger substituted compound (i.e. substitution at X with concomitant substitution at W, Y and Z). This is most likely due to increased cell permeability and hydrophobicity.

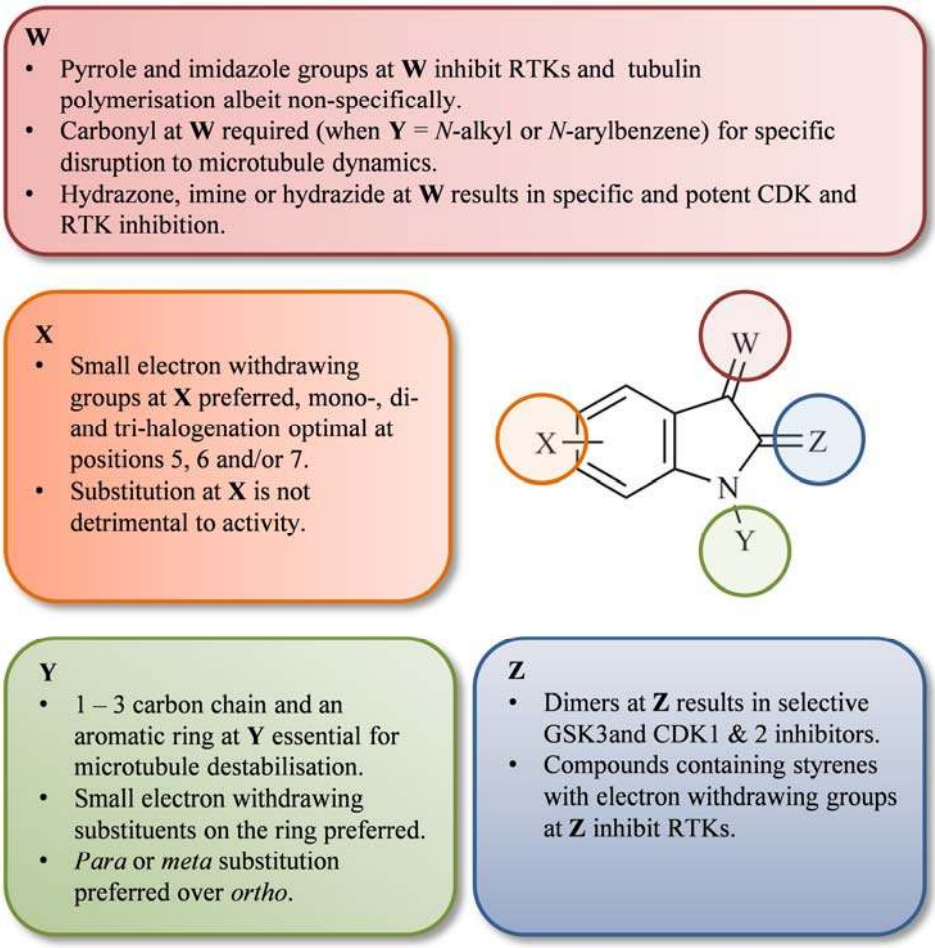


Fig. (27). A summary of the cytotoxic structure-activity relationship of isatin derivatives.

N-Alkylation at Y however, with no further substitution at W or Z, often results in cytotoxic compounds with sub-micromolar activity; inducing morphological change, G2/M cell cycle arrest and ultimately cell death via apoptosis (see section 2.2). The compounds discussed in this review appear to be tubulin specific and do not inhibit serine/threonine or tyrosine kinases [50]. A 1-3 carbon chain linker extending to an aromatic ring is optimal for potent microtubule disruption and small electron-withdrawing substituents on the ring in the *para* or *meta* position are favoured over the *ortho* position.

Most importantly, and together with its specificity, this series of novel molecules are >10 times more active than the conventional cancer chemotherapeutics; 5-FU, vinblastine and paclitaxel on human monocytic lymphoma cells. In addition to the possibility that these compounds may bind to a novel site on tubulin, it suggests that the development of anticancer agents based on the *N*-alkylisatin scaffold may be beneficial in combination with clinically used anticancer agents for the treatment of MDR tumours in the future. Further development and preclinical assessment of this particular series of isatins is therefore warranted.

Substitution at W has by far resulted in the generation of the most structurally and biochemically diverse isatin-based compounds to date (see section 2.4). Yet despite over 1500 cytotoxic C3 substituted compounds reported in the literature, only one has made it to market as an anticancer drug. This is primarily due to their non-specific toxicity, as these molecules often target the ATP binding site of multiple kinases, therefore affecting a magnitude of cellular targets. For example, substitution at W on the 3'- and 4'-positions of the pyrrole ring of some arylidene derivatives, are regarded as favourable for CDK2 inhibition, while substituents in the 5'-position are beneficial for VEGFR-2 and PDGFR-β inhibition. 3-Substituted indolinones possessing the *Z* configuration are potent FGFR inhibitors, while compounds adopting the *E* configuration inhibit EGFR.

Finally, dimerisation at Z often results in ATP-competitive inhibition of CDK1, CDK2 and GSK3, as well as reduction in the activation and expression of, c-Src kinase and NF-κB (the most renowned modulators being the indirubins, see section 2.3). Although the early indirubin derivatives were plagued by poor water solubility, the addition of basic diamine side chains and the incorporation of a quaternary centre into the 3'-position markedly improved their water solubility, while retaining potency towards CDK2. Such small modifications have led to large advances in the development of

this class of molecules as potential new anticancer agents. In general, the C2-substituted class of isatins is active in the micro to nanomolar range and extend from kinase inhibitors to potent anticancer antibiotics.

In summary, isatin has already proven to be an excellent scaffold for both the natural and synthetic construction of molecules with interesting biological activities. With the possibility of derivatising the N1, C2 and C3 positions, along with substitution on the aromatic ring, the synthetic permutations for isatin are almost endless. Despite the fact that isatins are well studied compounds, new derivatives are continually being discovered and known isatin-based compounds have been explored in combination therapy approaches, fused with other bioactive drug fragments and subsequently investigated as hybrid/dual action drugs and selectively targeted to cancer cells via conjugation to tumour targeting moieties. Isatin has proven to be a privileged scaffold in medicinal chemistry that warrants further exploration for the purpose of discovering new and exciting molecules with anticancer activity.

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5. ABBREVIATIONS[1]

A549 = human non-small cell lung adenocarcinoma

AML = acute mylogenous leukemia

CDK = cyclin dependent kinase

CML = chronic mylogenous leukemia

Colo-205 = human colon adenocarcinoma

DU-145 = hormone-independent prostate carcinoma

EAC = Ehrlich ascitic carcinoma

EC_{50} = half maximal effective concentration

ECA-109 = human oesophageal carcinoma

EGF/R = epidermal growth factor/receptor

EPA = eicosapentaenoic acid

ERK = extracellular signal-related protein kinase

GI_{50} = concentration required to inhibit the growth a cellular population by 50%

GSK3 = glycogen synthase kinase 3

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[1] Only abbreviations that appear more than twice in the text are listed here

HCT-116 = human colon carcinoma

HeLa = human cervical carcinoma

HepG2 = human liver hepatocellular carcinoma

HL60 = human promyleocytic leukemic cell line

HT-29 = human colon adenocarcinoma

IC_{50} = concentration required for 50% inhibition of a biological or biochemical process *in vitro*

K562 = human myelogenous leukemia

KB-3-1 = human cervical carcinoma

LD_{50} = median lethal dose

MCF-7 = human non-metastatic mammary gland adenocarcinoma

MDA-MB-231/468 = human metastatic/non-metastatic mammary gland adenocarcinoma

MDR = multidrug resistance

NCI = National Cancer Institute

PAI-2 = plasminogen activator inhibitor type-2

PC-3 = human prostate adenocarcinoma

PDGF/R = platelet-derived growth/receptor

P-gp = P-glycoprotein

RTK = receptor tyrosine kinase

SAR = structure activity relationship

SH-SY5Y = human neuroblastoma

Tf/R = transferrin/receptor

U937 = human monocyte-like histiocytic lymphoma

UCH-L1 = ubiquitin *C*-terminal hydrolase

uPA/R = urokinase plasminogen activator/receptor

VEGF/R = vascular endothelial growth factor/receptor

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