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Synthesis and evaluation of functionalized isoindigos as antiproliferative agents

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ABSTRACT

A series of functionalized isoindigos structurally related to meisoindigo (1-methylisoindigo), a therapeutic agent used for the treatment of a form of leukemia, were synthesized and evaluated for antiproliferative activities on a panel of human cancer cells. Two promising compounds (1-phenpropylisoindigo and 1-(*p*-methoxy-phenethyl)-isoindigo) that were more potent than meisoindigo and comparable to 6-bromoindirubin-3′-oxime on leukemic K562 and liver HuH7 cells were identified. Structure–activity relationships showed the importance of keeping one of the lactam NH in an unsubstituted state. Substitution of the other lactam NH with aryl or arylalkyl side chains retained or improved activity in most instances. An intact exocyclic double bond was also essential, possibly to maintain planarity and rigidity of the isoindigo scaffold. None of the compounds were found to inhibit CDK2 in an in vitro assay, in spite of reports linking the antiproliferative activities of meisoindigo and other isoindigos to CDK2 inhibition. Hence, these functionalized isoindigos disrupted cell growth and proliferation by other mechanistic pathways that did not involve CDK2 inhibition.

1. Introduction

The indigoids are a group of isomeric bisindoles that have recently emerged as a promising scaffold for anticancer activity. Interest in the indigoids was kindled when indirubin was identified as the active ingredient of a traditional Chinese recipe (Danggui Longhui Wan) that was used for the treatment of chronic myelogenous leukemia (CML).[1,2] The anti-tumor activity of indirubin and some of its analogs (notably indirubin-3′-oximes) were subsequently attributed to the inhibition of cyclin-dependant kinases (CDK).[3] Co-crystallized structures of CDK2 and various indirubin analogs showed that these compounds interacted with the ATP binding site via hydrogen bonds to key amino acids in the hinge region.[3,4] Unfortunately, indirubin was not considered a good candidate for drug development because its poor water solubility would adversely affect oral bioavailability. Moreover, there were reports of gastrointestinal side effects associated with its use.[2] Hence, more drug-like substitutes of indirubin were sought and some promising candidates identified were the functionalized indirubin-3′-oximes,[5–7] meisoindigo,[2,8–12] and Natura™ (1-β-D-triacetylxylopyranosylisoindigo)[13] (Fig. 1). Meisoindigo and Natura™ are derivatives of isoindigo (3,3′-bisindole) which is a regioisomer of indirubin (3,2′-bisindole). Unlike the indirubin scaffold which is widely investigated as an anti-cancer lead, isoindigos have attracted less attention.

Meisoindigo (1-methylisoindigo) is used as an indirubin substitute for the treatment of CML in China.[12] It has a multi-targeting profile that included inhibition of DNA biosynthesis and microtubule assembly in tumor cells,[10] induction of leukemic cell differentiation and c-myb gene downregulation,[11] and inhibition of angiogenesis.[12] Natura™ is an example of an isoindigo that is derivatized with a sugar moiety. Its anti-cancer agent activity was attributed to inhibition of several cyclin dependent kinases (CDK 2, 4, and 6).[13] The presence of the protected sugar moiety was pro-

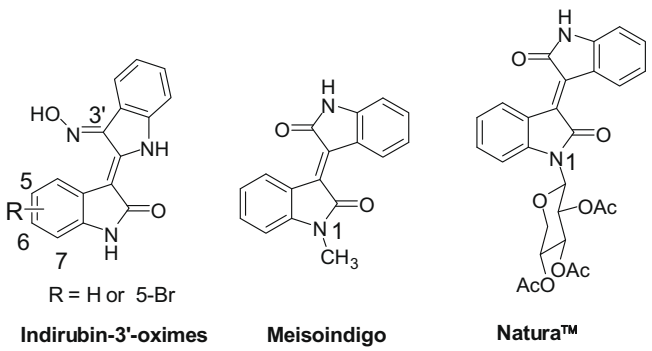

Figure 1. Structures of indirubin 3′-oximes, meisoindigo and Natura™.

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posed to enhance the interaction with the ATP binding site of the kinase, since isoindigo was essentially inactive as a CDK inhibitor.[14] When several 1-glycosylisoindigos were evaluated as CDK inhibitors, it was found that only compounds with *O*-benzyl groups on the sugar hydroxyl (OH) residues inhibited CDK while most entities with *O*-acetyl or unprotected OH groups on the sugar moiety were inactive.[14] Notwithstanding their CDK inhibitory activities, the *O*-benzyl 1-glycosylisoindigos had only weak antiproliferative activities,[15] possibly due to their poor aqueous solubilities which would affect cellular penetration.[14] As these compounds had molecular weights that exceeded 500 Da and lipophilicities (evaluated by *C* log *P*) that were greater than 5, their poor permeabilities are anticipated from property profiling rules like the Lipinski's rule of five.[16] The same would be true for Natura™ which was also poorly soluble in water.[13] Thus, designing pharmacologically active isoindigo derivatives with a desirable solubility–lipophilicity balance that would translate to an acceptable level of cellular permeability poses a formidable challenge. In this report, we describe our efforts to address this problem by synthesizing a series of isoindigos that have functionalized arylalkyl or alkyl substituents on the ring nitrogen. To maintain a pharmaceutically friendly profile, efforts were made to restrict the size and lipophilicity of the synthesized compounds. The compounds were evaluated for effects on the growth of several human cancer cell lines. As CDK2 inhibition has been widely associated with the growth inhibitory properties of isoindigos,[13–15] we carried out in silico docking to assess their potential as CDK2 inhibitors and followed this up with experimental determination of CDK2 inhibitory activity.

2. Results

2.1. Design of functionalized isoindigos

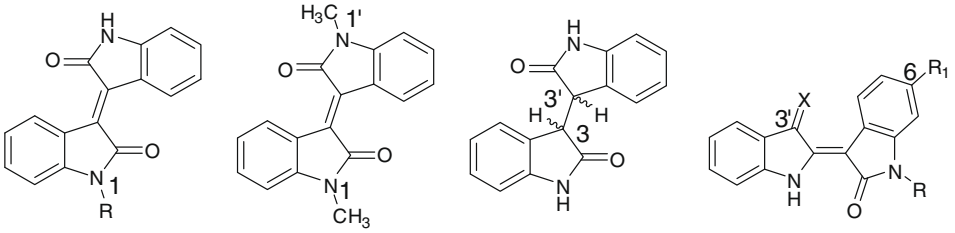
The structures of the functionalized isoindigos investigated in this study are given in Table 1. As the compounds were designed to be structural analogs of meisoindigo, modifications were restricted to the N-substituent. These were the replacement of *N*-methyl in meisoindigo (**2**) with arylalkyl substituents of varying alkyl chain lengths (**3–6**) and introducing different substituents to the phenyl ring of the phenethyl side chain (**7–11**). Analogs of meisoindigo with an additional *N*-methyl group (**12**) and a reduced exocyclic double bond (**13**) were also targeted for synthesis. In addition, indirubins (**14**, **15**) were included for comparison to corresponding isoindigos, as well as indirubin 3′-oximes (**16**, **17**) which are known CDK inhibitors.[3] In keeping with commonly employed property profiling rules,[16,17] these compounds had molecular weights (⩽500), *C* log *P* values (⩽5), total number of H bond donors and acceptors (⩽12) and rotatable bonds (⩽10) that were within threshold values.

2.2. Synthesis of test compounds

The isoindigos **1–5**, **7**, **8** were synthesized by an acid-catalyzed aldol condensation of an isatin (or N-substituted isatin) with an oxindole under microwave-assisted conditions (200 °C, 4 bar). The microwave-assisted method gave yields that were comparable to those obtained by refluxing[20,21] but had shorter reaction times. Except for 1-phenylisatin which was purchased, the other 1-substituted isatins were synthesized by reacting isatin with the appropriate alkyl halide in the presence of potassium carbonate in anhydrous DMF with microwave heating[22] (Scheme 1). An alternative approach was to react isoindigo with the alkyl halide under similar conditions to give the 1-substituted isoindigo. Isoindigos **6**, **9–11** were prepared in this way (Scheme 2). In the case of **9**, the phenolic hydroxyl group in 4-(2-bromoethyl)phenol was protected with dihydropyran, reacted with isoindigo and subsequently deprotected. Compound **11** was obtained by first reducing 2-bromoethyl-4-nitrobenzene to give the aniline, followed by acetylation of the amino function to give the corresponding amide and then reaction with isoindigo.

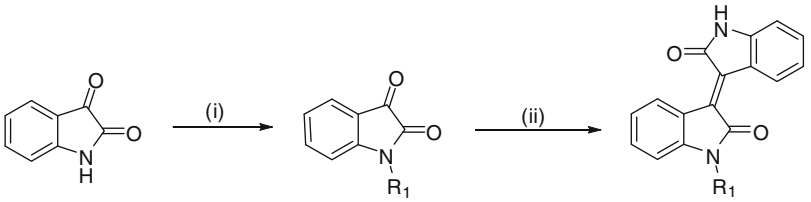
1,1′-Dimethylisoindigo (**12**) was prepared by reacting isoindigo with methyl iodide (ratio of 1:2). 3,3′-Dihydroisoindigo (**13**) was obtained as a mixture of isomers by catalytic reduction of isoindigo. The indirubins (**14**, **15**) were prepared by reacting isatin or 1-methylisatin with 3-acetoxyindole.[4] The indirubin oximes (**16**, **17**) were purchased. Compounds used for biological evaluation

Table 1
Structures of synthesized compounds and their *C* log *P* values

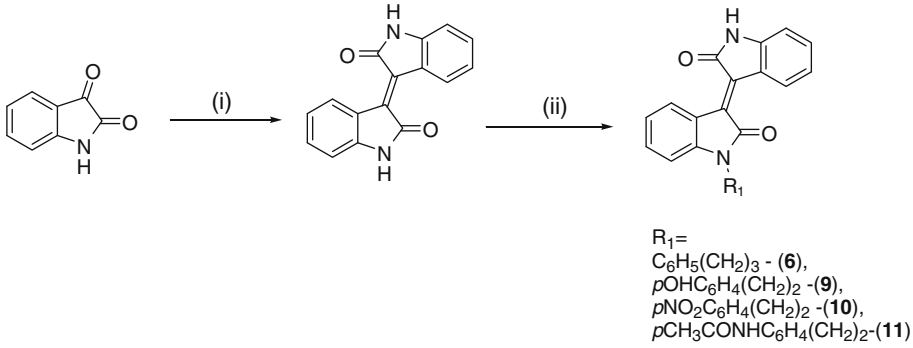


Compd	R	*C* log *P*[a]	Compd	R	X	R_1	*C* log *P*[a]
1	H	2.0	**12**	—	—	—	2.7
2	$-CH_3$	2.7	**13**	—	—	—	3.4
3	$-C_6H_5$	2.9	**14**	H	H	H	2.6
4	$-CH_2\ C_6H_5$	4.0	**15**	$-CH_3$	H	H	3.2
5	$-(CH_2)_2\ C_6H_5$	4.8	**16**	H	NOH	H	2.4
6	$-(CH_2)_3\ C_6H_5$	5.2	**17**	H	NOH	Br	3.5
7	$-(CH_2)_2\ C_6H_4$ (p-CH_3)	5.3					
8	$-(CH_2)_2\ C_6H_4$ (p-OCH_3)	4.7					
9	$-(CH_2)_2\ C_6H_4$ (p-OH)	3.2					
10	$-(CH_2)_2\ C_6H_4$ (p-NO_2)	4.6					
11	$-(CH_2)_2\ C_6H_4$ (p-$NHCOCH_3$)	3.8					

[a] *C* log *P* values were determined on ChemDraw Ultra 10.0, CambridgeSoft, Cambridge, MA.



Scheme 1. Reagents and conditions: (i) alkyl halide, K_2CO_3, anhydrous DMF, MW, 150 °C, 5–15 min; (ii) 2-oxindole, CH_3COOH, HCl, MW, 200 °C, 15–30 min.



Scheme 2. Reagents and conditions: (i) 2-oxindole, CH_3COOH, HCl, MW, 200 °C, 15–30 min; (ii) alkyl bromide, K_2CO_3, anhydrous DMF, MW, 150 °C, 5–15 min.

were at least 95% pure as determined by combustion analyses or reverse phase HPLC (Supplementary data).

2.3. Antiproliferative activity of test compounds

The antiproliferative activities of the test compounds were determined by the microculture tetrazolium assay using MTT as the indicator dye.[23,24] The compounds were tested on several human cancer lines (leukemic cell lines K562 and HL60, colon HCT116, hepatoma HuH7) and normal lung fibroblasts (IMR-90). The normal cell line was included to indicate the selective activity of the compounds. The antiproliferative IC_{50} values of **1–17** are listed in Table 2.

Although the compounds were tested on different cancer cell lines, several common features were observed in their antiproliferative activities. Notably, the test compounds were more active against one leukemic cell line (K562: IC_{50} 1.4–8.5 μM) than the other (HL60: IC_{50} 3.8–16.6 μM). The hepatoma cells HuH7 were

Table 2
Anti-proliferative IC_{50} values of target compounds on human cancer (K562, HL60, HCT116, HuH7) and normal (IMR-90) cells

Compd	MTT Assay IC_{50}[a] (μM)				
	K562	*HL60*	*HCT116*	*HuH7*	*IMR-90*
1	6.7 (*5.6–7.4*)	16.6 (*13.5–20.5*)	>30	28.9 (*26.5–31.6*)	16.3 (*14.0–19.1*)
2	6.7 (*5.7–7.8*)	12.2 (*11.3–13.1*)	15.4 (*11.5–20.6*)	18.3 (*16.6–20.1*)	7.7 (*6.8–8.6*)
3	8.5 (*8.2–8.9*)	10.4 (*8.1–13.2*)	15.4 (*13.8–17.2*)	13.2 (*12.5–14.1*)	6.0 (*5.6–6.6*)
4	>30[b]	>30[b]	10.9 (*9.0–13.1*)	9.0 (*8.4–9.7*)	4.9 (*4.4–5.5*)
5	7.3 (*6.7–7.9*)	11.9 (*10.8–13.0*)	12.2 (*11.5–13.0*)	12.0 (*10.3–13.9*)	6.8 (*6.1–7.6*)
6	1.8 (1.5–2.2)	3.8 (*3.5–4.2*)	7.7 (*6.3–9.4*)	8.2 (*7.5–9.0*)	3.9 (*3.1–4.8*)
7	>30	>30	>30	19.0 (*16.1–22.3*)	>30[b]
8	1.4 (1.1–1.7)	12.0 (*11.4–12.7*)	11.6 (*10.8–12.4*)	7.5 (*6.8–8.4*)	5.4 (*4.9–6.0*)
9	>30	>30	>30	19.9 (*16.5–24.0*)	>30[b]
10	>30	>30	>30	13.3 (*11.8–15.0*)	7.5 (*4.5–12.3*)
11	>30	>30	>30	13.3 (*11.5–15.3*)	19.5 (*18.0–21.1*)
12	>20[b]	>20[b]	>20[b]	>20[b]	>20[b]
13	>30	>30	>30	>30	>30
14	>30[b]	>30[b]	>30[b]	>30[b]	>30[b]
15	>20[b]	>20[b]	>20[b]	>20[b]	>20[b]
16	>30	15.7 (*11.2–21.6*)	12.0 (*10.2–14.2*)	—	18.2 (*16.8–19.7*)
17	1.3 (*1.2–1.5*)	5.4 (*4.8–5.9*)	5.2 (*4.9–5.7*)	6.2 (*5.8–6.7*)	1.9 (*1.6–2.2*)

[a] Concentration required to reduce viability by 50% compared to control untreated cells after incubation. Results of at least 4 independent determinations. Ninety five percent confidence limits are given in brackets.

[b] Could not be determined due to poor solubility of compound in vehicle. Compounds had less than 50% decrease in cell viability at the highest concentration tested. Precipitation of the compound was observed beyond this concentration.

also more susceptible to the isoindigos than the colon HCT116 cells as seen from the larger number of compounds with determinable IC_{50} values against HuH7 (**1–11**) than HCT116 (**2–6**, **8**). In spite of these differences, we noted that some compounds like **6** and **8** were consistently more potent than others across different cancer cell lines while poor activity was always found for certain members like **10–15**.

Some general structure–activity trends could be deduced from the antiproliferative activities. First, meisoindigo (**2**) was almost as potent as isoindigo (**1**) on leukemic cells but it was significantly more active than isoindigo on the solid tumor cell lines ($IC_{50\ HCT116}$ 15.4 μM, $IC_{50\ HuH7}$ 18.3 μM as compared to >30 μM and 28.9 μM for **1**). Meisoindigo and isoindigo were also more potent than their indirubin counterparts **14** and **15**, particularly against leukemic cells. Second, introducing another *N*-methyl group to meisoindigo (to give **12**) or reducing the exocyclic double bond (to give **13**) significantly reduced anti-proliferative activity. Clearly, activity depended on the presence of at least one lactam NH and an intact exocyclic double bond on the isoindigo scaffold. Third, replacing the methyl group of meisoindigo with phenyl (**3**), benzyl (**4**), phenethyl (**5**) or phenpropyl (**6**) side chain generally caused small increases in activity except for **4** (inactive against leukemic cell lines) and **6** (2–3 times more potent than meisoindigo). The antiproliferative activity of **6** was almost comparable to that of the indirubin 3′-oxime **17** whose outstanding activity had been reported by others.[7] Lengthening the side chain from methyl (**2**) to phenpropyl (**6**) caused a progressive increase in *C* log *P* (Table 1) and it may be that **6** owed its improved activity to its greater lipophilic character. Lastly, introducing substituents of different polarities and electronic effects to the phenyl ring of the phenethyl analog **5** did not yield compounds with improved activities. A notable exception was the *p*-methoxy analog **8** which was more active than **5** on the leukemic K562 cells ($IC_{50\ K562}$ 1.4 μM compared to 7.3 μM for **5**) and hepatoma HuH7 cells ($IC_{50\ K562}$ 7.5 μM compared to 12.0 μM for **5**). The good activity of **8** did not appear to be linked to its lipophilic character (*C* log *P* of **5** and **8** were comparable), its electron donating effect (σ_p of OCH_3 was between that of CH_3 and OH present in the less active analogs **7** and **9**, respectively)[25] or H bonding capability (NO_2 and OCH_3 were H bond acceptors and not H bond donors, but the NO_2 analog **9** was inactive).

The test compounds also affected the proliferation of the noncancerous fibroblasts IMR-90, often with lower IC_{50} values than those obtained against the cancer cell lines. Notably, the more promising isoindigos **6** and **8** were modestly selective against K562 cells by a 2–4-fold difference as seen from the ratios of their IC_{50} values ($IC_{50\ IMR\text{-}90}/IC_{50\ K562}$). These levels of selectivities compared favorably to those obtained for meisoindigo (**2**) and the indirubin 3′-oxime **17** ($IC_{50\ IMR\text{-}90}/IC_{50\ K562} < 2$).

2.4. Molecular docking

Meisoindigo and Natura™ were reported to suppress cyclin D mediated CDK4/6 activities of prostate cancer cells (LNCaP) at concentrations of 5 and 15 μM.[13] Almost complete inhibition was reported at 15 μM while at 5 μM, Natura™ was a slightly stronger inhibitor than meisoindigo (60% vs 40% inhibition).[13] Several *O*-benzyl 1-glycosylisoindigos inhibited CDK2 at submicromolar IC_{50} values and molecular modeling of one of the potent analogs revealed two H bonds with the critical hinge region amino acids (Glu 81, Leu 83) and hydrophobic interactions between the benzyl groups on the sugar moiety and a hydrophobic pocket in the CDK2 active site.[14] These findings prompted us to investigate the binding potential of our compounds (Table 1) to the CDK2 active site.

Docking was conducted using a co-crystallized complex of CDK2, cyclin A and adenosine-5′-triphosphate (ATP) (PDB code: 1FIN).[18] The interaction was scored by evaluating the probability of H bonding between the docked compound and selected residues on the apo-protein using the Protein Ligand Interaction Fingerprint (PLIF) module in Molecular Operating Environment (MOE, Version 2008.10). The residues in the hinge region (Glu 81 and Leu 83) of CDK2 were selected as these were widely reported to be involved in H bonding (Glu 81 as H bond donor, Leu 83 as both H bond donor and acceptor) with CDK2 inhibitors.[19] Three scores, one for each H bond interaction, were calculated for every compound, with each score providing a measure of the probability of H bonding at the stated residue. Summation of these scores gave the 'HingeSum' which reflected the total probability of H bonding at the 3 sites. As each compound docked with different poses on the apo-protein, only the docked pose with the highest HingeSum was evaluated (Table 3).

The indirubin-3′-oximes (**16**, **17**) had higher docking scores than most compounds, indicating strong H bonding with the active site residues which was compatible with their potent CDK2 inhibitory activities.[3,4] Interestingly, several isoindigos (**3**, **5**, **8**) had scores that were in the range of the 3′-oximes (**16**, **17**) and this raised the possibility that CDK inhibition could have contributed to their effects on cell growth and proliferation. Hence we investigated the CDK2 inhibitory activities of these compounds.

2.5. Inhibition of CDK2

The CDK2 inhibitory activities of **1–17** were evaluated by the immobilized metal Ion affinity-based fluorescence polarization (IMAP) assay.[26] Briefly, the assay measures the change in fluorescence polarization of a peptide substrate of CDK2 on phosphorylation. The phosphorylated substrate binds to the IMAP binding reagent and experiences a loss in molecular motion that translates to an increase in florescence polarization. In the presence of a CDK2 inhibitor, phosphorylation of the substrate was interrupted leading to a decrease in fluorescence polarization. The compounds were evaluated at a fixed concentration of 10 μM.

As seen from Figure 2, only the indirubin 3′-oximes **16** and **17** significantly inhibited CDK2 (70–80% inhibition), in keeping with their reported CDK2 inhibitory activities.[3] None of the isoindigos, including meisoindigo (**2**) and those with good docking scores (**3**, **5**, **8**) inhibited CDK2 to any significant extent. To confirm this finding, meisoindigo (**2**) and the phenpropyl analog **6** were screened for inhibitory activities against CDK2 and other kinases by a com-

Table 3
Docking scores of test compounds

Compound	HingeSum[a]	GLU81 as H donor	LEU83 as H donor	LEU83 as H acceptor
Natura™	0.35	0.13	0.00	0.21
1	0.34	0.14	0.00	0.20
2	0.33	0.20	0.00	0.13
3	0.60	0.00	0.22	0.38
4	0.47	0.00	0.21	0.27
5	0.63	0.00	0.34	0.28
6	0.38	0.00	0.24	0.15
7	0.44	0.00	0.26	0.19
8	0.64	0.00	0.38	0.26
9	0.45	0.00	0.21	0.24
10	0.47	0.00	0.19	0.29
11	0.46	0.34	0.00	0.12
12	0.22	0.00	0.00	0.22
14	0.79	0.33	0.04	0.42
15	0.55	0.00	0.06	0.49
16	0.63	0.16	0.05	0.43
17	0.72	0.00	0.34	0.38

[a] HingeSum is summation of hydrogen bonding probability scores with Glu81 as H donor, Leu83 as H bond donor, and Leu83 as H bond acceptor. The latter were calculated with the PLIF module in MOE.

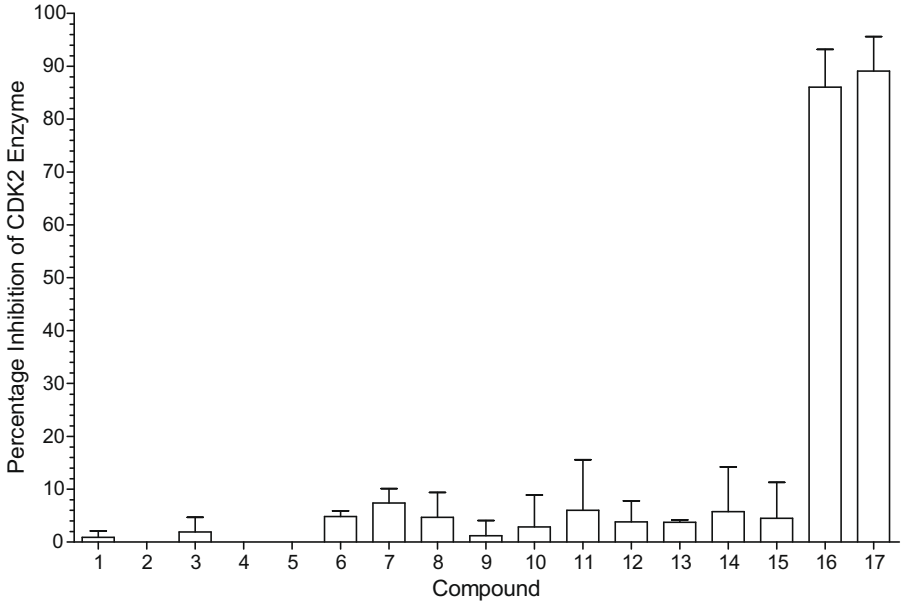


Figure 2. Percentage inhibition of CDK2 by test compounds (10 μM) in the IMAP assay. Vertical bars represent SD for $n = 3$ determinations.

mercial service provider (KINOMEScan™-ScanEdge™, Ambit Biosciences, San Diego, CA). The compounds were tested at 10 μM on a binding assay that quantified the ability of the compound to compete with an immobilized active site directed ligand.[27] The results confirmed that meisoindigo and **6** did not inhibit CDK2 at 10 μM. In fact, the **2** compounds failed to inhibit any kinase by more than 70% at this concentration. The kinase that was most susceptible to meisoindigo was CDK7 (56% inhibition). More kinases were inhibited by **6** (at 40–52% inhibition) and of these, 2/3rd were tyrosine kinases and none were serine–threonine kinases. The results of the screen for meisoindigo and **6** are given in Supplementary data.

3. Discussion

There are limited reports on the anti-cancer activity of isoindigo derivatives and those available to date centred on meisoindigo[2,8–12] and isoindigos that were derivatized with sugar residues like Natura™[13] and various 1-glycosylated isoindigos.[14,15] A major concern with these isoindigo derivatives was that they may not be sufficiently drug-like for clinical advancement. In this report, we have synthesized a series of functionalized isoindigos that had more desirable drug-like/lead-like features as defined by commonly used structure profiling guidelines. We found that fairly simple modifications of meisoindigo resulted in compounds that had greater antiproliferative activities against a panel of human cancer cell lines. Among the more promising analogs was 1-phenpropylisoindigo (**6**) which was more potent than meisoindigo and comparable to 6-bromoindirubin-3′-oxime (**17**) on the cancer cell lines, notably the leukemic K562 and hepatoma HuH7 cells. Compound **6** was also selectively more potent against leukemic K562 cells than normal fibroblast cells, unlike meisoindigo and the indirubin-3′-oxime (**17**). Another promising candidate was **8** which had a 1-(*p*-methoxy-phenylethyl) side chain. Like **6,** this compound was modestly selective for the leukemic K562 cells compared to normal fibroblasts.

In spite of the small number of compounds investigated, some useful structure–activity correlations were evident. The inactivity of 1,1′-dimethylisoindigo (**12**) highlighted the importance of keeping at least one free lactam NH. Another compound **13** may owe its lack of activity to the lost of planarity and increased flexibility arising from the reduction of the exocyclic double bond. Replacing 1-methyl of meisoindigo with longer and bulkier side chains as those found in **3**, **5**, and **6** generally maintained activities. Of the various substituents that were introduced at the *p*-position of the 1-phenethyl side chain, only the *p*-methoxy group (**8**) gave rise to good activity. Both **6** and **8** were more lipophilic than meisoindigo as assessed by their *C* log *P* values but more isoindigos with varied lipophilicities must be evaluated before the role of lipophilicity in determining antiproliferative activity is established.

In spite of reports linking meisoindigo with the inhibition of CDK2,[13] none of the isoindigos inhibited CDK2 on the IMAP assay. However, as anticipated, the indirubin 3′-oximes (**16**, **17**) had significant CDK2 inhibitory activities on the same assay. It is notable that meisoindigo had negligible effects on all the kinases in the KINOMEScan™ except CDK7, which it inhibited by 56% at 10 μM. Interestingly, CDK7 is known to regulate the activity of several CDKs (including CDK2) through phosphorylation of a threonine residue in the activation segment.[29] However, CDK2 was reported to be capable of autophosphorylation at Thr60 of its activation segment[30] which may mean that if CDK7 was inhibited, CDK2 would still retain its activity. Unlike meisoindigo, the phenpropyl analog **6** did not inhibit CDK7 but it affected a larger number (9) of kinases, most of which were tyrosine kinases. The antiproliferative activity of meisoindigo may arise from its multiple effects on cellular processes[10,11,13] and this may also be true for the functionalized isoindigos.

The lack of agreement between the in silico docking experiments and the experimentally determined CDK2 inhibitory activities raised the question as why this discrepancy was observed. A likely explanation may be that molecular docking was carried out with a CDK2 protein (1FIN) that was co-crystallized with ATP instead of an isoindigo derivative. A recent study showed that when compounds were docked onto protein structures derived from complexes co-crystallized with a different ligand ('non-native docking'), the results would be less reliable.[28] The problem would be further compounded if a rigid docking procedure was used, as in the present case. Thus, unless a flexible docking protocol was applied to a co-crystallized isoindigo-CDK2 complex of good resolution (not available as yet), the docking results of functionalized isoindigos should be interpreted with caution. *Subsequently, we*

*investigated the interaction of meisoindigo and indirubin-3′-oxime (**16**) with CDK2 by molecular dynamics (MD) simulation (Supplementary data) and found that meisoindigo was held by weak and transient H bonds to the CDK2 binding pocket. Its binding energy was considerably smaller than that of oxime **16** which established stronger interactions with the enzyme consistent with its strong CDK2 inhibitory activity.[3,4] Thus, the more rigorous MD simulation reflected the weak CDK2 inhibitory activity of meisoindigo more accurately than molecular docking.*

In conclusion, we have identified several functionalized isoindigos with antiproliferative activities against a panel of cancer cell lines. The most promising members (**6** and **8**) were more potent than meisoindigo and comparable to 6-bromoindirubin-3′-oxime (**16**) on leukemic K562 cells and hepatoma HuH7 cells but had less growth inhibitory activity against normal lung fibroblast cells. In spite of docking simulations that supported interactions of several isoindigos with CDK2, this was not borne out experimentally and none of the isoindigos (including meisoindigo) inhibited CDK2 to any significant extent. The antiproliferative activity of these compounds may arise from disruption of other pathways or enzymes involved in cell growth processes. Current reports on meisoindigo suggest that it has multiple targets in cancer cells[10–12] and the analogs **6** and **8** may share a similar profile. If this were so, activity against several targets combined with their pharmaceutically friendly features would make functionalized isoindigos promising lead structures for further investigation as anticancer agents.

4. Experimental

4.1. General details for chemical synthesis

All reagents were obtained from commercial suppliers and used without further purification. Melting points were determined on a Gallenkemp melting point apparatus 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 and ^{13}C NMR spectra were measured on Brucker ACF (DPX-300) (^{1}H at 300 MHz, ^{13}C at 75 MHz) magnetic resonance spectrometer. ^{1}H chemical shifts were reported in ppm using TMS as internal standard. Coupling constants (J) were reported in hertz (Hz). Proton decoupled ^{13}C NMR spectra were reported in ppm (δ) relative to residual $CHCl_3$ (δ 77.0) and DMSO (39.5). Nominal mass spectra were captured on a LcQ Finnigan MAT mass spectrometer with chemical ionization (APCI) as probe and m/z values for the molecular ion were reported. Microwave assisted synthesis was carried out using a Biotage Initiator™ microwave synthesizer. Purity of final compounds were verified by either combustion analysis (C, H) (Elementar Varlo Micro Cube) or by reverse phase HPLC on two different eluting systems (isocratic mode) (Supplementary data).

4.2. General method for the preparation of 1-methylisatin (2a), 1-benzylisatin (4a), 1-phenethylisatin (5a), 1-(*p*-methyl-phenethyl) isatin (7a) and 1-(*p*-methoxy-phenethyl)-isatin (8a)

Anhydrous K_2CO_3 (400 mg) was added to a solution of the isatin (2 mmol) dissolved in anhydrous DMF (1.5 ml) in a 5 ml microwave vessel. The vessel was sealed and the contents stirred at room temperature (27 °C) for 15 min, after which the alkyl halide (in excess) was injected into the vessel, stirred for another 5 min and then heated under pressure in a microwave reactor for 150 °C, 15 min. Deionized water (5 ml) was added to quench the reaction and the suspension was extracted with ethyl acetate (**2a**) or dichloromethane (**8a**). The organic layer was dried using anhydrous Na_2SO_4 and concentrated to give a residue which was recrystallization with ethanol to give the substituted isatin. For **4a**, **5a**, and **7a**, the suspension was shaken vigorously, cooled down to 5–10 °C for approximately 1 h and then filtered under reduced pressure. The residue was washed with ice-cold deionized water and recrystallized with ethanol to give the desired product. Yields and 1HNMR spectroscopic data of **2a**, **4a**, **5a**, **7a**, and **8a** are given in Supplementary data.

4.3. Isoindigo (1)

Isatin (2 g, 13.6 mmol) and 2-oxindole (1.81 g, 13.6 mmol) were dissolved in 15 ml glacial acetic acid in a 20 ml microwave vessel and three drops of concd hydrochloric acid was added. The seal vessel was subjected to microwave irradiation at 200 °C for 30 min. The resultant crude mixture was concentrated in vacuo. Toluene (30 ml) was added to aid in the removal of glacial acetic acid. Ethyl acetate (30 ml) was added to the residue, the suspension was stirred vigorously and allowed to stand at 4 °C, 1 h. The suspension was filtered under reduced pressure, rinsed with chilled ethyl acetate and dried in the oven. Compound **1** was obtained as a dark violet powder. Yield: 91.2%; mp: >350 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.87 (s, 2H), 9.04 (d, J = 8.1 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 6.95 (t, J = 7.8 Hz, 2H), 6.84 (d, J = 7.8 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 170.0 (CO–NH), 145.1, 134.4, 133.7, 130.3, 122.7, 122.2, 110.6; MS(APCI) m/z: 263.4 [(M+H$^+$) calculated for $C_{16}H_{11}N_2O_2$, 263.3]. Anal. Calcd for $C_{16}H_{10}N_2O_2$ (262.26): C, 73.27; H, 3.84. Found: C, 73.29; H, 4.01.

4.4. Meisoindigo (2)

1-Methyl-isatin (**2a**) (150 mg, 0.9 mmol) and 2-oxindole (149 mg, 1 mmol) were dissolved in 3 ml glacial acetic acid in a 5 ml microwave vessel and one drop of concentrated HCl was added. The sealed vessel was subjected to microwave irradiation at 200 °C for 30 min. The resultant crude mixture was concentrated in vacuo and purified using flash chromatography to give the desired product as dark violet powder. Yield: 40.6%; mp: 229 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.92 (s, 1H), 9.10 (dd, J_1 = 7.8, J_2 = 2.1 Hz, 2H), 7.44 (t, J = 7.5 Hz, 1H), 7.36 (t, J = 7.5 Hz, 1H), 7.05 (t, J = 8.4 Hz, 1H), 7.03 (d, J = 8.1, 1H), 6.98 (t, J = 7.8 Hz, 1H), 6.85 (d, J = 7.8 Hz, 1H), 3.23 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.9 (CO–NH), 168.3 (CO–NH), 146.0, 145.3, 134.8, 133.9, 133.6, 133.2, 130.4, 130.0, 122.8, 122.6, 122.2, 121.9, 110.7, 109.5; MS(APCI) m/z: 277.7 [(M+H$^+$) calculated for $C_{17}H_{13}N_2O_2$, 277.3]. Anal. Calcd for $C_{17}H_{12}N_2O_2$ (276.29): C, 73.90; H, 4.38. Found: C, 73.52; H, 4.43. Purity of **2** was verified by HPLC to be more than 95% (Supplementary data).

4.5. 1-Phenyl-isoindigo (3)

1-Phenyl-isoindigo (**3**) was synthesized by a similar method as **2** starting from 1 mmol quantities of 1-phenyl-isatin and 2-oxindole. Compound **3** was obtained as a dark red powder. Yield: 62.0%; mp: 273–274 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.95 (s, 1H), 9.17 (d, J = 7.8 Hz, 1H), 8.97 (d, J = 8.1 Hz, 1H), 7.64–7.59 (m,2H), 7.53–7.50 (m, 3H), 7.38–7.33 (m, 2H), 7.10 (t, J = 7.8 Hz, 1H), 6.95 (t, J = 7.8 Hz, 1H), 6.86 (d, J = 7.8 Hz, 1H), 6.68 (d, J = 8.1 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.8 (CO–NH), 166.7 (CO–NH), 144.8, 144.4, 134.4, 133.9, 133.1, 132.6, 131.9, 129.6, 129.5, 129.2, 128.4, 127.3, 122.3, 121.6, 121.2, 121.0, 109.7, 108.8; MS(APCI) m/z: 339.5 [(M+H$^+$) calculated for $C_{22}H_{15}N_2O_2$, 339.4]. Anal. Calcd for $C_{22}H_{14}N_2O_2$ (338.35): C, 78.09; H, 4.17. Found: C, 78.07; H, 4.14.

4.6. 1-Benzyl-isoindigo (4)

1-Benzyl-isoindigo (**4**) was synthesized by the method described for **2** starting from 1-benzyl-isatin (**4a**) (0.84 mmol, 200 mg) and 2-oxindole (1.1 mmol, 135 mg, 1.2 equiv). Compound **4** was obtained as dark red crystals. Yield: 43.1%; mp: 238–241 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.94 (s, 1H), 9.10 (dd, J_1 = 7.8 Hz, J_2 = 3.3 Hz, 2H), 7.36–7.27 (m, 7H), 7.06–6.95 (m, 3H), 6.86 (d, J = 7.8 Hz, 1H), 5.02 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.9 (CO–NH), 168.4 (CO–NH), 145.4, 145.0, 137.3, 135.4, 134.1, 133.5, 132.9, 130.6, 130.2, 129.8, 128.5, 128.3, 123.0, 122.7, 122.3, 122.0, 110.7, 110.0, 43.8; MS(APCI) m/z: 353.7 [(M+H$^+$) calculated for $C_{23}H_{17}N_2O_2$, 353.4]. Anal. Calcd for $C_{23}H_{16}N_2O_2$ (352.28): C, 78.39; H, 4.58. Found: C, 78.27; H, 4.63.

4.7. 1-(Phenethyl)-isoindigo (5)

1-(Phenethyl)-isoindigo (**5**) was synthesized by the method described for **2** starting from 1-(phenethyl)-isatin (**5a**) (0.80 mmol, 200 mg) and 2-oxindole (0.96 mmol, 127 mg, 1.2 equiv). Compound **5** was obtained as dark red crystals. Yield: 49%; mp: 216–217 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.91 (s, 1H), 9.09 (d, J = 7.8 Hz, 1H), 8.98 (d, J = 7.8 Hz, 1H), 7.40 (t, J = 7.5 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 7.29–7.18 (m, 5H), 7.08 (d, J = 7.8 Hz, 1H), 7.03 (t, J = 8.1 Hz, 1H), 6.96 (t, J = 7.8 Hz, 1H), 6.84 (d, J = 7.8 Hz, 1H), 4.00 (t, J = 7.5 Hz, 2H), 2.94 (t, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.9 (CO–NH), 168.1 (CO–NH), 145.2, 145.1, 139.4, 134.9, 134.0, 133.6, 133.1, 130.4, 130.1, 129.9, 129.4, 127.5, 122.7, 122.6, 122.2, 121.8, 110.7, 109.7, 41.9, 34.0; MS(APCI) m/z 367.8 [(M+H$^+$) calculated for $C_{24}H_{19}N_2O_2$, 367.4]. Anal. Calcd for $C_{24}H_{18}N_2O_2$ (366.40): C, 78.67; H, 4.95. Found: C, 78.40; H, 4.95.

4.8. 1-(Phenpropyl)-isoindigo (6)

Anhydrous potassium carbonate (1 mmol) and isoindigo (1 mmol) were dissolved in 1.5 ml of anhydrous DMF in a 5 ml microwave vessel and allowed to stir at room temperature for 15 min. Phenpropyl bromide (167 μl, 1.1 mmol) was injected into the mixture which was then subjected to microwave irradiation for 30 min at 150 °C. The mixture was extracted with ethyl acetate/brine, the organic fraction was dried (anhydrous Na_2SO_4), concentrated in vacuo and the residue purified with flash chromatography (4:1 hexane/ethyl acetate as eluting solvent). Compound **6** was recrystallized from ethanol. Yield: 42%; mp: 173–174 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.91 (s, 1H), 9.08 (t, J = 7.5 Hz, 2H), 7.41 (t, J = 7.8 Hz, 1H), 7.35 (t, J = 7.5 Hz, 1H), 7.29–7.17 (m, 5H), 7.01 (t, J = 6.6 Hz, 2H), 6.97 (t, J = 7.8 Hz, 1H), 6.85 (d, J = 7.8 Hz, 1H), 3.80 (t, J = 6.9 Hz,2H), 2.67 (t, J = 7.5 Hz, 2H), 1.93 (p, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.9 (CO–NH), 167.1 (CO–NH), 144.2, 141.2, 133.8, 132.9, 132.6, 132.2, 129.5, 129.1, 128.3, 128.2, 125.9, 121.7, 121.6, 121.2, 120.9, 109.6, 108.5, 40.3, 32.5, 28.6; MS(APCI) m/z: 381.5 [(M+H$^+$) calculated for $C_{25}H_{21}N_2O_2$, 381.4]. Anal. Calcd for $C_{25}H_{20}N_2O_2$ (380.43): C, 78.93; H, 5.30. Found: C, 78.75; H, 5.25.

4.9. 1-(*p*-Methyl-phenethyl)-isoindigo (7)

1-(*p*-Methyl-phenethyl)-isoindigo (**7**) was synthesized by a similar method as **2** starting from 1-(*p*-methyl-phenethyl)-isatin (**7a**) (0.75 mmol, 200 mg) and 2-oxindole (0.91 mmol, 121 mg, 1.2 equiv). Compound **11** was obtained as dark red crystals. Yield: 49%; mp: 242–243 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.90 (s, 1H), 9.08 (d, J = 7.8 Hz, 1H), 8.94 (d, J = 8.1 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 7.16–7.00 (m, 6H), 6.95 (t, J = 7.8 Hz, 1H), 6.84 (d, J = 7.5 Hz, 1H), 3.96 (t, J = 7.2 Hz, 2H), 2.88 (t, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.9 (CO–NH), 168.0 (CO–NH), 145.2, 145.1, 136.4, 136.3, 134.9, 134.0, 133.7, 133.1, 130.3, 130.0, 129.8, 122.7, 122.6, 122.2, 121.9, 110.7, 109.7, 42.0, 33.5, 21.7; MS(APCI) m/z: 381.7 [(M+H$^+$) calculated for $C_{25}H_{21}N_2O_2$, 381.4]. Anal. Calcd for $C_{25}H_{20}N_2O_2$ (380.43): C, 78.93; H, 5.30. Found: C, 78.85; H, 5.29.

4.10. 1-(*p*-Methoxy-phenethyl)-isoindigo (8)

1-(*p*-Methoxy-phenethyl)-isoindigo (**8**) was synthesized by a similar method as **2** starting from 1-(*p*-methoxy-phenethyl)-isatin (**8a**) (0.71 mmol, 200 mg) and 2-oxindole (0.85 mmol, 114 mg, 1.2 equiv). Yield: 34%; mp: 216–217 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.89 (s, 1H), 9.09 (d, J = 8.1 Hz, 1H), 8.97 (d, J = 8.1 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.35 (t, J = 7.5 Hz, 1H), 7.20 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 7.8 Hz, 1H), 7.03 (t, J = 7.8 Hz, 1H), 6.96 (t, J = 7.8 Hz, 1H,), 6.83–6.82 (m, 3H), 3.95 (t, J = 7.2 Hz, 2H,), 3.69 (s, 3H), 2.87 (t, J = 7.5 Hz, 2,); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.9 (CO–NH), 168.0 (CO–NH), 158.9, 145.2, 145.1, 134.9, 133.9, 133.6, 133.2, 131.2, 130.9, 130.4, 130.1, 122.7, 122.6, 122.2, 121.9, 114.9, 110.7, 109.7, 56.0, 42.2, 33.1; MS(APCI) m/z: 397.4 [(M+H$^+$) calculated for $C_{25}H_{21}N_2O_3$, 397.4]. Anal. Calcd for $C_{25}H_{20}N_2O_3$ (396.43): C, 75.74; H, 5.08. Found: C, 75.48; H, 5.04.

4.11. 1-(*p*-Hydroxy-phenethyl)-isoindigo (9)

p-Hydroxyphenethyl bromide (600 mg, 3 mmol), dihydro-2*H*-pyran (411 μl, 4.5 mmol), and pyridinium *p*-toluenesulfonate (PPTS, 75 mg, 0.3 mmol) were dissolved in 5 ml DCM and stirred for 16 h at room temperature. The mixture was extracted with DCM/brine, the organic fraction was dried over anhydrous sodium sulfate, concentrated in vacuo, and the residue was purified by flash chromatography with 20:1 hexane/ethyl acetate as eluting solvent. The tetrahydropyranyl ether was obtained as a colorless liquid in 80% yield. It was then added to a previously stirred mixture of K_2CO_3 (310 mg, 2.25 mmol) and 393 mg of isoindigo (390 mg, 1.5 mmol) in 2 ml anhydrous DMF contained in a 5 ml microwave vessel, as described for **6.** The mixture was irradiated by microwave for 30 min at 150 °C and worked up as described for **6**. The tetrahydropyranyl protecting group was removed by refluxing the product from column chromatography (155 mg) with 20 mg of PPTS in 50 ml of ethanol for 3 h. The solution was concentrated under reduced pressure and extracted using ethyl acetate/brine. The organic fraction was dried (anhydrous Na_2SO_4), concentrated in vacuo, and recrystallized using ethanol to give the desired product. Yield: 34%; mp: 282–285 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.90 (s, 1H), 9.20 (br s, 1H), 9.08 (d, J = 8.1 Hz, 1H), 9.00 (d, J = 7.8 Hz, 1H), 7.39 (t, J = 7.8 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.07–7.01 (m, 4H), 6.96 (t, J = 7.8 Hz, 1H), 6.84 (d, J = 7.5 Hz, 1H), 6.65 (d, J = 8.4, 2H), 3.91 (t, J = 7.2 Hz, 2H), 2.80 (t, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.8 (CO–NH), 166.9 (CO–NH), 155.8, 144.14, 144.08, 133.8, 132.8, 132.5, 132.1, 129.7, 129.3, 129.0, 128.3, 121.6, 121.1, 120.8, 115.1, 109.6, 108.6, 41.2, 32.0. MS(APCI) m/z: 383.4 [(M+H$^+$) calculated for $C_{24}H_{19}N_2O_3$, 383.4]. Anal. Calcd for $C_{24}H_{18}N_2O_3$ (382.40): C, 75.38; H, 4.74. Found: C, 75.24; H, 4.75.

4.12. 1-(*p*-Nitro-phenethyl)isoindigo (10)

1-(*p*-Nitro-phenethyl)isoindigo (**10**) was synthesized by the method described for **6** starting from isoindigo (**1**) (5 mmol, 1310 mg) and 1-(*p*-nitro-phenethyl)-isatin (6 mmol, 1380 mg, 1.2 equiv). Yield: 33%; mp: 241–242 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.90 (s, 1H), 9.09 (d, J = 7.8 Hz, 1H), 8.85 (d, J = 7.8 Hz, 1H), 8.13 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.41 (t, J = 7.5 Hz, 1H), 7.33 (t, J = 7.5 Hz, 1H), 7.11 (d, J = 7.8 Hz, 2H), 7.03 (t, J = 7.8 Hz, 1H), 6.90 (t, J = 7.5 Hz, 1H), 6.83 (d,

J = 7.5 Hz, 1H), 4.07 (t, J = 6.9 Hz, 2H), 3.10 (t, J = 6.9 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.8 (CO–NH), 167.0 (CO–NH), 146.9, 146.2, 144.2, 143.8, 133.9, 132.9, 132.5, 131.9, 130.3, 129.1, 129.0, 123.4, 121.8, 121.5, 121.1, 120.8, 109.6, 108.7, 42.0, 32.7; MS(APCI) m/z: 412.7 [(M+H$^+$) calculated for $C_{24}H_{17}N_3O_4$, 412.4]. Anal. Calcd for $C_{24}H_{16}N_3O_4$ (411.41): C, 70.07; H, 4.16. Found: C, 68.64; H, 4.46. Purity of **10** was verified by HPLC to be more than 95% (Supplementary data).

4.13. *N*-(4-(2-Bromoethyl)phenyl)acetamide

4-Nitrophenethyl bromide (1 g, 4.3 mmol) was dissolved in a solution of 16 ml glacial acetic acid and 7.5 ml methanol. The solution was heated to 65 °C in an oil bath, and iron powder (870 mg) was added in 2 batches, 30 min apart. The mixture was then stirred for 2.5 h at 65 °C. It was then concentrated in vacuo, with toluene (30 ml) added to aid in the removal of acetic acid. DCM (50 ml) was added to the residue, followed by concd ammonia solution and extraction with DCM/brine. The organic solvent was dried with anhydrous sodium sulfate and concentrated to give a colorless liquid which was then dissolved in dry DCM (4 ml) and triethylamine (600 μl, 2.2 equiv). Acetyl chloride (300 μl, 2.2 equiv) was added to the solution, followed by refluxing for 1 h. NaOH (0.1 M) was added to quench the reaction. The reaction mixture was then extracted with DCM/brine, washed successively with 0.1 M $NaHCO_3$ and 0.1 M HCl, the organic fraction was dried (anhydrous Na_2SO_4), concentrated in vacuo and the residue purified by flash chromatography with 2:1 hexane/ethyl acetate as eluting solvent. Target compound was obtained as a beige powder. Yield: 31%; mp: 136–137 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.45 (d, J = 8.4 Hz, 2H), 7.26 (s, 1H), 7.16 (d, J = 8.1 Hz, 2H), 3.54 (t, J = 7.5 Hz, 2H), 3.12 (t, J = 7.5 Hz, 2H), 2.17 (s, 3H).

4.14. 1-(*p*-Acetylamino-phenethyl)-isoindigo (11)

Isoindigo (262 mg, 1 mmol) and K_2CO_3 (166 mg, 1.2 mmol) were dissolved in 2 ml of anhydrous DMF in a 5 ml microwave vessel and allowed to stir at room temperature for 15 min. *N*-(4-(2-Bromoethyl)phenyl)acetamide (266 mg, 1.1 mmol) was dissolved in anhydrous DMF (1 ml) and injected into the vessel. The mixture was subjected to microwave irradiation for 30 min at 150 °C. The mixture was then extracted with ethyl acetate/brine, the organic fraction was dried (anhydrous Na_2SO_4), concentrated in vacuo and the residue was purified by column chromatography with 1:1 hexane/ethyl acetate as eluting solvent. Compound **11** was recrystallized in ethanol to give red crystals. Yield 21%; mp: 278–279 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 10.90 (s, 1H), 9.85 (s, 1H), 9.08 (d, J = 8.1 Hz, 1H), 8.95 (d, J = 7.8 Hz, 1H), 7.46 (d, J = 8.4 Hz, 2H), 7.41 (t, J = 8.1 Hz, 1H), 7.35 (t, J = 7.8 Hz, 1H), 7.18 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.1 Hz, 1H), 7.02 (t, J = 7.8 Hz, 1H), 6.95 (t, J = 7.5 Hz, 1H), 6.84 (d, J = 7.8 Hz, 1H), 3.96 (t, J = 6.9 Hz, 2H), 2.87 (t, J = 6.6 Hz, 2H), 2.00 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.9 (CO–NH), 168.3 (CO–NH), 167.0 (C=O), 144.2, 137.7, 133.9, 133.0, 132.9, 132.7, 132.2, 129.3, 129.1, 121.7, 121.6, 121.2, 120.8, 119.0, 109.7, 108.7, 41.0, 32.3; MS(APCI) m/z 424.4 [(M+H$^+$) calculated for $C_{26}H_{22}N_3O_3$, 424.5]. Anal. Calcd for $C_{26}H_{21}N_3O_3$ (423.45): C, 73.74; H, 5.00. Found: C, 73.56; H, 5.20.

4.15. 1,1′-Dimethyl-isoindigo (12)

Four hundred milligrams of dry K_2CO_3 (excess) were added to isoindigo (265 mg,1 mmol) dissolved in 2.5 ml anhydrous DMF in a 5 ml microwave vessel and allowed to stir at room temperature for 15 min. Methyl iodide (624 μl, 10 mmol) was injected and the mixture was stirred at room temperature for 5 min before microwave irradiation at 150 °C for 10 min. Five milliliters of water were added to quench the reaction and the suspension was shaken vigorously and left to stand in the refrigerator at 4 °C for 1 h. The suspension was filtered under reduced pressure, rinsed with excess water and dried in the oven. Recrystallization with ethanol **12** as dark violet powder. Yield: 95%; mp: 274–275 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.20 (d, J = 8.1 Hz, 2H), 7.37 (t, J = 7.8 Hz, 2H), 7.07 (t, J = 8.1 Hz, 2H), 6.78 (d, J = 7.8 Hz, 2H), 3.29 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 164.8, 146.2, 133.9, 130.1, 122.9, 121.8, 109.6, 100.6; MS(APCI) m/z: 291.7 [(M+H$^+$) calculated for $C_{18}H_{15}N_2O_2$, 291.3]. Anal. Calcd for $C_{18}H_{14}N_2O_2$ (291.32): C, 74.47; H, 4.86. Found: C, 74.43; H, 4.87.

4.16. 3, 3′-Dihydroisoindigo (13)

Isoindigo (**1**, 350 mg, 1.3 mmol) was dissolved in 20 ml of ethanol and 35 mg of 10% Pd/C was added. Hydrogen gas was bubble into the solution and stirred at room temperature (27 °C) for 2 h. The suspension was filtered and the solvent removed under reduced pressure to give a pale yellow powder which gave two spots on the TLC. Compound **13** was obtained as a mixture of isomers which was not separated. The methyl protons at 3 and 3′ positions were detected at 4.16 (major isomer) and 4.07 (minor isomer) in the ratio of 2:1 in the ^{1}H NMR (300 MHz). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 177.2, 176.1, 143.2, 142.7, 128.19, 128.12, 127.4, 125.9, 123.3, 122.9, 121.3, 121.2, 109.4, 109.3, 45.7, 45.6; MS(APCI) m/z 265.5 [(M+H$^+$) calculated for $C_{16}H_{13}N_2O_2$, 265.3]. Anal. Calcd for $C_{16}H_{12}N_2O_2$ (264.28): C, 72.72; H, 4.58. Found: C, 72.78; H, 4.61. Compound **13** was tested as a mixture of stereoisomers.

4.17. Determination of anti-proliferative activity by MTT assay

The cell lines K562 (human chronic myelogenous leukemia lymphoblast), HL60 (human acute promyelocytic leukemia myeloblast), HCT116 (human colon carcinoma) and IMR-90 (normal human lung fibroblast) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and grown in media recommended by ATCC. HuH7 (human hepatoma) cells were a gift from Dr Ho Han Kiat of A*STAR, Singapore. K562 and HL60 cells were sub-cultured when they reached a density of 10^6 cells/ml and cells in their 6–12 passages were used for the experiments. HCT116 and IMR-90 cells were sub-cultured when they reached 90% confluency and used within 4–10 passages for the assay.

An aliquot (99 μl, 1.0×10^5 cell/ml) of K562 cells in media was added to each well in a microtitre plate, followed by an aliquot (1 μl) of the test compound that was previously prepared at a 100-fold concentration. The final concentration of DMSO in each well was 0.4% v/v. The same procedure was followed for the HL60 cells except that a higher cell density (2.0×10^5 cells/ml) was used. The test compounds were incubated with K562 cells for 72 h at 37 °C, 5% CO_2, while the HL60 cells were incubated for five days due to their slower growth rate. After the incubation period, an aliquot (25 μl) of MTT in media was added to give a final concentration of 0.5 mg/ml per well. The plate was gently agitated, incubated for 3 h at 37 °C, after which it was centrifuged (1200 g, 10 min), the supernatant removed by aspiration and DMSO (100 μl) added to dissolve the formazan crystals. The plate was read at 550 nm. Controls were wells of similar composition except for the exclusion of test compound ('vehicle') and wells containing only 0.4% DMSO in media ('blank'). A slightly different protocol was followed for the adherent HCT116, HuH7 and IMR-90 cells. HCT116, HUH7 and IMR-90 cells in media (100 μl) were seeded at 10,000 cells/well, 6000 cells/well, and 4000 cells/well, respectively. The cells were allowed to adhere to the well over 24 h after which media containing the test compound (prepared in 0.5% v/v DMSO-media) was added and the plates incubated for 72 h at 37 °C, 5% CO_2. After incubation, the supernatant in each well was

removed by aspiration and the cell layer gently rinsed with PBS. An aliquot of MTT (100 μl, 0.5 mg/ml in phosphate buffer) was added to each well, incubated for 4 h, 37 °C, after which the MTT solution was removed and DMSO (100 μl) added to dissolve the formazan crystals. Absorbances were measured within 30 min at 590 nm on a microplate reader. Control wells were maintained as described earlier. The viability of cells at a given concentration of test compound was determined from the following expression:

$$\text{Percentage viability} = (A_{\text{drug}} - A_{\text{blank}}/A_{\text{vehicle}} - A_{\text{blank}}) \times 100,$$

where A_{drug} = absorbance of wells with cells grown in vehicle (DMSO/media) and treated with test compound, A_{blank} = absorbance of wells with only 100 μl DMSO and A_{vehicle} = absorbance of wells with cells grown in vehicle (DMSO/media). Each concentration of test compound was evaluated at least four times on separate occasions and different cell passages. The IC_{50} (concentration that inhibited 50% of cell growth) was determined from the sigmoidal curve obtained by plotting percentage viability versus logarithmic concentration of test compound using Graphpad Prism 4 (San Diego, CA).

4.18. Determination of CDK2 inhibitory activity using the IMAP assay

The IMAP 800-tp kit (Molecular Devices R8155) was purchased for the assay. The EC_{70} value of the CDK2/cyclinA (Millipore #14–448) enzyme was first determined using the protocol described in the kit. Aliquots (5 μl) of the following: (i) complete reaction buffer, (ii) CDK2/cyclinA enzyme (3.96, 1.32, 0.44, 0.15, 0.05, 0.02, 0.005 unit/ml), (iii) 120 μM ATP and (iv) 400nM lyophilized FAM-Histone H1-derived peptide (5FAM-GGGPATPKKAKKL-COOH) (Molecular Devices, R7252) were added in sequence to into a well (384-well black microplate, Sigma, CLS3571) such that the final concentration of ATP and peptide substrate were 30 μM and 100 nM, respectively. The mixture was incubated for 1 h for the phosphorlyation reaction to take place, after which 60 μl of the progressive binding solution (1/400 dilution of binding reagent) was added and incubated for another hour. Fluorescent polarization (FP) readings were taken on a microplate reader (MDC Spectramax M5) at $\lambda_{\text{Excitation}}$ 485 nm and $\lambda_{\text{Emission}}$ 530 nm. The EC_{70} of CDK2/cyclin A was determined from the sigmoidal curve obtained by plotting FP versus logarithmic enzyme concentration (GraphPad Prism 4, San Diego, CA). The assay was repeated in the presence of 10 μM test compound. Briefly, 5 μl aliquots of the test compound (40 μM in 4% v/v DMSO), CDK2/cyclin A at 4× EC_{70} concentration, 120 μM ATP and 400nM lyophilized FAM-Histone H1-derived peptide was added to each well. The final concentration of DMSO in the well was maintained at 1% v/v. Control wells contained complete reaction buffer (5 μl) in place of the same volume of enzyme solution and the 'vehicle' control were wells that did not contain test compound but with other reagents unchanged. The enzyme inhibition activity of test compound at 10 μM was calculated using the following equation:

$$\text{Percentage inhibition} = (\text{FP}_{\text{vehicle}} - \text{FP}_{\text{drug}}/\text{FP}_{\text{vehicle}} - \text{FP}_{\text{control}}) \times 100,$$

where FP is the fluorescent polarization units (mP) measured for the test compound, vehicle control and control. Each test compound was evaluated at least twice on separate occasions.

4.19. Docking with CDK2

The CDK2 protein complex (1FIN) was protonated in the presence of its ligand ATP using the Protonate3D process in Molecular Operating Environment (MOE, version 2007.09, Montreal, Canada) at 300 K and pH 7.4. The non-heavy atoms were minimized using the AMBER force field and unbound water molecules at the active site were deleted. No force field minimization was carried out on the heavy atoms. The co-crystallized ATP was extracted from the crystal structure (1FIN) to give an apo-CDK2/CyclinA structure. *The extracted ATP structure was then re-docked onto the apo-CDK2/cyclin A structure with the software GOLD 4.0 (CCDC, Cambridge, UK). The variation (measured in terms of the heavy atom root mean square deviation RMSD) between the most favored re-docked pose of ATP and the original ATP in 1FIN was 1.84 Å, which was an acceptable variation considering that ATP has a large number of rotatable bonds. This process served as a means of validating the subsequent docking of the test compounds as it showed that the software parameters selected for the process were appropriate.*

The test compounds used in the docking exercise were drawn with MOE. As the bisindole ring of isoindigo was reported to be planar[31], semi-empirical PM3 method was used to energy minimize (gradient 0.00001 kcal/mol) the isoindigo ring. Thereafter, the potential of the heavy atoms of the isoindigo ring were constrained using fixed potential and the substituents groups were added and minimized with MMFF94× forcefield (gradient 0.00001 kcal/mol) and saved as mol2 files. This procedure was found to be necessary to ensure planarity of the isoindigo ring as the usual forcefield minimization was found to cause the ring to assume a non-planar conformation. Partial charges were added using the Gasteiger and Hückel charge calculation method using Sybyl8.0 (Tripos). Docking of the test compounds was conducted on the apo-CDK2/CyclinA protein using GOLD 4.0. The geometric center of the co-crystallized ATP was determined using MOE. All CDK2 atoms within 12 Å spherical radius of the geometric center were defined as the docking pocket and it included the 'hinge' region amino acids of the kinase active site. All ligands were added as a single input in one GOLD run. The number of genetic operations performed were determined by using the automatic (ligand-dependent) genetic algorithm parameter settings. Search efficiency was set at the default 100%. The number of poses returned by GOLD was determined by the default settings. Atom and bond types were assigned automatically for the ligands. No constraints were set for the GOLD run.

The CDK2–ligand interactions of all docked poses were analyzed using the Protein Ligand Interaction Fingerprint (PLIF) module in MOE. PLIF uses a fingerprint scheme comprising of various interactions. Hydrogen bonds, ionic interactions and surface contacts were considered according to the residue of origin. Since known inhibitors of CDK2 formed hydrogen bonds with Glu81 and/or Leu83 in the hinge region of the active site, the BitSelector function of PLIF was used to filter out those ligands that had no probability of forming interactions with Glu81 and Leu83 in any of their docked poses. Among those ligands that were likely to form interactions with Glu81 and Leu83, the probability of each ligand forming such interactions were scored using PLIF for each ligand. PLIF calculated hydrogen bonds between polar atoms using a method based on protein contact statistics,[32] whereby a pair of atoms were scored by their distances and orientation. The score was expressed as a percentage probability of forming a H bond. The individual score for each interaction (Glu81 as donor, Leu83 as donor, Leu83 as acceptor) was obtained and summed up to produce a new score called HingeSum which reflected the total probability of a ligand establishing either one of the three possible interactions with Glu8a/Leu83 at the hinge region of CDK2. Only the docked pose of each compound with the highest HingeSum was retained while the other poses were discarded. This method of selecting poses placed emphasis on poses that formed significant interactions with the hinge region of CDK2.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.008.

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