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Discovery of substituted N′-(2-oxoindolin-3-ylidene)benzohydrazides as new apoptosis inducers using a cell- and caspase-based HTS assay

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ABSTRACT

We report the discovery of a series of substituted N′-(2-oxoindolin-3-ylidene)benzohydrazides as inducers of apoptosis using our proprietary cell- and caspase-based ASAP HTS assay. Through SAR studies, N′-(4-bromo-5-methyl-2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazide (**3g**) was identified as a potent apoptosis inducer with an EC_{50} value of 0.24 μM in human colorectal carcinoma HCT116 cells, more than a 40-fold increase in potency from the initial screening hit N′-(5-bromo-2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazide **(2a)**. Compound **3g** also was found to be highly active in a growth inhibition assay with a GI_{50} value of 0.056 μM in HCT116 cells. A group of potentially more aqueous soluble analogs were prepared and found to be highly active. Among them, compound **4e** incorporating a methyl piperazine moiety was found to have EC_{50} values of 0.17, 0.088 and 0.14 μM in human colorectal carcinoma cells HCT116, hepatocellular carcinoma cancer SNU398 cells and human colon cancer RKO cells, respectively. Compounds **3g** and **4e** were found to function as inhibitors of tubulin polymerization.

Apoptosis, or program cell death, plays a crucial role in normal cell development and tissue homeostasis.[1] Apoptosis is used by organisms to control their cell numbers and to eliminate unneeded or damaged cells.[2] Inappropriate apoptosis induction is known to result in excessive cell death, and could cause degenerative diseases.[3] Inadequate apoptosis, however, could lead to over proliferation of cells and cancer.[4] In addition, it is known that the antitumor efficacy of many chemotherapeutical agents is correlated to their apoptosis inducing ability.[5] Identification of compounds that promote or induce apoptosis in cancer cells, therefore, is an attractive approach for anticancer research.[6]

We have been interested in the discovery and development of apoptosis inducers as potential anticancer agents.[7] Applying our novel caspase-3 substrates,[8] we have developed a caspase- and cell-based, high throughput screening technology, termed Apoptosis Screening and AntiCancer Platform (ASAP), for the identification of apoptosis inducers.[9] We have reported the discovery of several novel series of apoptosis inducers, including 4-aryl-4*H*-chromenes (**1a**),[10] gambogic acid (**1b**),[11] 3-aryl-5-aryl-1,2,4-oxadiazoles (**1c**),[12] *N*-phenyl-1*H*-pyrazolo[3,4-*b*]quinolin-4-amines (**1d**),[13] 4-anilinoquinazolines (**1e**)[14,15] and 4-aryl-3-(3-aryl-1-oxo-2-propenyl)-2(1*H*)-quinolinones (**1f**)[16] (Chart 1). Herein we report the discovery of substituted N′-(2-oxoindolin-3-ylidene)-benzohydrazide (**2a**), an isatin derivative, as an apoptosis inducer using our HTS assay. SAR study of **2a** led to the discovery of N′-(4-bromo-5-methyl-2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazide (**3g**) and analogs as potent apoptosis inducers.

Many isatins and isatin derivatives have been synthesized and reported to have a variety of biological activities, including as SARS coronavirus 3C-like protease inhibitors,[17] caspase-3 inhibitors,[18] and as inhibitors of Src homology-2 domain containing protein tyrosine phosphatase-2.[19] More recently, *N*-alkyl isatin acylhydrazone derivatives such as **7a** (Chart 2) have been reported to be potent and selective cannabinoid receptor 2 inverse agonists for the potential treatment of neuropathic pain.[20] In addition, N-substituted isatins such as **7b** have been reported to be cytotoxic with a mode of action that includes inhibition of tubulin polymerization, induction of G_2/M cell cycle arrest and activation of caspase-3 and -7.[21]

Substituted N′-(5-bromo-2-oxoindolin-3-ylidene)-benzohydrazides **2a**–**2f** were obtained from ChemDiv and Asinex, and their structures were confirmed by ^{1}H NMR and MS. Substituted N′-(2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazides **3a**–**3m** were prepared from condensation of the corresponding substituted isatin (**5**)[19] with substituted 3,4,5-trimethoxybenzohydrazide (**6**) according to reported procedures.[20,22] The *N*-substituted analogs **4a**–**4h** were prepared from condensation of **2a**, **3a**, **3g** and **3h** with formaldehyde and an amine following literature procedures (Scheme 1).[23,24]

The apoptosis inducing activity of substituted N′-(2-oxoindolin-3-ylidene)-benzohydrazides was measured using our cell- and cas-

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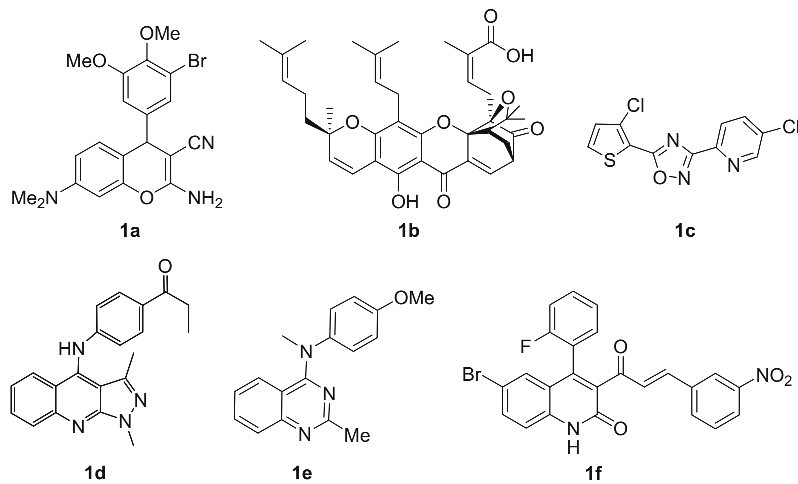


Chart 1.

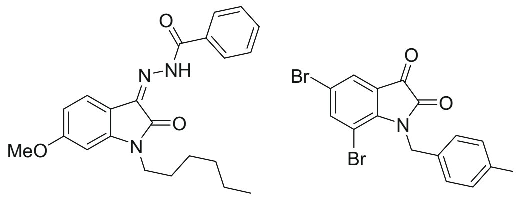
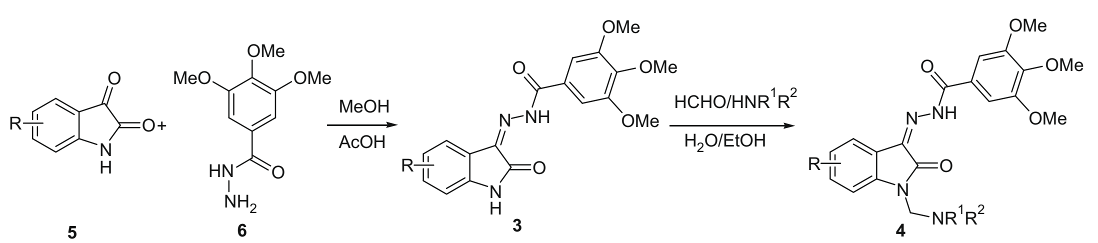


Chart 2.

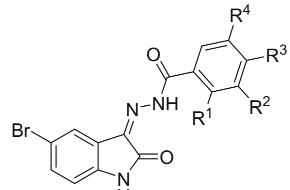
pase-based HTS assay[7] in human colorectal carcinoma cells HCT116, hepatocellular carcinoma cancer SNU398 cells and human colon cancer RKO cells, and the results are summarized in Tables 1–3. Compound **2a** was found to have EC_{50} values of 4–10 μM in the three cell lines tested. By maintaining the 5-bromo group in the isatin, we explored replacement of the 3,4,5-trimethoxy groups in the benzoyl group of **2a** by other groups. Table 1 showed that, except for compound **2b**, all these compounds (**2c**–**2f**) were inactive up to 20 μM in all the three cell lines, indicating that the 3,4,5-trimethoxy group is preferred.

By maintaining the 3,4,5-trimethoxybenzoyl group, we then explored substitutions in the 4- to 7-positions of isatin ring (Table 2). The 5-methoxy (**3a**) and 5-iodo (**3b**) analogs were similar or slightly more active than **2a**. The 5-trifluoromethoxy (**3c**), 5-amino (**3d**) and 5-acetylamino (**3e**) analogs were less active or inactive in



Scheme 1.

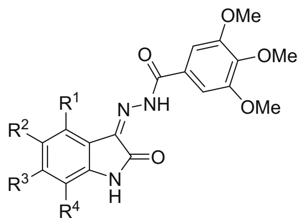
Table 1
Activity of substituted N′-(5-bromo-2-oxoindolin-3-ylidene)-benzohydrazides in the caspase activation assay



Compound #	R^1	R^2	R^3	R^4	EC_{50}[a] (μM)		
					HCT116	SNU398	RKO
2a	H	OMe	OMe	OMe	10.7 ± 0.5	8.9 ± 0.2	4.4 ± 0.5
2b	H	OCH_2O		H	>20	>20	9.7 ± 0.6
2c	OMe	H	H	H	>20	>20	>20
2d	H	F	H	H	>20	>20	>20
2e	H	Br	H	H	>20	>20	>20
2f	H	H	NO_2	H	>20	>20	>20

[a] Cells were treated with the test compounds for 48 h, and data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

Table 2
Activity of substituted *N′*-(2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazides in the caspase activation assay



Compound #	R^1	R^2	R^3	R^4	EC_{50} (μM)[a]		
					HCT116	SNU398	RKO
3a	H	OMe	H	H	6.6 ± 0.8	4.1 ± 0.8	4.0 ± 0.5
3b	H	I	H	H	10.5 ± 0.2	4.1 ± 0.1	3.6 ± 0.4
3c	H	OCF_3	H	H	>20	>20	8.3 ± 1.5
3d	H	NH_2	H	H	>20	>20	>20
3e	H	AcNH	H	H	>20	>20	>20
3f	Cl	H	H	H	3.8 ± 0.3	2.1 ± 0.2	2.0 ± 0.2
3g	Br	Me	H	H	0.24 ± 0.03	0.13 ± 0.01	0.23 ± 0.03
3h	Cl	Cl	H	H	0.64 ± 0.02	0.57 ± 0.11	0.73 ± 0.12
3i	Ph	Me	H	H	>20	>20	>20
3j	H	Me	Br	H	8.4 ± 1.1	3.7 ± 0.7	4.6 ± 0.8
3k	H	OMe	Br	H	>20	>20	>20
3l	H	Cl	H	Cl	>20	>20	>20
3m	H	Me	H	Br	10.6 ± 0.1	>20	4.9 ± 0.4

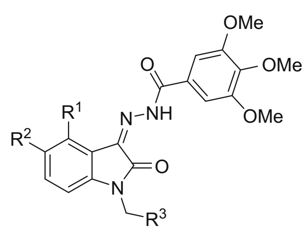
[a] Cells were treated with the test compounds for 48 h, and data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

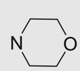
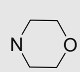
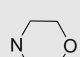
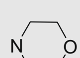
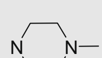
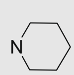
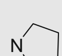
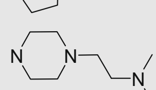
the three cell lines. These data suggested that strong electron withdrawing, or hydrophilic, or large groups are not favored at the 5-position. The 4-chloro analog **3f** was >2-fold more potent than **2a** in all the three cell lines, indicating that a small group at the 4-position might increase potency. Combination of substitutions at both the 4- and 5-positions led to the 4-bromo-5-methyl analog **3g** that was highly potent with an EC_{50} value of 0.24 μM in HCT116 cells, >40-fold more potent than **2a**. The 4,5-dichloro analog **3h** also was highly active. Interestingly, 5-methyl-4-phenyl analog **3i** was inactive up to 20 μM, indicating that a large group is not tolerated at 4-position. 5,6-Di-substituted analogs (**3j** and **3k**) and 5,7-di-substituted analogs (**3l** and **3m**) were found to have low activity or inactive, suggesting that substitutions at the 6- and 7-positions may not be preferred.

To explore the SAR further and to improve the aqueous solubility, we introduced an *N*-morpholinomethyl group into the nitrogen of the isatin of compounds **2a**, **3a**, **3g** and **3h**. Table 3 showed that compounds **4a**–**4d** had activities similar to the corresponding non-N-substituted analogs. Several compounds with various aminomethyl groups were prepared from **3g** and found to be highly active. Compounds **4e** and **4f** were the most potent ones, both with EC_{50} values of 0.17 μM. These data indicated that substitution at the nitrogen of isatin is tolerated and it could be used to introduce aqueous solubility enhancing groups.

Overall, the apoptosis inducing activities of these compounds in human colon cancer HCT116 cells were similar to that observed in hepatocellular carcinoma cancer SNU398 cells and human colon cancer RKO cells (Tables 1–3). Compound **3g**, 4e and **4f**, three of

Table 3
Activity of N-substituted *N′*-(2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazides in the caspase activation assay



Compound #	R^1	R^2	R^3	EC_{50}[a] (μM)		
				HCT116	SNU398	RKO
4a	H	Br		>20	>20	5.1 ± 0.8
4b	H	OMe		>20	>20	>20
4c	Cl	Cl		0.49 ± 0.09	0.47 ± 0.11	0.66 ± 0.10
4d	Br	Me		0.31 ± 0.04	0.12 ± 0.01	0.20 ± 0.02
4e	Br	Me		0.17 ± 0.02	0.088 ± 0.010	0.13 ± 0.02
4f	Br	Me		0.17 ± 0.03	0.087 ± 0.016	0.14 ± 0.04
4g	Br	Me		0.25 ± 0.01	0.14 ± 0.01	0.21 ± 0.03
4h	Br	Me		0.25 ± 0.02	0.13 ± 0.01	0.23 ± 0.03

[a] Cells were treated with the test compounds for 48 h, and data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

Table 4
Growth inhibition activity of substituted *N*′-(2-oxoindolin-3-ylidene)-3,4,5-trimethoxybenzohydrazides

Compound #	GI_{50}[a] (μM)		
	HCT116	SNU398	RKO
2a	7.1 ± 1.1	2.7 ± 0.1	2.7 ± 1.2
3g	0.056 ± 0.015	0.022 ± 0.008	0.019 ± 0.003
3h	0.32 ± 0.14	0.14 ± 0.04	0.054 ± 0.012
4d	0.062 ± 0.008	0.026 ± 0.011	0.019 ± 0.003
4e	0.051 ± 0.018	0.023 ± 0.008	0.018 ± 0.004
4f	0.088 ± 0.043	0.024 ± 0.006	0.036 ± 0.012
4h	0.086 ± 0.008	0.029 ± 0.014	0.043 ± 0.016

[a] Cells were treated with the test compounds for 48 h, and data are the mean of three experiments and are reported as mean ± standard error of the mean (SEM).

the most active compounds in HCT116 cells, also were the most active ones in SNU398 and RKO cells, suggesting that these compounds most probably will be broadly active against many cancer cell lines.

Representative compounds were assayed in a traditional growth inhibition (GI_{50}) assay to confirm that the active compounds in the caspase induction assay also inhibit tumor cell growth. The growth inhibition assays in T47D, HCT116 and SNU398 cells were run in a 96-well microtiter plate as described previously[7] and the data are summarized in Table 4. Compound **3g** had GI_{50} values of 0.056, 0.022 and 0.019 μM in HCT116, SNU398 and RKO cells, respectively, which are >100-fold more active than the original hit compound **2a**. Compounds **4d**–**4f** and **4h** were also highly active with GI_{50} values similar to that of **3g**. Compound **3h** was less active than **3g**. These data confirmed that the cell-based caspase activation HTS assay is not only useful for the identification of inducers of apoptosis, but also for subsequent optimization and SAR studies.

The potent compounds in this series of substituted *N*′-(2-oxoindolin-3-ylidene)-benzohydrazides, such as compounds **3g** and **4e**, were tested by cell cycle analysis[10] and found to arrest HCT116 cells in G_2/M followed by apoptosis, which is similar to what was reported for compound **7b** (Chart 2).[21] We suspected that compounds **3g** and **4e** might be tubulin inhibitors. In a tubulin polymerization assay,[25] compounds **3g** and **4e** were found to inhibit tubulin polymerization with IC_{50} values of 0.97 and 0.19 μM. In comparison, the IC_{50} values for vinblastine and colchicine were 0.5 μM. These data suggest that inhibition of tubulin polymerization might be the main mechanism of action for these compounds as apoptosis inducers.

In conclusion, a series of substituted *N*′-(2-oxoindolin-3-ylidene)-benzohydrazides were identified as apoptosis inducers utilizing our ASAP assay. Through SAR studies, potent compound **3g** and significantly more aqueous soluble compound **4e** were identified with EC_{50} values in HCT116 cells >40-fold more potent than the screening hit **2a**. The mode of action for the potent compounds **3g** and **4e** was found to be inhibition of tubulin polymerization.

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