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Exploring the Anticancer Activity of Functionalized Isoindigos: Synthesis, Drug-like Potential, Mode of Action and Effect on Tumor-Induced Xenografts

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Meisoindigo has been used as an indirubin substitute for the treatment of chronic myeloid leukemia (CML) for several years. In view of its poor solubility and erratic absorption, several investigations have focused on developing analogues with more desirable physicochemical profiles. Here, we investigated the structure–activity relationship (SAR) of meisoindigo with respect to its antiproliferative activity on leukemic K562 cells and found that appending a phenalkyl side chain onto the lactam NH resulted in analogues that retained good activity. Furthermore, analogues in which the phenyl ring was substituted with a basic heterocycle were significantly more soluble than meisoindigo while retaining acceptable antiproliferative profiles. The most promising analogue (*E*)-1-(2-(4-methylpiperazin-1-yl)ethyl)-[3,3′-biindolinylidene]-2,2′-dione (**5-4**) is more potent than meisoindigo across a panel of malignant cells, with at least 40 times greater solubility than meisoindigo, little or no tendency to aggregate in solution and capable of significantly extending the lifespans of animals with K562 induced xenografts. Mechanistically, it induced apoptotic cell death and disrupted the progression of K562 cells from the G_1 to G_2 phase. Taken together, our findings highlighted the feasibility of addressing the physicochemical deficits of the isoindigo scaffold by systematic modifications which was achieved without overt loss of growth inhibitory activity.

Introduction

The isomeric bisindoles (indigo, indirubin and isoindigo), collectively known as indigoids, are associated with varied pharmaceutical and chemical applications (Figure 1). The indigos are valuable textile dyes that have been used since antiquity.[1] Functionalized indirubins are widely reported to be potent inhibitors of various kinases including CDK,[2–5] GSK3β[4,6] and aurora kinases,[7] and thus are potential therapeutic leads for cancer. In comparison, the isoindigo scaffold has received scant attention, although an isoindigo, meisoindigo (**1**) (Figure 1), has been used for the treatment of chronic myeloid leukemia (CML) in the People's Republic of China since 1992.[8–11] Reports on the mode of action of **1** suggests that it has several targets. It inhibits DNA and RNA biosynthesis in rat carcinosarcoma cells,[12] and in common with other DNA-targeting agents, induces differentiation of cancer cells by decreasing the expression of the oncogene *c-myb*.[13] On the cell cycle, **1** arrests the progression of leukemic cells through the G_0/G_1 phase and increases levels of the cell cycle inhibitor proteins p21 and p27.[14] There are conflicting views on whether **1** inhibits the phosphorylation mediated by cyclin-dependent kinases,[15–17] possibly due to different assay protocols used to obtain the results. Its effects on apoptosis are pronounced and involve the intrinsic mitochondrial pathway, with up-regulation of pro-apoptotic proteins and concurrent down-regulation of antiapoptotic proteins.[14] Inhibition of angiogenesis through diminished VEGF secretion has been observed.[18]

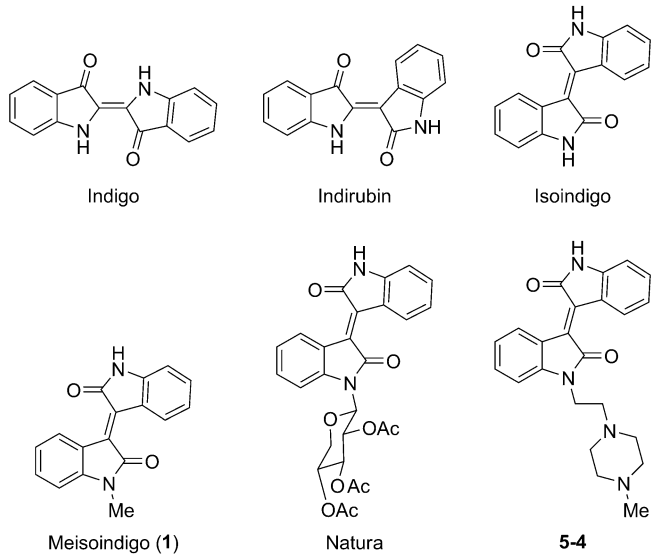

Figure 1. Structures of bisindoles, meisoindigo, natura and **5-4**.

A significant drawback of functionalized indigoids is their poor aqueous solubility, due in part to the "brick-dust" nature of the scaffold, which promotes tight crystal packing. Not unexpectedly, efforts in medicinal chemistry have thus far focused on modifications that would enhance solubility. One approach has been to attach sugar moieties to the bisindole,

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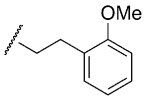
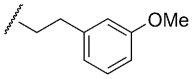
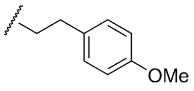
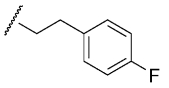
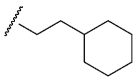
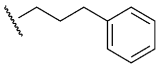
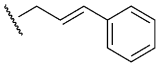
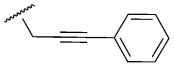
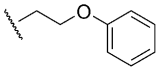
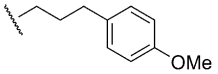
giving rise to *N*-glycosides, and in the case of indirubins, *O*-glycosides from glycosylation of the 3′-oxime derivatives.[16,19–22] Unfortunately, greater solubility is often achieved at the expense of biological activity. For example, the water-soluble *N*-xylosyl analogue of isoindigo has negligible antiproliferative activity which was restored when the hydroxy groups on the sugar moiety was acetylated.[16,19] The resulting compound (natura, Figure 1) was apparently designed to increase the bioavailability and bioactivity of **1**, although it has reportedly poor solubility in water.[16,19] A large number of *N*-glycosylisoindigos have been found to be kinase inhibitors but only when the hydroxy groups on the sugar moiety were O-benzylated or O-acetylated.[21] It is clear that designing biologically active bisindoles that combine acceptable solubility–permeability profiles with potent activity is a daunting task.

In an earlier study, we have evaluated several isoindigos, including **1**, for their antiproliferative activities on a panel of human cancer cells.[17] The structure–activity relationship (SAR) revealed key positions on the scaffold that were sensitive to modification, notably, the *N*-methyl of **1**, which could be replaced by *N*-phenpropyl or *N*-(*p*-methoxy)phenethyl leading to significant improvements in activity. In order to expand the nascent SAR derived from that investigation, we have undertaken a systematic exploration of the isoindigo scaffold with the aim of identifying analogues that reconcile potency (assessed in terms of antiproliferative activity on the CML cell line K562) with acceptable aqueous solubility. In this regard, we found that the isoindigo scaffold could be modified to accommodate these dual requirements. The most promising compound **5-4** is more potent than **1** on K562 cells, has improved solubility and shows no tendency to form aggregates in solution. **5-4** increased the survival of K562 xenograft-bearing nude mice on intratumoral administration. Mechanistically, it induces cell cycle arrest at G_1 and promotes apoptosis of K562 cells.

Table 1. Series 1: $IC_{50,\,K562}$ values, Clog *P* and estimated solubilities (pH 7.4).

	R^1	Clog *P*[a]	Solubility [μM][b]	IC_{50} [μM][c]
1	Me	2.04	673	7.75 ± 0.71
1-1		4.37	7.6	5.30 ± 0.97
1-2		4.73	7.6	4.55 ± 0.06[d]
1-3		4.73	7.6	6.09 ± 1.01
1-4		4.95	5.2	18.95 ± 3.29
1-5		5.89	11.8	3.79 ± 0.57[d]
1-6		5.19	5.3	1.70 ± 0.22[d]
1-7		5.08	2.6	2.34 ± 0.33[d]
1-8		4.43	< 1.0	9.31 ± 1.18
1-9		4.48	10.5	3.84 ± 0.53[d]
1-10		5.11	4.9	1.75 ± 0.14[d]

[a] Determined with ChemDraw 7.0. [b] Determined with ACD/Labs 12.0 Solubility DB. [c] Values represent the mean ± SD for $n \geq 3$ determinations. [d] Significantly lower than meisoindigo **1** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc).

Results

Design and synthesis of target compounds

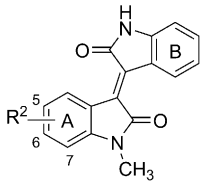
The target compounds were broadly divided into five groups (series 1–5) which are presented in Tables 1–5, respectively. Series 1, shown in Table 1, comprises compounds with varying phenalkyl side chains attached to the NH at position 1 (N^1) and is based on two hits that were identified earlier,[17] namely 1-(*p*-methoxyphenethyl) analogue **1-3** and 1-(3-phenylpropyl) analogue **1-6**. Here, **1-3** was modified to give its *o*-methoxy and *m*-methoxy regioisomers **1-1** and **1-2**), respectively, the *p*-fluoro analogue **1-4** and the cyclohexyl analogue **1-5**. The alkyl side chain of **1-6** was rigidified by unsaturation (**1-7**, **1-8**) or rendered less lipophilic by inserting an ether linkage (**1-9**). A *p*-methoxy substituent was also introduced to the phenyl ring of **1-6** to give **1-10**.

Series 2 comprises ring substituted analogues of **1** (Table 2). The substituents (fluoro, chloro, bromo and methoxy) were introduced at positions 5, 6 or 7 of ring A (**2-1** to **2-12**) or the corresponding positions on ring B (**2-13** to **2-24**). The choice of substituents was determined largely by the commercial availability of reagents and, in the case of bromine, its striking association with antiproliferative activity, when attached to the indirubin scaffold.[23] As each series was synthesized and evaluated iteratively, inputs from one series determined the modifications made to the next series. Thus, having noted the exceptional activity of analogue **2-20** with 6′-methoxy in series 2 (Table 2), this group was incorporated in series 3 (Table 3), which also intended the replacement of the N^1-methyl substituent with side chains present in some of the more potent compounds of series 1. In a related manner, having deduced that the optimal substituent at N^1 is the *p*-methoxyphenethyl side chain from series 3, this feature was kept constant in series 4 (Table 4), which now saw other groups (methoxy, fluoro, chloro or bromo) introduced to either ring A or B of the scaffold. Thus, based on the activities of compounds from

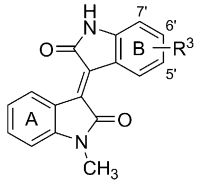
series 3 and 4, the optimal combination of groups on rings A/B and N^1 could be deduced.

Series 5 shown in Table 5 comprises compounds that were designed to have greater aqueous solubility. Having observed acceptable activity in several variants of the phenethyl side chain (series 1, Table 1), we speculated that a viable approach would be, to replace the lipophilic aromatic ring of the side chain with a solubilizing basic heterocyclic ring, such as, morpholine, thiomorpholine, piperidine or 1-methylpiperazine. Inserting an azomethine nitrogen in the isoindigo scaffold to give azaisoindigo was also considered, as estimated values point to a potential decrease in lipophilicity (log $D_{7.4}$ value of 1.03 and 0.6 for isoindigo and azaisoindigo, respectively) and an increase in solubility (aqueous solubility of 62 μM and 330 μM for isoindigo and azaisoindigo, respectively) with this modification.

Table 2. Series 2: $IC_{50,\,K562}$ values, Clog *P* and estimated solubilities (pH 7.4).



2-1 to **2-12**



2-13 to **2-24**

	R^2/R^3	Clog *P*[a]	Solubility [μM][b]	IC_{50} [μM][c]
2-1	5-F	2.86	642.2	19.24 ± 1.30
2-2	5-Cl	3.43	209.2	32.23 ± 2.46
2-3	5-Br	3.58	259.0	> 30
2-4	5-OMe	2.63	692.1	5.18 ± 0.91
2-5	6-F	2.86	339.8	10.87 ± 0.93
2-6	6-Cl	3.43	157.7	11.08 ± 1.27
2-7	6-Br	3.58	166.1	9.84 ± 0.69
2-8	6-OMe	2.63	297.1	9.74 ± 1.45
2-9	7-F	2.86	727.2	13.45 ± 1.38
2-10	7-Cl	3.43	234.9	17.12 ± 0.96
2-11	7-Br	3.58	284.4	20.82 ± 0.65
2-12	7-OMe	2.63	639.9	5.78 ± 0.96
2-13	5′-F	3.11	713.6	18.20 ± 0.89
2-14	5′-Cl	3.68	231.7	21.85 ± 3.55
2-15	5′-Br	3.83	287.2	14.41 ± 1.88
2-16	5′-OMe	2.79	656.2	5.93 ± 1.13
2-17	6′-F	3.11	951.5	14.82 ± 1.65
2-18	6′-Cl	3.68	238.1	18.45 ± 2.40
2-19	6′-Br	3.83	191.4	6.71 ± 1.03
2-20	6′-OMe	2.79	470.1	3.18[d] ± 0.34
2-21	7′-F	3.11	744.2	32.89 ± 2.67
2-22	7′-Cl	3.68	241.4	> 30[e]
2-23	7′-Br	3.83	292.8	> 30[e]
2-24	7′-OMe	2.79	669.2	> 30[e]

[a] Determined with ChemDraw 7.0. [b] Determined with ACD/Labs 12.0 Solubility DB. [c] Values represent the mean ± SD for $n \geq 3$ determinations. [d] Significantly lower than **1** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [e] Could not be determined due to limited solubility. > 50% viability was observed at the stated concentration.

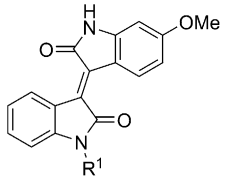
Three synthetic routes were adopted for the synthesis of series 1–5. The first approach involves the N-alkylation of isatin (or ring-substituted isatin) under basic conditions, followed by aldol condensation with oxindole (or ring-substituted oxindole) under acidic conditions (Scheme 1). This route was adopted for the synthesis of series 3 and 4, and selected compounds in series 1 (**1-1** to **1-5**, **1-7**, **1-8**, **1-10**) and series 2 (**2-1** to **2-7**, **2-9** to **2-11**, **2-20**).

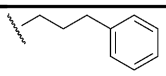
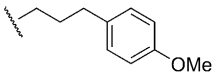
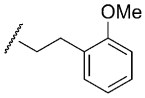
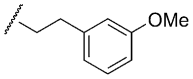
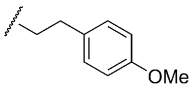
In the second approach, which is essentially a reversal of the first approach, commercially available 1-methyloxindole is reacted with a ring-substituted isatin in an acid-catalyzed aldol condensation reaction (Scheme 2). Most of the series 2 compounds (**2-8**, **2-12** to **2-19**, **2-21** to **2-24**) were synthesized by this route. In the case of **2-8** and **2-12**, the substituted 1-methyloxindole was synthesized by a methylation reaction with dimethylsulfate.

The third approach involves condensing isatin and oxindole to give isoindigo, followed by alkylation of the latter under basic conditions (Scheme 3). Direct alkylation of isoindigo is a short and convenient approach, especially as isoindigo is obtained in excellent yields (91%).[17] However, the separation of the target compound from unreacted isoindigo by column chromatography was cumbersome and, consequently, this route was adopted only when other approaches had failed to give the target compounds in good yields, as in the case of **1-6**, **1-9**, **5-2** and **5-3**.

Unlike in series 1–4, the alkyl halides used for series 5 (**5-1** to **5-3**, **5-5**) had to be synthesized. As shown in Scheme 4, 2-bromoethanol was reacted with the basic heterocycle (morpholine, thiomorpholine, or piperidine) to attach the hydroxyethyl side chain to the basic nitrogen, followed by displacement of the hydroxy group by chlorine from thionyl chloride. 1-(2-Chloroethyl)morpholine was then reacted with isatin to give compound **41**, which subsequently was condensed with oxindole (or 6-methoxyoxindiole) to give **5-1** and **5-5**. In the case of 1-(2-chloroethyl)th-

Table 3. Series 3: $IC_{50,\,K562}$ values, Clog *P* and estimated solubilities (pH 7.4).



	R^1	Clog *P*[a]	Solubility [μM][b]	IC_{50} [μM][c]
3-1		5.26	4.9	2.75 ± 0.33[d,e]
3-2		5.18	4.5	4.67 ± 0.77[d]
3-3		4.80	4.7	> 20[f]
3-4		4.80	4.7	11.43 ± 0.66
3-5		4.80	7.0	0.50 ± 0.06[d,e,g]

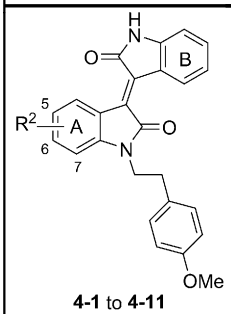
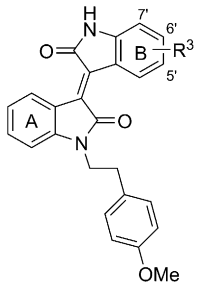
[a] Determined with ChemDraw 7.0. [b] Determined with ACD/Labs 12.0 Solubility DB. [c] Values represent the mean ± SD for $n \geq 3$ determinations. [d] Significantly lower than **1** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [e] Significantly lower than **2-20** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [f] Could not be determined due to limited solubility. > 50% viability was observed at the stated concentration. [g] Significantly lower than corresponding series 1 analogue **1-3** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc).

iomorpholine and 1-(2-chloroethyl)piperidine, these were directly reacted with isoindigo to give **5-2** and **5-3**.

A different approach was followed for **5-4** and **5-6** (Scheme 5), which both have a 4-methyl-1-piperazinylethyl side chain. Here, isatin was alkylated with dibromoethane to give 1-(2-bromoethyl)indolin-2,3-dione **42** and then reacted with 2-oxindole to give the corresponding isoindigo, after which the bromine atom in the side chain was displaced by 1-methylpiperazine. This approach has the advantage of facilitating the separation of the less polar intermediate from the final product by column chromatography.

The azaisoindigos **5-7** and **5-8** were prepared from the common intermediate 7-aza-2-oxindole **46** (Scheme 6) which was synthesized by a reported method.[24] The intermediate was reacted with 1-methylisatin or 1-(*p*-methoxyphenethyl)isatin to give the desired products **5-7** and **5-8**.

Table 4. Series 4: $IC_{50,\,K562}$ values, Clog *P* and estimated solubilities (pH 7.4).

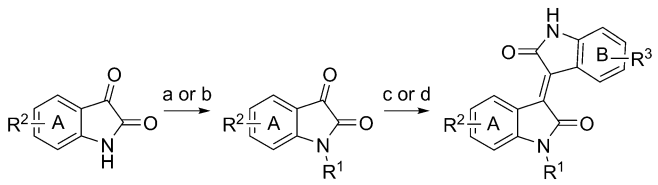
		R^2 or R^3	Clog *P*[a]	Solubility [μM][b]	IC_{50} [μM][c]
 **4-1** to **4-11**	**4-1**	5-F	4.87	7.2	18.58 ± 0.82
	4-2	5-Cl	5.44	2.3	> 30[d]
	4-3	5-Br	5.59	4.2	> 30[d]
	4-4	5-OMe	4.65	9.4	> 30[d]
	4-5	6-F	4.87	4.8	> 30[d]
	4-6	6-Cl	5.44	2.3	> 30[d]
	4-7	6-Br	5.59	2.1	10.75 ± 1.62
	4-8	6-OMe	4.65	4.7	5.15 ± 0.90[e]
	4-9	7-F	4.87	9.7	22.24 ± 2.51
	4-10	7-Cl	5.44	2.3	> 30[d]
	4-11	7-Br	5.59	4.2	> 30[d]
 **4-12** to **4-25**	**4-12**	5′-F	5.13	9.7	14.03 ± 1.53
	4-13	5′-Cl	5.70	2.3	12.01 ± 0.51
	4-14	5′-Br	5.85	4.2	12.04 ± 0.60
	4-15	6′-F	5.13	12.0	2.42 ± 0.28[e,f]
	4-16	6′-Cl	5.70	2.3	1.05 ± 0.10[e,f]
	4-17	6′-Br	5.85	2.1	1.06 ± 0.17[e,f]
	3-5	6′-OMe	4.80	7.0	0.50 ± 0.06[e,f]
	4-18	7′-F	5.13	9.7	> 10[d]
	4-19	7′-Cl	5.70	2.3	> 30[d]
	4-20	7′-Br	5.85	4.2	> 10[d]
	4-21	6′-NMe_2	4.89	4.6	> 30[d]
	4-22	6′-CF_3	6.06	< 1.0	9.81 ± 1.21
	4-23	6′-CN	4.76	2.4	13.82 ± 0.14
	4-24	6′-SO_2Me	3.73	12.6	10.30 ± 0.54
	4-25	6′-NH(C=O)Me	3.75	6.6	9.79 ± 1.54

[a] Determined with ChemDraw 7.0. [b] Determined with ACD/Labs 12.0 Solubility DB. [c] Values represent the mean ± SD for $n \geq 3$ determinations. [d] Could not be determined due to limited solubility. > 50% viability was observed at the stated concentration. [e] Significantly lower than **1** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [f] Significantly lower than **1-3** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc).

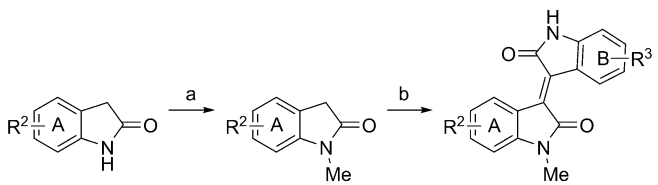
Antiproliferative activity on K562 cells

The antiproliferative activities of the target compounds were determined on K562 cells and expressed in terms of half maximal growth inhibitory concentrations (IC_{50}). Tables 1–5 list the IC_{50} values for series 1–5 compounds as well as their estimated lipophilicities (Clog *P*) and aqueous solubilities.

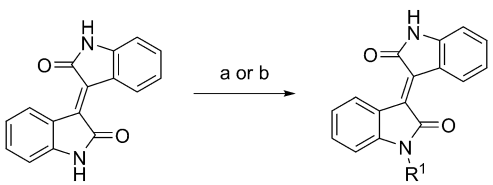
Initially, we had identified **1-3** and **1-6** as the most promising compounds, with $IC_{50,\,K562}$ values of 1.4 μM and 1.6 μM, respectively, relative to 6.7 μM for **1**.[17] A re-determination of the $IC_{50,\,K562}$ values gave comparable values for **1** (7.75 μM) and **1-6** (1.70 μM), but not for **1-3** (6.09 μM). Interestingly, **1-6** was again the most potent compound in series 1, followed closely by its *p*-methoxyphenpropyl analogue (**1-10**, $IC_{50,\,K562} = 1.75$ μM). The SAR for **1-6** showed that the activity was significantly decreased only when a triple bond was inserted into the phenpropyl side chain (**1-8**). Other modifications, such as introducing a double bond (**1-7**) or an ether linkage (**1-9**) in the side chain of **1-6** were well tolerated, with only incremental losses to activity. In the case of **1-3**, which was initially thought to be a potent hit, the replacement of the *p*-methoxyphenethyl side chain with a non-aromatic cyclohexylethyl side chain (**1-5**) significantly improved activity, while a relocation of the *p*-methoxy substituent to the *ortho* or *meta* position of the ring (**1-1**, **1-2**) did not adversely affect activity. On the other hand, inserting a *p*-fluoro group on the phenyl ring of **1-3** was detrimental, which was in line with earlier observations, where a ring substitution with *p*-methyl, *p*-hydroxy or *p*-nitro groups resulted in sharp losses in activity.[17]



Scheme 1. *Reagents and conditions*: a) Alkyl halide R^1–X, DMF, CaH_2, 80 °C, 2–4 h; b) Alkyl halide R^1–X, DMF, K_2CO_3, MW, 150 °C, 15–40 min; c) AcOH, HCl, reflux, 16 h; d) AcOH, HCl, MW, 200 °C, 30 min.



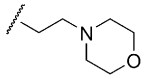
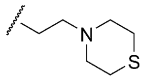
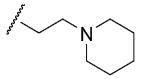
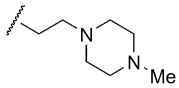
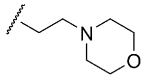
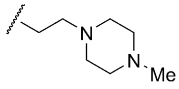
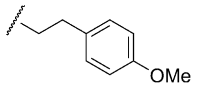
Scheme 2. *Reagents and conditions*: a) NaH, Me_2SO_4, xylene, 50 °C, 3 h; b) AcOH, HCl, reflux, 16 h.

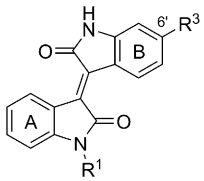


Scheme 3. *Reagents and conditions*: a) Alkyl halide R^1–X, DMF, K_2CO_3, 80 °C, 3 h; b) Alkyl halide R^1–X, DMF, K_2CO_3, MW, 150 °C, 15–40 min

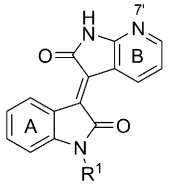
The results for series 2 (Table 2) showed that there was generally little benefit in introducing substituents on ring A or B of meisoindigo **1**. Only compound **2-20** (6′-methoxy) was more potent than **1** ($IC_{50,\,K562} = 3.18\ \mu M$). Curiously, the methoxy substitutent fared better than the halogens when introduced at every position on rings A and B, except position 7′ on ring B, where both halogens and methoxy were poorly tolerated. Notionally, this might be related to the proximity of position 7′ to the NH of the isoindigo scaffold. It could be that substitution at this position sterically hinders hydrogen bonding at this site. Indeed, methylation of this NH group in **1** to give dimethylisoindigo was found to abolish antiproliferative activity on K562 cells,[17] supporting a critical hydrogen bonding role for the NH moiety.

Table 5. Series 5: $IC_{50,\,K562}$ values, Clog *D* and estimated solubilities (pH 7.4).

	R^1	R^3	Clog $D_{7.4}$[a]	Solubility [μM][b]	IC_{50} [μM][c]
5-1		H	1.13	258.4	6.81 ± 0.14
5-2		H	1.87	81.7	7.91 ± 0.51
5-3		H	1.47	720.3	7.70 ± 0.74
5-4		H	0.88	602.4	3.97 ± 0.21[d]
5-5		OMe	1.15	192.7	3.78 ± 0.51[d]
5-6		OMe	0.90	456.4	5.89 ± 0.67[d]
5-7	Me	–	0.85	1644.5	17.25 ± 0.51
5-8		–	3.03	20.1	> 20[e]



5-1 to **5-6**

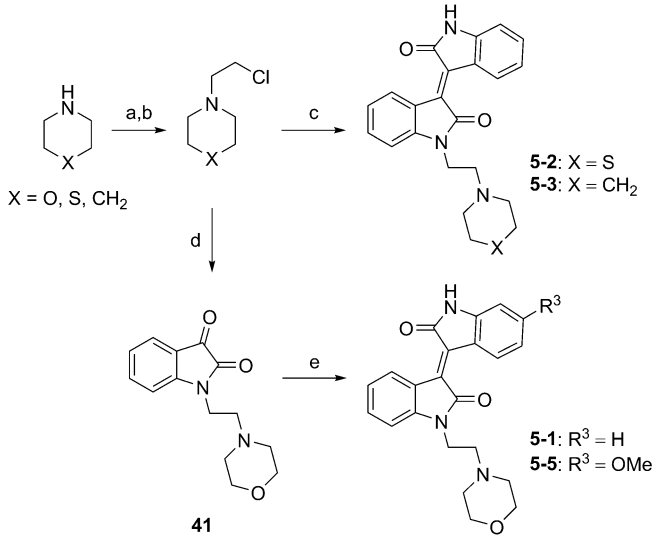


5-7 and **5-8**

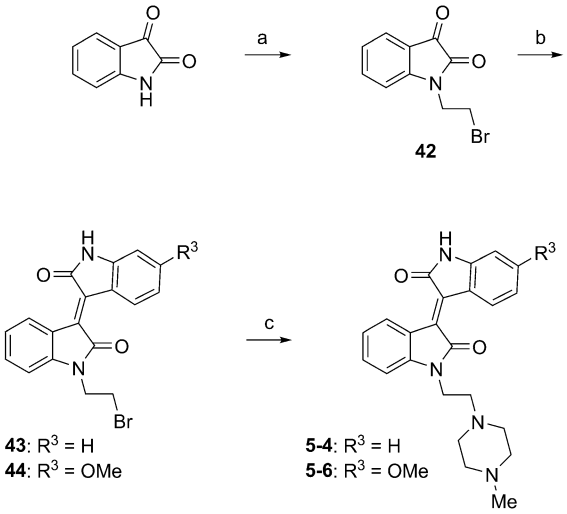
[a] Determined with ACD/Labs 12.0 Log *D*. [b] Determined with ACD/Labs 12.0 Solubility DB. [c] Values represent the mean ± SD for $n \geq 3$ determinations. [d] Significantly lower than **1** ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [e] Could not be determined due to limited solubility. > 50% viability was observed at the stated concentration.

Series 3 is to determine, whether the activity would be improved in analogues that carry both the optimal 6′-methoxy group and the favored sidechain(s) identified in series 1. Disappointingly, this was not found to be so. In fact, only analogue **3-5** ($IC_{50,\,K562} = 0.50\ \mu M$), which bears the 6′-methoxy group and the *p*-methoxyphenethyl side chain, was more active than its series 1 analogue **1-3** ($IC_{50,\,K562} = 6.09\ \mu M$). The remaining compounds in series 3 showed sharp losses in activity when the 6′-methoxy group was introduced.

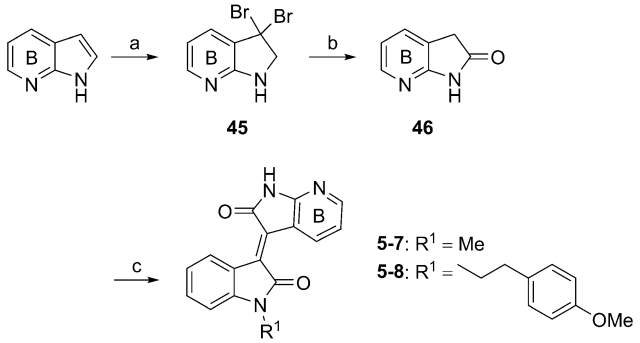
The SAR of **3-5** was further explored in series 4 by varying the position and type of substitution on rings A and B (Table 4). As in series 2, there was no added benefit arising from substitution of either ring A or B. However, unlike series 2, substitution on ring B of series 4 resulted in more active analogues than substitution



Scheme 4. *Reagents and conditions*: a) 2-Bromoethanol, toluene, triethylamine, reflux, 4 h; b) Thionyl chloride, 0–80 °C, 2 h; c) Isoindigo, K_2CO_3, DMF, 80 °C, 4 h; d) Isatin, DMF, 80 °C, 4 h; e) 2-Oxindole or 6-methoxy-2-oxindole, AcOH, HCl, reflux, 16 h.



Scheme 5. *Reagents and conditions*: a) 1,2-Dibromoethane, K_2CO_3, DMF, RT, 4 h; b) 2-Oxindole or 6-methoxy-2-oxindole, AcOH, HCl, reflux, 16 h; c) *N*-Methylpiperazine, triethylamine, DMF, 80 °C.



Scheme 6. *Reagent and conditions*: a) PBPB, *t*BuOH, RT, 6 h; b) Pd/C, H_2, 50 psi, RT, 4 h; c) 1-Methylisatin or **4**, EtOH, HCl, reflux, 4 h.

on ring A. Position 6′ was again the most favored position in series 4, with both 6′-methoxy and 6′-halogen modifications giving rise to exceptionally active analogues. However, the poor activity of analogues with other substituents at position 6′, such as, dimethylamino (**4-21**), trifluoromethyl, (**4-22**), cyano (**4-23**), methylsulfonyl (**4-24**) and acetamide (**4-25**) bears consideration, as it suggests that the type of group at position 6′, and not just its occupancy, plays a decisive role in influencing the activity.

Of the compounds of series 5, only the three analogues **5-4**, **5-5**, and **5-6** were significantly more potent than **1** (Table 5). A unique role might be inferred for the 4-methyl-1-piperazinylethyl side chain, as it is present in two of the three active compounds (**5-4**, **5-5**). However, both compounds did not achieve the submicromolar growth inhibitory activity of **3-5**, which remains the most active analogue identified thus far. The azaisoindigos **5-7** and **5-8** fared poorly in terms of activity ($IC_{50,\,K562} > 15$ μM). In both these compounds, the azomethine nitrogen is at position 7′, which has been found to be generally intolerant of substitution. Wang and co-workers[25] have reported more promising activity for their series of functionalized azaindigos, in which the azomethine nitrogen was placed at position 7. Their best compound had an IC_{50} value of 10.5 μM on the prostate cancer cell line DU145, compared to 17.4 μM for meisoindigo **1**. Possibly, more active analogues could be obtained, if the azomethine nitrogen is introduced at positions other than 7′ of ring B. Even so, there are doubts whether the azaisoindigo scaffold would indeed improve water solubility, as **5-8** was too insoluble to permit determination of its IC_{50} value.

Antiproliferative activity on other malignant and non-malignant cell lines

Next, selected analogues from each series were screened for growth inhibitory effects on other human malignant cell lines, namely NB4 (an acute promyelocytic leukemic cell line), HuH7 (a hepatocellular carcinoma cell line), RCC786 (a renal carcinoma cell line) and HCT116 (a colon carcinoma cell line). They were also screened against non-malignant IMR90 (human lung fibroblasts) to establish selective activity. Seven analogues, including **1**, were shortlisted for screening, namely those that were most active on K562 cells in their respective series: **1-6**, **1-10** of series 1, **2-20** of series 2, **3-5** of series 3 and **5-4** of series 5. Compound **1-3** was included to represent a compound with moderate potency. The results are given in Table 6.

Two general trends are noted. First, the isoindigos were distinctly more potent on leukemic cells (K562, NB4) than on malignant cells derived from solid tumors (HCT116, HuH7, RCC786). Notably, **1-3**, **1-6**, **1-10**, and **3-5** had submicromolar IC_{50} values on leukemic NB4, but not on solid tumor cell lines. Second, many of the isoindigos maintained significantly higher activity than **1** across the panel of malignant cells (K562, NB4, HuH7, HCT116). Exceptions were **2-20** and **5-4** on leukemic NB4 cells, which were not more active than **1**, and compound **5-4**, which was the only compound that was more potent than **1** on RCC786 cells. When the compounds were tested on the non-malignant cell line IMR90, selective targeting against the leukemic cells (K562, NB4) was evident, with selectivity for the malignant cells ranging from 1.6–9.6-fold (K562) and 2.2–27-fold (NB4). Compound **3-5** exhibited impressive selective activity on the leukemic cells relative to IMR90.

Table 6. IC_{50} values [μM] of selected compounds on K562, NB4, HuH7, RCC786, HCT116 and IMR90 cells.[a]

Compd	K562	NB4	HuH7	RCC786	HCT116	IMR90
1	7.75 ± 0.71	2.38 ± 0.27	20.43 ± 0.95	5.53 ± 0.33	31.96 ± 1.61	12.01 ± 0.27
1-3	6.09 ± 1.01	0.68 ± 0.05[b]	7.51 ± 0.31[b]	5.22 ± 0.39	12.76 ± 0.44[b]	7.94 ± 0.34[b]
1-6	1.70 ± 0.22[b]	0.89 ± 0.07[b]	7.87 ± 0.33[b]	4.46 ± 0.54	6.76 ± 0.83[b]	5.76 ± 0.16[b]
1-10	1.75 ± 0.14[b]	0.98 ± 0.11[b]	6.62 ± 0.32[b]	3.99 ± 0.44	7.03 ± 0.46[b]	5.83 ± 0.63[b]
2-20	3.18 ± 0.34[b]	2.52 ± 0.23	7.39 ± 0.61[b]	> 20[c]	17.57 ± 1.60[b]	> 20[c]
3-5	0.50 ± 0.06[b]	0.18 ± 0.01[b]	5.16 ± 0.94[b]	7.13 ± 1.30	4.96 ± 0.32[b]	4.80 ± 0.83[b]
5-4	3.97 ± 0.21[b]	4.32 ± 0.16	9.49 ± 0.51[b]	3.52 ± 0.21[b]	15.69 ± 1.14[b]	9.42 ± 0.42[b]

[a] Values represent the mean ± SD for $n \geq 3$ determinations. [b] Significantly lower than **1** on the specified cell line ($p < 0.05$, one-way ANOVA, Dunnett's post hoc). [c] Could not be determined due to limited solubility. > 50% viability was observed at the stated concentration.

Aqueous solubilities of 3-5 and 5-4

Thus far, we have identified **3-5** as the most potent analogue and **5-4** as the most promising water soluble analogue against K562 cells. Notwithstanding its potency, **3-5** is predicted to have poor solubility at pH 7.4 (7.0 μM), while estimates suggest that **5-4** is likely to be as soluble as **1** (**5-4**: 602 μM; **1**: 673 μM, Tables 1 and 5). Hence, it was of interest to determine the aqueous solubilities (pH 7.4) of **3-5**, **5-4**, and **1**. Determinations were made on multiscreen solubility filter plates by a method which involved measuring the amount of compound dissolved in phosphate buffer (pH 7.4) after 24 h of agitation. As seen in Table 7, both **3-5** and **5-4** were more soluble than **1** by almost 50-fold. The inclusion of a solubilizing piperazine ring in **5-4**

Table 7. Solubility and light scattering properties of meisoindigo **1**, **3-5** and **5-4**.

Compd	Exptl Solubility [μM][a]	Calcd Solubility [μM][b]	Light Scattering Count [kcps][c]
1[d]	8.34 ± 1.48	673	480 ± 15 (10 μM) 11 ± 1 (1 μM)
3-5[d]	22.36 ± 3.65	7	455 ± 14 (10 μM) 4 ± 1 (0.5 μM)
5-4[e]	348.38 ± 3.23[f]	602	11 (10 μM) 1 (1 μM)

[a] Determined in universal buffer (pH 7.4) containing 1% *v/v* DMSO, 24 h agitation, 28 °C. Values represent the mean ± SD for $n \geq 3$ determinations. [b] Calculated by ACD/Labs 12.0 Solubility DB, pH 7.4. [c] Determined in phosphate buffer (5 mM, pH 7.4) containing 1% *v/v* DMSO. Counts represent the mean ± SD for $n \geq 3$ independent determinations. [d] Concentration of initial solution from which filtrate was derived = 100 μM. [e] Concentration of initial solution from which filtrate was derived = 400 μM. [f] Solubility can exceed 348 μM because no precipitate was observed in the starting solutions (400 μM).

has indeed dramatically escalated solubility to the millimolar range. In the case of meisoindigo (**1**), its solubility has been grossly overestimated, possibly because the algorithms used in the software did not take into account the brick-dust character of the molecules.

Aggregation potential of 3-5 and 5-4

There is growing interest in the ability of bioactive substances to form colloid-like aggregates in aqueous solutions.[26–32] These "aggregators" might inhibit proteins in a nonspecific manner and lead to the misinterpretation of biological data. Common characteristics shared by aggregators are low solubility, high lipophilicity and extended conjugation.[29] Noting that indirubin and indigo have been reported to form aggregates,[28] there was concern that this could be a shared property of the present series of functionalized isoindigos. The aggregation phenomenon can be monitored by measuring the light scattering capacity of a known concentration of the test compound in phosphate buffer. In the presence of aggregates, significant scattering is observed, giving rise to a high count rate. Table 7 gives the results for **3-5**, **5-4** and **1**. Benzyl benzoate, a known aggregator, gave a count rate of 680 kilocounts per second (kcps) at 250 μM. The scattering capacities of the test compounds were monitored at a lower concentration of 10 μM. At this concentration, **1** and **3-5** registered counts of 480 kcps and 455 kcps, respectively. The scattering effect is concentration dependent and was negligible at the lowest test concentrations (1 μM for **1** and 0.5 μM for **3-5**). In the case of **5-4**, negligible scattering was observed at 10 μM, indicating that its improved solubility has served to negate the aggregating potential of the isoindigo scaffold.

In vivo effects of 3-5 and 5-4 on mice bearing K562 xenografts

3-5 and **5-4** were administered to xenograft-bearing animals to assess their in vivo effects. Meisoindigo **1** was tested under similar conditions. K562 cells were injected into the flank of immunocompromised mice to induce tumor formation. Once a palpable tumor was observed (day 0), the compound (10 μM) was administered directly into the tumor on days 0, 3 and 10. The animals were monitored up to day 28, barring the absence of censored events that would require euthanizing the animals.

Here, we chose to test the three compounds at the same dose of 10 μM, in spite of differences in their growth inhibitory properties ($IC_{50,\,K562}$ value of **1** > **5-4** > **3-5**). The original intent was to arbitrarily test at concentrations approximately twice the $IC_{50,\,K562}$ value, which would be 16 μM for **1**, 10 μM for **5-4**, and 1 μM for **3-5**. However, this was not possible, due to the poor solubility of **1**, which did not allow dosing at the proposed concentration. As **1** was readily administered at 10 μM, it was decided that all three compounds would be evaluated at this dose, although it would mean that **3-5** would be given at almost 20 times its IC_{50} value, exceeding by far that of **1** and **5-4**.

Table 8 summarizes the mean and median survival times of the xenograft bearing mice in the control and treatment groups. Mice in the control group (treated with saline-DMSO)

Table 8. Kaplan–Meier analysis for comparison between treatment groups in xenograft-bearing mice.

	Control	**1**	**3-5**	**5-4**
No. of animals	8	5	5	6
No. survived after 28 d	2	1	0	5
Median survival time [d]	19	26	16	>28[a]
Mean survival time [d]	19.6 ± 1.9	23.8 ± 1.8	19.6 ± 2.5	26.7 ± 1.2
No. of censored events[b]	2	1	0	5
ILS [%][c]	–	+21.3	−0.1	+35.9
Log-rank test (p)[d]	–	0.631	0.658	0.028

[a] Median could not be determined because 5 of 6 animals in this group survived beyond 28 d. [b] The only censored event was the survival of animals beyond 28 d. [c] Increase in mean life span (ILS) = [(mean survival time of treated group − mean survival time of control group)/mean survival time of control group]×100%. [d] p value gives the statistical significance between control and treated animals.

survived for 19.6 days, while those treated with **1** and **5-4** lived longer at 23.8 days and 26.7 days, respectively. Animals treated with **3-5** survived as long as the control animals. To determine if the increases in mean lifespans observed with **1** and **5-4** were significant, the data were analyzed by the log-rank test (pair-wise) comparison statistic p in the Kaplan–Meier analysis. The results show that only mice treated with **5-4** had significantly longer mean life spans ($p = 0.028$). While animals treated with **3-5** or **1** were not significantly long-lived, neither were their life spans prematurely curtailed, indicating that these compounds were relatively nontoxic at the given doses. The survival curves of animals treated with the three test compounds are given in Supporting Information.

The body weights of the treated animals were monitored daily and were found to remain constant throughout the test period (not shown). Changes in the tumor volumes were also tracked over 28 days (Figure 2). It is seen that tumor volumes

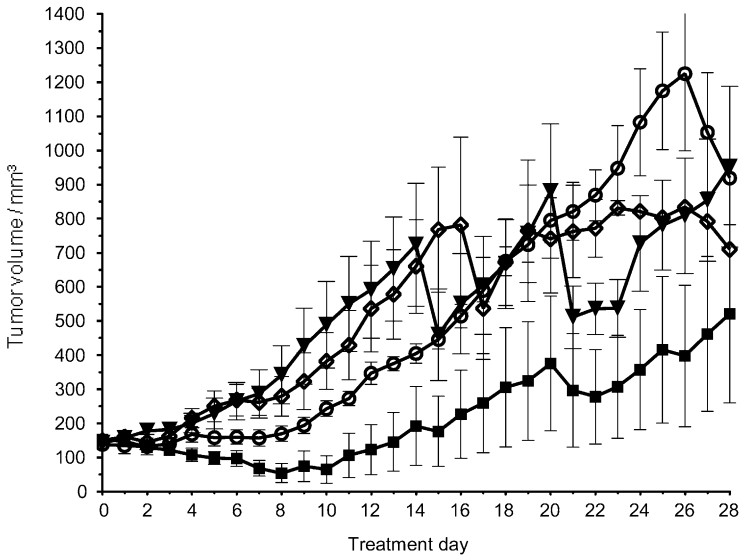


Figure 2. Changes in tumor size (mm^3) of xenograft-bearing mice treated with the vehicle control (▼) and test compounds, meisoindigo (○), **3-5** (◇), **5-4** (■).

of control animals and those treated with **1** and **3-5** increased steadily from day 0 to day 14, but showed greater variability and less defined trends thereafter, due to the progressive demise of test animals after day 14. In the case of animals treated with **5-4**, tumor volumes actually decreased from day 0 to day 10 before resuming a gradual increase. When changes in tumor volumes were analyzed for statistical difference from control animals by one-way ANOVA (Dunnett's post hoc), only animals treated with **5-4** between day 6 ($p = 0.026$) to day 14 ($p = 0.036$) had tumors that were statistically smaller than those of control animals over the same period. It was good to note that of the six mice treated with **5-4**, the tumors of two mice completely regressed and remained as such until day 28. In a single case, the tumor shrunk initially for the first 10 days, but increased thereafter. The tumors of the remaining animals grew steadily up to day 28, but did not exceed the tumor diameter of 1.5 cm, which was the threshold for euthanasia.

Effects of 3-5 and 5-4 on the cell cycle of K562 cells

Having established that **3-5** and **5-4** are promising hits with good potencies and aqueous solubilities, we proceeded to examine their effects on the cell cycle of K562 cells. The intent is to derive a better understanding of the mechanistic basis underlying their antiproliferative activities. Cell cycle arrest was investigated by fluorescence activated cell sorter analysis (FACS) using flow cytometry. K562 cells were incubated with the test compound for 24 h, after which cellular DNA content was analyzed to determine the proportion of cells in the different phases of the cell cycle. Each compound was tested over a range of concentrations that spanned its $IC_{50,\,K562}$ value and up to approximately four times this concentration. The results are listed in Table 9.

The control K562 cells were not synchronized in this analysis and showed a high cell density at the G_1 phase and about equal amounts of cells in the S and G_2 phases. As seen from Table 9, meisoindigo **1** ($IC_{50} = 7.75$ μM) caused minimal disruption to the cell cycle, notwithstanding a small but significant decrease in the proportion of cells in the G_2 phase at the highest concentration tested (20 μM). However, a concurrent increase of cells in the G_1 phase, typical of a G_1 arrest, was not observed.

3-5 was investigated at lower concentrations (0.1–10 μM) in view of its potent growth inhibitory effects ($IC_{50} = 0.5$ μM). The results showed that it induced a concentration dependent decrease in the proportion of cells in G_1 while concurrently increasing those in the G_2 phase, a profile which attests to G_2 arrest. There was also a significant increase in the proportion of cells in the sub-G_1 phase which is associated with cellular debris arising from necrotic or apoptotic cells.

In the case of **5-4**, which was investigated at 1–15 μM, disruption of the cell cycle at the G_1 phase (increase in G_1, decrease in G_2) was evident only at the highest concentration (15 μM). There was a concurrent increase in the proportion of cells in the sub-G_1 phase. Thus, in spite of their structural similarity, all three compounds had very different effects on the cell cycle, namely G_2 arrest for **3-5**, G_1 arrest in the case of **5-4** and no clear cut effects for **1**.

Effects of 3-5 and 5-4 on apoptosis of K562 cells

Meisoindigo **1** is known to induce apoptosis in leukemic cells,[14] and it was of interest to determine if **3-5** and **5-4** have similar effects. Hence, apoptosis was investigated by double

Table 9. FACS analyses of the various phases of the cell cycle of leukemic K562 cells on exposure to meisoindigo, **3-5** and **5-4**.

	c [μM]	Sub-G_1 [%][a]	G_1 [%][a]	S [%][a]	G_2 [%][a]
Control[b]	–	1.80 ± 0.46	40.02 ± 4.49	29.81 ± 1.09	26.76 ± 2.29
1	1	1.42 ± 0.25	43.92 ± 1.37	29.37 ± 0.22	26.08 ± 1.29
	5	1.91 ± 0.71	45.71 ± 2.29	29.63 ± 0.92	23.46 ± 2.58
	10	2.56 ± 0.39	40.16 ± 0.54	30.27 ± 0.20	27.52 ± 0.62
	15	1.89 ± 0.15	41.64 ± 0.82	30.82 ± 0.16	22.44 ± 0.65[c]
	20	2.23 ± 0.20	39.09 ± 0.61	31.59 ± 0.18[c]	23.70 ± 0.44[c]
3-5	0.1	1.38 ± 0.11	41.90 ± 0.81	29.61 ± 0.50	24.11 ± 0.91
	0.5	2.15 ± 0.24	32.44 ± 0.90[c]	25.90 ± 0.62[c]	35.68 ± 1.61[c]
	1	4.97 ± 0.20[c]	19.88 ± 0.71[c]	35.76 ± 0.82[c]	34.57 ± 1.31[c]
	5	14.11 ± 0.31[c]	9.99 ± 0.11[c]	9.03 ± 0.19[c]	57.24 ± 0.22[c]
	10	9.87 ± 0.44[c]	4.17 ± 0.20[c]	6.18 ± 0.23[c]	71.31 ± 0.82[c]
5-4	1	1.26 ± 0.02	44.34 ± 1.06	29.37 ± 0.68	25.41 ± 1.37
	5	1.24 ± 0.10	44.07 ± 1.30	28.79 ± 0.57	26.43 ± 1.62
	10	2.18 ± 0.11	42.41 ± 0.55	28.69 ± 0.18	27.16 ± 0.77
	15	9.76 ± 0.46[c]	45.30 ± 0.23[c]	22.40 ± 0.29[c]	18.19 ± 0.59[c]

[a] Values represent the mean ± SD for $n \geq 3$ determinations. [b] Control cells treated with media (0.4% *v/v* DMSO) in the absence of test compound. [c] Significantly different ($p < 0.05$) from control (one-way ANOVA, Dunnett's post hoc).

staining the K562 cells with annexin V and propidium iodide at different concentrations of the test compounds. Cells that were positively stained by annexin V, but not propidium iodide, were deemed to be apoptotic, while those that were positively stained by both annexin V and propidium iodide were likely to be necrotic. Representative plots are given in the Supporting Information. The distribution of treated cells in each quadrant of the plot was monitored for significant variations from the control untreated cells (Table 10). The results showed that both **3-5** and **5-4** increased the proportion of apoptotic cells, and interestingly, at concentrations that closely corresponded to those required to disrupt the cell cycle, namely at the IC_{50} value of **3-5** and at a higher concentration of 15 μM for **5-4.** **1** also increased the proportion of apoptotic cells but only at higher concentrations (15 μM and 20 μM).

Table 10. Distribution of normal, apoptototic and necrotic K562 cells treated with different concentrations of **1**, **3-5** and **5-4**.

	c [μM]	Normal [%][a]	Apoptotic [%][a]	Necrotic [%][a]
Control[b]		93.77 ± 1.67	3.42 ± 0.73	1.92 ± 0.62
1	1	93.25	3.70	2.42
	5	93.36	3.48	2.51
	10	93.58	3.77	2.07
	15	83.50 ± 0.88	10.15 ± 0.32[c]	5.11 ± 0.42[c]
	20	82.90 ± 0.47	11.27 ± 0.31[c]	5.04 ± 0.33[c]
3-5	0.1	95.56 ± 0.03	2.74 ± 0.10	1.28 ± 0.62
	0.5	87.27 ± 1.31	8.91 ± 0.79[c]	2.77 ± 1.06
	1	90.26 ± 0.12	7.72 ± 0.19[c]	1.62 ± 0.08
	5	82.34 ± 0.14	14.03 ± 0.27[c]	3.05 ± 0.26[c]
	10	78.18 ± 0.05	16.76 ± 0.08[c]	4.57 ± 0.14[c]
5-4	1	92.72	3.74	2.63
	5	93.29	3.43	2.42
	10	94.02	3.73	1.59
	15	79.84 ± 0.87	14.06 ± 1.24[c]	5.41 ± 0.14[c]

[a] Proportion of normal, apoptotic and necrotic cells were deduced from the FACS analysis of cell populations in the lower left, lower right and upper right quandrants, respectively. [b] Control cells treated with media (0.4% *v/v* DMSO) in the absence of test compound, $n = 5$ determinations. [c] Significantly different ($p < 0.05$) from control (one-way ANOVA, Dunnett's post hoc). Values represent the mean ± SD for $n \geq 3$ determinations. For $n = 2$ determinations, no SD was calculated.

Discussion

Meisoindigo **1** and its glycosylated analogue, natura, are arguably the most widely reported isoindigos with anticancer properties. **1** is used in China as an efficacious and well-tolerated substitute for indirubin in the treatment of CML,[9] while natura was designed to improve the bioavailability and potency of **1**.[16,19] Both compounds reportedly induce apoptosis, inhibit cyclin-dependent kinases and arrest tumor growth in mice transplanted with Walker 256 cancer cells.[16,19] The aim of the present study is to investigate the SAR of **1** with reference to its antiproliferative activity, and to use these findings to identify key features on the scaffold that could be modified to enhance the physicochemical profile without overt loss of activity. To this end, we have obtained a reasonably comprehensive understanding of the critical features of the scaffold that contribute significantly to the activity on K562 cells. An intact 3,3′-double bond to ensure planarity and a methyl substitution at only one of the lactam NH (N^1) groups are key features deduced from our earlier report.[17] Here we show that replacing the methyl group of **1** with phenalkyl side chains, notably, phenpropyl, is a viable means of improving activity. Moreover, with the exception of a selected ring substitution (**1-4**) and restricted flexibility of the alkyl side chain (**1-8**), most modifications of the phenalkyl moiety were well tolerated, with many of the resulting analogues (series 1) displaying greater activity than **1**. In order to negate the adverse increases in lipophilicity associated with these modifications, the phenyl ring was replaced by several basic heterocycles. This strategy did indeed address the physicochemical deficits of the phenalkyl analogues, in spite of modest antiproliferative activities (**5-4** to **5-6**), which did not match the submicromolar potencies of **3-5**. The other approach of inserting an azomethine nitrogen to improve solubility was less successful, as the resulting 7′-azaisoindigos showed dramatic losses in activity and were possibly less soluble than **1**.

We also examined the effect of ring substitutions on rings A/B of the scaffold and found that substitutions on rings A and B produced differential effects on activity, even with the same substituent. There is a general preference for a substitution on ring B, particularly at position 6′. At this position, the association of 6′-methoxy with good activity is striking, but contingent on the side chain present at the lactam nitrogen (N^1). Thus, activity was optimal when 6′-methoxy was paired with 1-methyl (**2-20**) and 1-(*p*-methoxyphenethyl) (**3-5**), but not with side chains present in **3-1** to **3-4** or the water soluble analogue **5-6**.

It is encouraging to note that the antiproliferative activities of the potent functionalized isoindigos (**1-6**, **1-10**, **2-20**, **3-5**, **5-4**) were not limited to K562 alone, but were also observed at even lower IC_{50} values (except for **2-20**, **5-4**) on another leukemic cell line, NB4. Another positive finding is the selective activity on leukemic cells vis-à-vis the non-malignant human lung fibroblast cells, IMR90. More modest antiproliferative activities were observed against malignant cells derived from solid tumors, but even then, most of the synthesized compounds maintained significantly greater potencies than **1**. Notably, **5-4** was the only compound that was consistently more potent than **1** on the solid tumor cell lines HuH7, HCT116 and RCC786.

Previous approaches at improving the water solubility of the isoindigo scaffold have focused almost exclusively on substituting the lactam NH with sugar moieties. Here we showed that significant improvements in aqueous solubility and a concurrent decrease in aggregation potential can be achieved by appending solubilizing basic heterocycles to the NH moiety. Through this approach, we were able to identify analogues (**5-4**, **5-5**, **5-6**) that achieve drug-like profiles, as well as antiproliferative activities exceeding that of **1**. In fact, it is tempting to attribute the in vivo efficacy of **5-4** to its improved physicochemical profile and to further propose that the modest potency of **5-4** has been compensated or even overcome by its favorable solubility. In comparison, **3-5** with greater potency

but poorer solubility failed to demonstrate activity, in spite of being administered at a dose (10 μM) that was almost 20 times its IC_{50} value. We were not able to demonstrate the in vivo efficacy of **1** on the K562 xenografts. Such a study has not been reported, although **1** is reputed to be effective on xenografts induced by HT29 colon cancer cells[33] and HL60 acute myeloid leukemic cells,[13,14] but at higher doses and at greater frequencies. Criticism might be levied at the intratumoral route employed, but this was necessitated by the absence of pharmacokinetic data of the test compounds. Nonetheless, care was taken to ensure that the injection was made directly to the palpable tumor and that no damage was afflicted to neighboring tissues.

Meisoindigo **1** has been variously reported to arrest the cell cycle at either the G_1[13,14] or G_2 phase,[33] depending on the type of cells investigated. It has also been noted to induce apoptosis in multiple leukemic cell lines, possibly by down regulating antiapoptotic Bcl-2, and upregulating pro-apoptotic (Bak, Bax) and cell cycle-related proteins (p21,p27).[14] Here, we found that **1** (20 μM) decreased the proportion of cells in the G_2 phase but did not increase those in the G_1 phase, which is characteristic of G_1 arrest. Apoptosis was observed at a similar concentration range (10–20 μM). Strikingly different profiles were observed for **3-5** (G_2 arrest at IC_{50}) and **5-4** (G_1 arrest at concentrations $> IC_{50}$), indicating that relatively minor modifications can significantly alter the mechanistic pathways involved in growth inhibition. The potent G_2 arrest associated with **3-5** is interesting, as it has been shown not to inhibit CDK2, which is involved in progression through the G_2 phase.[17] Thus, the disruption of G_2 progression by **3-5** could be caused by other factors, such as an increase in the production of endogenous cell cycle inhibitors, down regulation of cyclins required for CDK activation or induction of arylhydrocarbon receptor (AhR) signaling pathways leading to cell cycle arrest. The latter is a plausible alternative, as isoindigo has been reported to be an AhR agonist,[34] and the antiproliferative activity of 1-methylated indirubins has been linked to the activation of AhR signaling pathways.[35] At this stage of our investigations, the mechanistic basis underlying the ability of **5-4** to induce apoptosis and cause G_1 arrest in K562 cells remains to be resolved.

In conclusion, our findings have provided a better understanding of the SAR of the isoindigo scaffold with regards to its antiproliferative activity and have shown that the physicochemical deficits of the scaffold are surmountable by systematic modifications of structure and could be achieved without overt loss of growth inhibitory activity.

Experimental Section

Chemistry

General details for chemical synthesis. Reagents were obtained from commercial suppliers and used without further purification. Melting points were determined on a Gallenkamp melting point apparatus (Weiss–Gallenkamp, Loughborough, UK) and reported as uncorrected values. Reactions were routinely monitored by thin layer chromatography (TLC) using pre-coated plates (Silica Gel 60, F254, Merck) and visualized with UV light. Flash column chromatography was carried out using Silica Gel 60 (230–400 mesh, Merck). ^{1}H NMR spectra were collected on a Spectrospin 300 Ultrashield (Bruker, Billerca, MA, USA) and referenced to residual solvent peaks ($CDCl_3$ at $\delta = 7.26$, [D_6]DMSO at $\delta = 2.50$) as internal standards. ^{13}C NMR spectra (75 MHz) were determined on the same instrument and are reported in ppm (δ) relative to residual $CDCl_3$ ($\delta = 77.0$) and [D_6]DMSO ($\delta = 39.5$). Coupling constants (J) are reported in Hertz (Hz). Nominal mass spectra were captured on a LcQ mass spectrometer (Thermo Finnigan MAT GmbH, Bremen, Germany) with chemical ionization (APCI) and m/z values for the molecular ion are reported. High resolution accurate mass spectra were analyzed on a micrOTOF-QII mass spectrometer (Bruker, Billerca, MA, USA) by electrospray ionization (ESI). Microwave-assisted reactions were carried out on a Biotage® Initiator microwave synthesizer (Biotage AB, Uppsala, Sweden). Purity of final compounds were verified by either combustion analysis (C, H) (Vario Micro Cube, Elementar, Hanau, Germany) or by reverse phase HPLC on two different eluting systems (isocratic mode). Analytical and purity data of final compounds are provided in the Supporting Information.

General method for the preparation of 1-alkyl isatin intermediates, 2–4, 6, 7, and 11. Isatin (1 equiv), anhyd K_2CO_3 (1.2 equiv) and anhyd DMF (2–4 mL) were added to a sealed microwave vessel (5 mL) and stirred at RT for 15 min, followed by injection of the alkyl halide (1.1 equiv). Stirring was continued for another 5 min before irradiation in a microwave reactor at 150 °C for 15–30 min. After H_2O was added to the mixture, it was either filtered to remove the crude product, which was then rinsed with deionized H_2O, or extracted with EtOAc/CH_2Cl_2. For the latter, the organic layer was dried with anhyd Na_2SO_4 and filtered, and the organic solvent was removed in vacuo. Purification by column chromatography followed by recrystallization with EtOH gave the desired product.

General method for the preparation of 1-alkyl isatin intermediates, 5, 8, and 9). Isatin (1 equiv), anhyd CaH_2 (1.2 equiv) and anhyd DMF (2–4 mL) were stirred in a flask for 15 min at RT. The alkyl halide (1.1 equiv) was added and the mixture heated at 80 °C for 2–4 h. Extraction was performed using EtOAc or CH_2Cl_2 with brine and purified as described for compound **2**.

(2-Chloroethoxy)benzene (10). Phenol (10 mmol), anhyd K_2CO_3 (15 mmol) and MeOH (8 mL) were stirred in a sealed vessel (20 mL) at RT for 15 min and then heated in a microwave reactor to 140 °C for 30 min. The mixture was extracted with CH_2Cl_2 and the organic layer was washed with an aq K_2CO_3 solution, dried (anhyd Na_2SO4) and filtered. The solvent was removed in vacuo and the residue purified by column chromatography (isocratic, 100% *n*-hexane) to give **10** as a colorless oil (674 mg, 43%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.21$ (t, $J = 8.1$ Hz, 2H), 6.89 (t, $J = 7.5$ Hz, 2H), 6.83 (d, $J = 8.4$ Hz, 2H), 4.13 (t, $J = 5.7$ Hz, 2H), 3.71 ppm (t, $J = 5.7$ Hz, 2H); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta = 158.1$, 129.5, 121.4, 114.7, 77.4, 77.0, 76.6, 67.9, 41.9 ppm.

General method for the preparation of substituted 1-methylisatins, 12–18 and 20–22. The 1-methylated isatins were prepared by reacting the corresponding substituted isatin with methyl iodide as described earlier.[17]

General method for the preparation of substituted 1-methyl-2-oxindoles, 19 and 23. To a solution of substituted 2-oxindole (2 mmol) in xylene (4 mL) was added NaH (2 mmol), followed by dimethylsulfate (2 mmol). The solution was allowed to stir at RT for 15 min, and then at 50 °C for 3 h. The mixture was concentrated in vacuo and extracted with EtOAc/brine. The organic layer was dried

(anhyd Na_2SO_4), filtered, concentrated, and purified by column chromatography (isocratic, hexane/EtOAc 4:1) to give the desired product.

6-Aminoindolin-2-one (24). The synthesis was performed as reported by Khanwelkar et al.[36] Briefly, a solution of 2,4-dinitrophenylacetic acid (10 mmol) in MeOH (100 mL) was hydrogenated in a Parr hydrogenator with 10% Pd/C (500 mg) at RT for 2.5 h. After MeOH (100 mL) was added, the mixture was sonicated at 50 °C for 5 min and filtered through celite under reduced pressure. The filtrate was concentrated to ca. 30 mL, followed by the addition of 2.5 N HCl (6 mL) and held at reflux for 16 h. The solution was adjusted to pH 10 with K_2CO_3 and then extracted with EtOAc/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered and concentrated in vacuo to give desired product **24** as a pale brown powder (53%, lit. 65%), which darkened rapidly on exposure to air and light.

6-(Dimethylamino)indolin-2-one (25). Sodium cyanoborohydride (4.5 mmol) was added to a solution of **24** (2 mmol) in glacial acetic acid (3 mL) followed by formaldehyde (4.5 mmol), and the mixture was stirred at RT for 24 h. The suspension was concentrated in vacuo and extracted with EtOAc/brine. The organic layer was dried (anhyd Na_2SO_4), filtered, concentrated in vacuo and purified by column chromatography (CH_2Cl_2/EtOH 100:0 to 98:2) to give the desired product **25** as an off-white powder (296 mg, 84%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 10.17$ (s, 1H), 6.97 (d, $J = 8.1$ Hz, 1H), 6.26 (dd, $^1J = 8.1$ Hz, $^2J = 2.1$ Hz, 1H), 6.19 (d, $J = 2.1$ Hz, 1H), 3.31 (s, 2H), 2.85 ppm (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 177.1$, 150.4, 144.5, 124.5, 112.7, 105.2, 94.3, 40.3, 35.0 ppm; MS(APCI+): m/z $[M+H]^+$ calcd for $C_{10}H_{12}N_2O$: 177.09, found: 177.1

***N*-(2-Oxoindolin-6-yl)acetamide (26)**. Concd H_2SO_4 (2 drops) was added to a solution of **24** (2 mmol) in acetic anhydride (4 mmol) and stirred at RT for 2 h, followed by the removal of the solvent in vacuo. The residue was extracted with EtOAc/brine, and the organic fraction was dried (anhyd Na_2SO_4), filtered and concentrated to give the desired product **26** as light brown solid (262 mg, 69%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 10.33$ (s, 1H), 9.88 (s, 1H), 7.35 (s, 1H), 7.08 (d, $J = 7.8$ Hz, 1H), 6.97 (d, $J = 8.1$ Hz, 1H), 3.23 (s, 2H), 2.02 ppm (s, 3H); MS(APCI+): m/z $[M+H]^+$ calcd for $C_{10}H_{10}N_2O_2$: 191.07, found: 191.1.

General method for the preparation of substituted 1-(4-methoxyphenethyl)isatins for series 4 (27–37). The method described for **5** was followed. Briefly, the substituted isatin was reacted with *p*-methoxyphenethyl bromide in the presence of CaH_2 in DMF by holding at reflux (80 °C) for 2–4 h.

General method for the preparation of chloroethylmorpholine (38), chloroethylthiomorpholine (39) and chloroethylpiperidine (40). The basic heterocycle (10 mmol morpholine, thiomorpholine or piperidine, Et_3N (12 mmol) and 2-bromoethanol (12 mmol) were dissolved in toluene (5 mL) and stirred at 80 °C for 4 h. The reaction mixture was concentrated in vacuo, and CH_2Cl_2 (5 mL) was added. The solution was cooled to 0 °C and $SOCl_2$ (30 mmol) was added dropwise. After effervescence had subsided, the mixture was heated at 80 °C for 2 h. The crude mixture was adjusted to pH 10 with K_2CO_3 and extracted using CH_2Cl_2/brine. The product was obtained by column chromatography as an oil which solidified on standing.

1-(2-Morpholinoethyl)indoline-2,3-dione (41). Isatin (1 equiv) and anhyd K_2CO_3 (1.1 equiv) were dissolved in anhyd DMF and stirred at RT for 15 min. **38** (1.1 equiv) was added, and after the reaction was continued for 4 h at 80 °C, the pH was adjusted to 8–9 and the mixture was extracted with CH_2Cl_2/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated and purified by column chromatography (*n*-hexane/EtOAc 3:1) to give the desired product **41** as orange crystals (192 mg, 74%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.63$–7.59 (m, 2H), 7.12 (t, $J = 7.8$ Hz, 1H), 6.92 (d, $J = 8.1$ Hz, 1H), 3.86 (t, $J = 6.6$ Hz, 2H), 3.66 (t, $J = 4.5$ Hz, 4H), 2.64 (t, $J = 6.6$ Hz, 2H), 2.53 ppm (t, $J = 4.5$ Hz, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 183.6$, 158.2, 150.8, 138.4, 124.5, 123.3, 117.4, 111.0, 66.2, 54.7, 53.2, 37.0 ppm; MS(APCI+): m/z $[M+H]^+$ calcd for $C_{14}H_{16}N_2O_3$: 261.12, found: 260.9.

1-(2-Bromoethyl)indoline-2,3-dione (42). Isatin (10 mmol) and anhyd K_2CO_3 (20 mmol) were dissolved in anhyd DMF (12 mL) and stirred at RT for 15 min. 1,2-Dibromoethane (12 mmol) was added to the solution, and stirring was continued at RT for 16 h. The suspension was filtered under reduced pressure and the residue rinsed with DMF to give the desired compound. The filtrate was concentrated in vacuo and H_2O (20 mL) was added. A precipitate was obtained, which was removed by filtration under reduced pressure, washed with H_2O, combined with the earlier obtained material and dried in an oven at 100 °C. **42** was obtained as a red solid (5.44 g, 67%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.64$–7.53 (m, 2H), 7.14 (t, $J = 7.5$ Hz, 1H), 7.00 (d, $J = 8.1$ Hz, 1H), 4.14 (t, $J = 6.6$ Hz, 2H), 3.61 ppm (t, $J = 6.6$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 182.6$, 158.2, 150.4, 138.4, 125.7, 124.0, 117.6, 110.2, 41.9, 27.0 ppm.

(*E*)-1-(2-Bromoethyl)-[3,3′-biindolinylidene]-2,2′-dione (43) and (*E*)-1-(2-bromoethyl)-6′-methoxy-[3,3′-biindolinylidene]-2,2′-dione (44). Compounds **43** and **44** were prepared by reacting **42** (2 mmol) with 2-oxindole (2 mmol) or 6-methoxy-2-oxindole (2 mmol), respectively, in acetic acid at reflux as described for compound **2-1. 43** (254 mg, 35%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.13$ (d, $J = 7.8$ Hz, 1H), 7.67 (s, 1H), 7.38–7.30 (m, 2H), 7.11–7.03 (m, 2H), 6.87 (d, $J = 7.8$ Hz, 1H), 6.81 (d, $J = 7.8$ Hz, 1H), 4.20 (t, $J = 7.2$ Hz, 2H), 3.60 ppm (t, $J = 7.2$ Hz, 2H). **44** (55 mg, 7%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 9.12$ (d, $J = 9.0$ Hz, 1H), 9.07 (d, $J = 8.1$ Hz, 1H), 7.98 (s, 1H), 7.34 (t, $J = 7.8$ Hz, 1H), 7.06 (t, $J = 7.8$ Hz, 1H), 6.86 (d, $J = 7.8$ Hz, 1H), 6.54 (dd, $^1J = 8.7$ Hz, $^2J = 1.8$ Hz, 1H), 6.85 (d, $J = 1.8$ Hz, 1H), 4.20 (t, $J = 7.2$ Hz, 2H), 3.86 (s, 3H), 3.59 ppm (t, $J = 6.9$ Hz, 2H).

3,3-Dibromo-1H-pyrrolo[2,3-b]pyridin-2(3H)-one (45). The synthesis was performed as reported by Marfat et al.[24] Briefly, pyridinium bromide perbromide (PBPB, 4 equiv) was added to a stirred solution of 7-azaindole (10 mmol) dissolved in *tert*-butanol (70 mL) in small portions over 6 h at RT. The solvent was removed in vacuo, and the residue extracted with EtOAc/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered and concentrated to give the crude residue, which was thrn dissolved in CH_2Cl_2, sonicated for 2 min, and filtered under reduced pressure. Recrystallization in toluene gave product **45** as a light brown solid which darkens on standing (2.34 g, 80%, lit. 86%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta =$ 12.0 (brs, 1H), 8.21 (d, $J = 5.1$ Hz, 1H), 8.00 (d, $J = 7.5$ Hz, 1H), 7.18 ppm (dd, $^1J = 7.5$ Hz, $^2J = 5.1$ Hz, 1H).

1H-Pyrrolo[2,3-b]pyridin-2(3H)-one (46). The synthesis was performed as reported by Marfat et al.[24] 10% Pd/C (200 mg) was added to a solution of **45** (1 mmol) in EtOH. Hydrogenation was performed at 50 psi H_2 and RT for 4 h in a Parr hydrogenator. The mixture was then filtered over celite, the solvent was removed in vacuo and the residue was used for the proceeding reaction without further purification (97 mg, 72%, lit. 75%): ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 11.42$ (brs, 1H), 8.06 (d, $J = 5.4$ Hz, 1H), 7.71 (d, $J = 7.2$ Hz, 1H), 7.05 (t, $J = 6.0$ Hz, 1H), 3.60 ppm (s, 2H); ^{13}C NMR

(75 MHz, $CDCl_3$): $\delta = 175.7$, 156.8, 142.7, 133.8, 122.0, 117.6, 35.1 ppm; MS(APCI+): *m/z* $[M+H]^+$ calcd for $C_7H_6N_2O$: 135.05, found: 134.9.

General method for the preparation of 1-alkylated isoindigos 1-1, 1-2, 1-5. The alkylated isatin (1 equiv **2, 3** or **6**) and 2-oxindole (1.1 equiv) were dissolved in acetic acid (1–3 mL) containing concd HCl (2 drops) in a sealed vessel and stirred at RT for 5 min. The mixture was heated to 200 °C, 20–30 min in a microwave reactor and after cooling, concentrated in vacuo and extracted with CH_2Cl_2/brine or EtOAc/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated and purified by column chromatography to give the desired product.

General method for the preparation of 1-alkylated isoindigos (1-3, 1-4, 1-7, 1-8, 1-10). The alkylated isatin (1 equiv **4**, **5**, **8**, **9** or **11**) and 2-oxindole (1.1 equiv) were dissolved in acetic acid (2–5 mL) containing concd HCl (2 drops) and held at reflux for 12–14 h. The mixture was purified as described for compound **1-1**.

General method for the preparation of 1-alkylated isoindigos (1-6, 1-9). Isoindigo (1 equiv) and anhyd K_2CO_3 (1.1 equiv) were dissolved in anhyd DMF (2–4 mL) in a sealed microwave vessel and stirred at RT for 15 min. The appropriate alkyl halide (1.1 equiv) was added and the mixture heated in a microwave reactor to 150 °C for 30 min. Extraction was performed using either CH_2Cl_2/brine or EtAc/brine and the organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated in vacuo and purified by column chromatography to give the desired product.

General method for the preparation of series 2 compounds (2-1 to 2-7 and 2-9 to 2-11). The appropriate substituted 1-methyl isatin (1 equiv **12-18**, **20-22**) and 2-oxindole (1 equiv) were dissolved in acetic acid (2–5 mL) containing concd HCl (2 drops). The mixture was held at reflux for 16 h. If the desired product precipitated from the reaction mixture on cooling, it was removed by filtration under reduced pressure, rinsed with cold acetic acid and recrystallized in EtOH. If no precipitate was observed, the solution was concentrated in vacuo and extracted with CH_2Cl_2/brine or EtOAc/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated and purified by column chromatography to give the desired product.

General method for the preparation series 2 compounds (2-8, 2-12). The appropriate substituted 1-methyl-2-oxindole (1 equiv **19**, **23**) and isatin (1 equiv) were reacted in acetic acid and worked up as described for compound **2-1**.

General method for the preparation of series 2 compounds (2-20, 2-24). The appropriate commercially available R^3-substituted 2-oxindole (1 equiv) and 1-methylisatin (1 equiv) were reacted in acetic acid and worked up as described for compound **2-1**.

General method for the preparation of series 2 compounds (2-13 to 2-19, 2-21 to 2-23). The appropriate commercially available R^3-substituted isatin (1 equiv) and 1-methyl-2-oxindole (1 equiv) were reacted in acetic acid and purified as described for compound **2-1**.

General method for synthesis of series 3 compounds (3-1 to 3-5). The method described for compound **1-1** was used. Briefly, the alkylated isatin (**7**, **11**, **2**, **3** or **4**) was reacted with 6-methoxy-2-oxindole in acetic acid at reflux to give the desired product on workup and purification.

General method for the preparation of series 4 compounds (4-1 to 4-11). The method described for compound **1-3** was applied. Briefly, the appropriate substituted 1-(*p*-methoxyphenethyl)isatin (**27**–**37**) was reacted with 2-oxindole in acetic acid at reflux to give the desired product on workup and purification.

General method for the preparation of series 4 compounds (4-12 to 4-25). Compounds **4-12** to **4-25** were prepared following the method described for compound **2-1**. Briefly, 1-(*p*-methoxyphenethyl)isatin (**4**) was reacted with the appropriate substituted 2-oxindole in acetic acid at reflux.

General method for the preparation of 5-1 and 5-5. Compound **41** (1 equiv) and 2-oxindole (1.1 equiv) were dissolved in acetic acid (2–5 mL) containing concd HCl (2 drops). The mixture was held at reflux at 140 °C for 12–16 h, then concentrated in vacuo, adjusted to pH 10 and extracted with CH_2Cl_2/brine. The organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated in vacuo and purified by column chromatography to give **5-1. 5-5** was prepared in a similar manner by reacting **41** with 6′-methoxy-2-oxindole instead of 2-oxindole.

General method for the preparation of 5-2 and 5-3. Isoindigo (1 equiv) and anhyd K_2CO_3 (1.1 equiv) were dissolved in anhyd DMF (4–8 mL) and stirred at RT for 15 min. **39** or **40** (1.1 equiv) was added to the mixture, which was then heated to 80 °C for 3 h. The mixture was extracted with CH_2Cl_2/brine and adjusted to pH 14. The organic fraction was dried (anhyd Na_2SO_4), filtered, concentrated and purified by column chromatography to give the desired product.

General procedure for synthesis of 5-4 and 5-6. A solution of *N*-methylpiperazine (1 equiv) and Et_3N (2 equiv) was stirred in anhyd DMF (4 mL) at RT for 15 min. **43** or **44** (1 equiv) was added and the mixture was heated to 80 °C for 16 h. The reaction mixture was extracted with CH_2Cl_2/brine at pH 10, and the organic layer dried (anhyd Na_2SO_4), filtered and concentrated in vacuo. The product was purifed by column chromatography.

General method for the preparation of 5-7 and 5-8. 46 (1 equiv) was reacted with 1-methylisatin or 1-(*p*-methoxyphenethyl)isatin (**4**) (1 equiv) as described for compound **2-1**. The mixture was held at reflux in EtOH for 16 h. The precipitate was removed by filtration under reduced pressure and rinsed with ice-cold EtOH to give the desired product.

Biology

Cell lines. Human chronic myelogenous leukemic cells K562, human normal lung fibroblast cells IMR90 and human colon carcinoma cells HCT116 were purchased from American Type Culture Collection (Rockville, MD, USA). Human acute promyelocytic leukemia cells NB4, human liver carcinoma cells HuH7 and human renal carcinoma cells RCC786 were gifts from Dr. Matiullah Khan (National University of Singapore), Dr. Ho Han-Kiat (National University of Singapore) and Dr. John Yuen (Singapore General Hospital) respectively. Iscove's modified Dulbecco's medium (IMDM, catalog no. I7633), high glucose Dulbecco modified eagle's medium (DMEM, catalog no. D1152) and Eagle's minimum essential medium (EMEM, catalog no. M0268) were purchased from Sigma–Aldrich in powder form and reconstituted according to manufacturer's instructions. RPMI-1640 (Hyclone) and phosphate buffered saline (PBS) were purchased from the National University Medical Institutes (NUMI, Laboratory Supplies, National University of Singapore). Fetal bovine serum (FBS) was obtained from Gibco (South America). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Duchefa Biochemie (Haarlem, Netherlands). All other chemicals were purchased from local suppliers and were of molecular biology or cell culture grade.

Propagation of cells. K562 cells were cultured in IMDM supplemented with FBS (10% *v/v*), penicillin G (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). The cells were sub-cultured by a ratio of 1:10 when a cell density of 10^6 cells mL^{-1} was attained and passages 4–12 were used for viability experiments. NB4 and HCT116 cells were grown in RPMI-1640 supplemented with FBS (10% *v/v*), penicillin G (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). NB4 cells were sub-cultured at a split ratio of 1:8 when the cell density reached 10^6 cells mL^{-1}. Only cells of passages 8–14 were used for experiments. HCT116 cells were subcultured (ratio of 1:5) when 90% confluent and used within passages 9–18 for experiments. HuH7 cells were propagated in high glucose DMEM supplemented with FBS (10% *v/v*), sodium pyruvate (1 mM), penicillin G (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). The cells were subcultured (ratio of 1:4) when 90% confluent and used within passages 8–16 for experiments. RCC786 cells were cultured in high glucose DMEM supplemented with FBS (10% *v/v*), penicillin G (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). The cells were subcultured (ratio of 1:4) when 90% confluent and used within passages 9–16 for experiments. IMR90 cells were grown in EMEM supplemented with FBS (10% *v/v*), penicillin G (100 mg L^{-1}) and streptomycin (100 mg L^{-1}). The cells were subcultured (ratio of 1:3) when 90% confluent and used within passages 6–13 for viability experiments.

MTT assay for determination of cell viability. The MTT assay for K562 cells was described earlier.[17] The same method was applied to NB4 cells except that cells were seeded at a lower density of 4000 cells per well in 96-well plates. For the non-adherent cells (HuH7, HCT116, RCC786, IMR90), the MTT assay was modified as follows: The cells in their respective media were seeded at densities of 6000 (HuH7), 2500 (HCT116), 10000 (RCC-786) and 4000 (IMR90) cells per well. Cells adhered to the base of the wells after a 16–18 h incubation period at 37 °C and 5% CO_2. The media was removed by aspiration and replaced by fresh media containing the test compound at varying concentrations. The final concentration of DMSO vehicle was kept at 0.4% *v/v* per well for all cell lines. After the cells were incubated for 72 h at 37 °C and 5% CO_2, the media was removed by aspiration, and the cells were gently rinsed with PBS to remove residual compound. MTT (100 μL, 0.5 mg mL^{-1} in PBS) was added to each well and incubated for 4 h at 37 °C. The MTT solution was removed gently by aspiration and the formazan crystals dissolved in DMSO (100 μL). Absorbances were measured within 30 min at 595 nm on a microplate reader as described earlier. Cell viability at a given concentration is determined from Equation (1), where $A_{\text{test compound}}$ = absorbance of wells with cells exposed to test compound in media; A_{vehicle} = absorbance of wells with cells in media; and A_{blank} = absorbance of wells with DMSO only.

$$\textit{Viability}\ [\%] = \left(\frac{A_{\text{test compound}} - A_{\text{blank}}}{A_{\text{vehicle}} - A_{\text{blank}}}\right) \times 100 \qquad (1)$$

Each concentration was tested at least three times on separate occasions, using two different stock solutions, and on cells of different passage numbers. The IC_{50} value was determined from the sigmoidal curve obtained by plotting the percentage viability versus logarithmic concentration of the test compound using GraphPad Prism 5.0 (San Diego, CA, USA).

Determination of aqueous solubility: Determination of aqueous solubility was carried out on Multiscreen® Solubility filter plates (MSSLBPC10, Millipore Billerca, MA, USA). The manufacturer's protocol was followed. Briefly, various concentrations of the test compound were prepared in universal buffer (pH 7.4)/acetonitrile/DMSO. The UV absorbances of these solutions were obtained at a pre-determined wavelength and used to construct a calibration curve for the test compound. Next, a stock solution of the test compound in DMSO was prepared at a known concentration, diluted with universal buffer (pH 7.4), dispensed into wells in the Multiscreen® Solubility filter plate, and agitated for a period of time. The suspension was filtered, the filtrate collected and diluted with acetonitrile to give the same solvent composition used to prepare the calibration solutions. The absorbance of the diluted filtrate was read at the predetermined wavelength and the concentration of the filtrate (equivalent to the solubility of the test compound) was determined from the calibration curve.

Assessment of aggregation tendency by dynamic light scattering. Stock solutions (10 mM) of test compounds were prepared in DMSO and serially diluted with potassium phosphate buffer (5 mM, pH 7.4, pre-filtered before use) to give final concentrations of 0.5, 1, 5 and 10 μM. The concentration of DMSO was maintained at 1% *v/v* in the final solutions. Measurements were carried out using a Zetasizer Nano ZS system (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He-Ne laser at 633 nm. The detector angle was 90°. Three or more independent measurements of derived count rates (in kilocounts per second, kcps) were made for each concentration of test compound, using at least two separately prepared solutions. Results are represented as mean values ± standard deviation (SD). Data collection and quality assessment were carried out using the software supplied with the instrument.

Cell cycle analysis. K562 cells were seeded at a density of 2.0×10^5 cells mL^{-1} in each well of a 6-well plate and treated with a known concentration of test compound for 24 h at 37 °C and 5% CO_2. The cells were then harvested, centrifuged at 200 *g* for 10 min and rinsed once with chilled PBS. 10^6 cells were suspended in 300 μL of PBS and ice cold abs EtOH (700 uL) was added dropwise to the cell suspension with intermittent vortexing between additions. The cell sample was kept at 4 °C overnight and subsequently stored at −20 °C prior to analysis. For analysis, the sample was centrifuged at 200 *g* for 10 min to remove the fixing solution and the cell pellet was rinsed with chilled PBS and resuspended in staining solution (1 mL), which contained 0.2 mg mL^{-1} RNase A (MP Biomedicals, Illkirch Cedex, France) and 20 μg mL^{-1} propidium iodide (Sigma–Aldrich, catalog no. P4170) in PBS. The cell suspension was then kept in the dark at RT for 15 min, after which it was immediately analyzed for cell cycle distribution on a Cytomation Cyan LX instrument (Dako, Fort Collins, CO, USA) equipped with an Ar solid state laser (488 nm) using Summit (Version 4.3) software. 20000 cells were read for each determination. Each test compound was evaluated at four or five concentrations with at least three repetitions per concentration.

Apoptosis. The Annexin V-FITC Apoptosis Detection Kit (Sigma–Aldrich, catalog no. APOAF) was used. K562 cells were seeded at a density of 2.0×10^5 cells mL^{-1} in each well of a 6-well plate and treated with a known concentration of test compound for 24 h (or 48 h) at 37 °C and 5% CO_2. Cells were harvested, centrifuged (200 *g*, 10 min), the cell pellet rinsed with chilled PBS and resuspended in 1× binding buffer at a density of 10^6 cells mL^{-1}. Annexin V-FITC conjugate protein (5 μL) and propidium iodide solution (10 μL) were added to an aliquot (500 μL) of cell suspension and incubated at RT for 10 min in the dark, after which flow cytometric analysis was immediately carried out on the Cytomation Cyan LX instrument (Dako, Fort Collins, CO, USA) using the Summit (Version 4.8) software. Unstained treated cells, treated cells stained with annexin V-FITC only and treated cells stained with propidium iodide only were used for calibration and compensation. 20000 cells were read for each sample determination. Each test compound was eval-

uated at five concentrations with at least three repetitions for those concentrations that were associated with apoptosis.

In vivo studies. Experimental protocols for the in vivo study were approved by the National University of Singapore (NUS) Institutional Animal Care and Use Committee (IACUC, 112/10) and were in compliance with guidelines for the care and use of animals for scientific purposes issued by the National Advisory Committee for Laboratory Animal Research, Republic of Singapore. Balb/c female nude athymic nude mice (16–20 g body weight, 5–8 weeks old) were obtained from the Biological Resource Center (Singapore). Animals were kept under controlled environmental conditions (19–26 °C, relative humidity <70%, 12 h dark-light cycle, Center for Life Science vivarium, NUS) and given free access to H_2O and standard feed. They were randomly assigned to one of four groups, namely control animals to be treated with vehicle (1% DMSO *v/v* saline), and treated animals earmarked to receive meisoindigo, **3**-**5** or **5**-**4**. Each group comprised 5–8 mice. To establish the xenograft, K562 cells (2.0×10^7 in 150 μL in saline) were injected subcutaneously in the right dorsal flank of the animal previously sedated with gaseous isoflurane (5% *v/v* in O_2 for induction of anaesthesia and 2–3% *v/v* in O_2 for maintenance). Tumor formation at the site was monitored carefully and treatment was initiated when a palpable tumor of approximately 150 mm^3 was evident (day 0). The test compound (10 μM in 100 μL saline containing 1% *v/v* DMSO) was injected into the tumor on three separate occasions—day 0, day 3 and day 10. Animals in the control group were similarly injected with saline containing 1% *v/v* DMSO on days 0, 3 and 10. Solutions of test compounds were freshly prepared prior to injection and sterilized by filtration using a 0.22 μm DMSO-safe Acrodisc® syringe filter (Pall Life Sciences, Ann Arbor, USA). The animals were monitored daily for changes in body weight, tumor volume and signs of distress. Tumor volume was calculated using the formula for the volume of an ellipsoid [Equation (2)].

$$V = \frac{(\pi \times L \times W^2)}{6} \quad (2)$$

Length (*L*) and width (*W*) of the tumor were measured with electronic vernier calipers and reported to two decimal places. The mice were monitored for 28 d after the first injection of test compound or vehicle (day 0), after which they were euthanized with CO_2. Animals were also euthanized (before day 28) if they complied with the following criteria: (i) tumor diameter >1.5 cm, (ii) tumor ulceration, infection or inflammation, (iii) ruffled fur, hunched back appearance or inappetent state, (iv) 10% or more body weight loss over 24 h or 20% body weight loss relative to control group, or (v) moribund or pre-moribund state. For the purpose of analyzing the results using the Kaplan–Meier survival analysis, censored events were recorded. These are events in which the period of survival of the animal could not be determined due to the following: (i) animal remained alive after the study period (28 d), (ii) death of animal due to unrelated causes such as sudden death or infection and (iii) conditions which warrants euthanasia due to unrelated disease pathology or infection (e.g., pin-worm infestation in animal).

Statistical Analysis. Data was analyzed for statistical significance by one-way ANOVA followed by Dunnett's post-hoc test on SPSS 18.0 for Windows (SPPS Inc., Chicago, IL, USA). $p < 0.05$ was taken as the criterion for significance. Survival data were analyzed by Kaplan–Meier (with log-rank test, pairwise comparison) on IBM SPSS Statistics 19.0 (SPPS Inc., Chicago, IL, USA) following the protocol described by Chan and co-workers.[37] Tumor size data were analyzed by One-way ANOVA with Dunnet post-hoc test on the same software. *p*-values <0.05 were considered significant for both tests. Graphical representations were plotted with GraphPad Prism 5.0 (San Diego, CA, USA).

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