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Discovery and SAR of indole-2-carboxylic acid benzylidene-hydrazides as a new series of potent apoptosis inducers using a cell-based HTS assay

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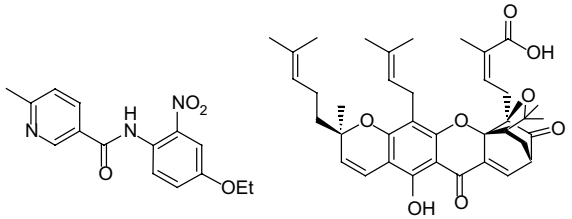
Abstract—A series of indole-2-carboxylic acid benzylidene-hydrazides has been identified as a new class of potent apoptosis inducers through a novel cell-based caspase HTS assay. The screening hit, 5-chloro-3-methyl-indole-2-carboxylic acid (4-nitrobenzylidene)-hydrazide (**3a**), was found to arrest T47D cells in G_2/M and to induce apoptosis as measured by the flow cytometric analysis assay. A SAR study was carried out by modification of the substitutions on the indole and benzene rings. Substitution at the 3-position of the indole ring was found to be important for apoptotic activity. A 20-fold increase of apoptotic activity was achieved from screening hit **3a** to 5-methyl-3-phenyl-indole-2-carboxylic acid (4-methylbenzylidene)-hydrazide (**9a**) and 5-chloro-3-phenyl-indole-2-carboxylic acid (4-nitrobenzylidene)-hydrazide (**9b**), with EC_{50} value of 0.1 μM in the caspase activation assay in T47D breast cancer cells. Compound **9b** also was found to be highly active in a standard growth inhibition assay with a GI_{50} value of 0.9 μM in T47D cells. Compound **3a** and its analogs were found to inhibit tubulin polymerization, which is the most probable primary mechanism of action of these compounds.

1. Introduction

Apoptosis, or programmed cell death, plays a vital role in normal embryonic development as well as adult life, such as elimination of dispensable or excess cells. It has been known that defects of apoptosis pathways and the ability to evade cell death is one of the hallmarks of cancers, which results in uncontrollable tumor cell growth, as well as tumor resistance to chemotherapeutic treatment.[1] Therefore the discovery and development of apoptosis inducers as new chemotherapeutic agents is a promising approach and has been a focus of research recently.[2–7] Caspases, especially caspase-3, are known to be the executioners of apoptosis by cleavage of protein substrates, leading to apoptotic cell death.[8] We have therefore developed a cell-based high throughput screening assay (HTS) for apoptosis inducers, using our proprietary fluorescent caspase-3 substrate.[9] The assay measures the phenotypic induction of apoptosis by measuring caspase-3 activity, regardless of mechanism of apoptosis induction and has been run with several cell types. Utilizing this assay, we reported recently the discovery and SAR studies of substituted *N*-phenyl nicotinamides, exemplified by 6-methyl-*N*-(4-ethoxy-2-nitro-phenyl)-pyridine-3-carboxamide (**1**),[10] and gambogic acid (**2**)[11] as potent apoptosis inducers. Herein we report the discovery, characterization, and SAR of substituted indole-2-carboxylic acid benzylidene-hydrazides as a new series of potent inducers of apoptosis using our cell-based caspase-3 HTS assay.



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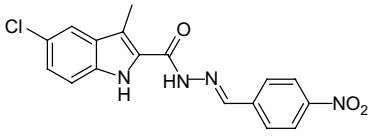

3a

Keywords: Apoptosis inducers; Anticancer drugs.
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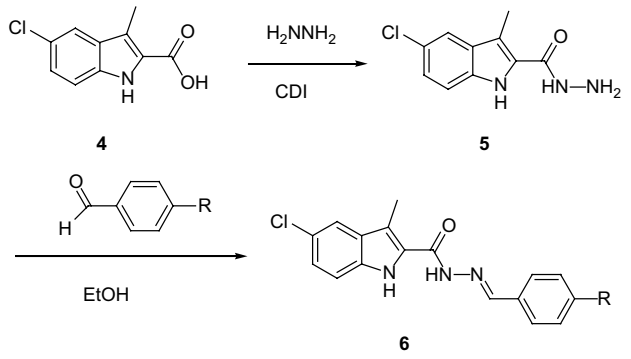


Scheme 1.

2. Results and discussion

2.1. Chemistry

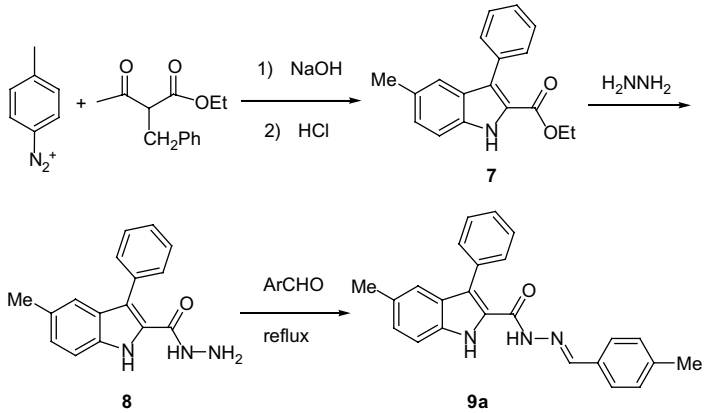
5-Chloro-3-methyl-indole-2-carboxylic acid (4-nitro-benzylidene)-hydrazide (**3a**) was obtained from a commercial source (ChemDiv, San Diego) and its structure was confirmed by NMR and MS. Indole-2-carboxylic acid benzylidene-hydrazides (**6a**–**j**) in general were prepared in two steps as shown in Scheme 1 from the corresponding indole-2-carboxylic acid. Coupling of indole-2-carboxylic acid **4** with hydrazine produced the hydrazide **5**,[12] which was condensed with aromatic aldehydes to produce **6**. 3-Phenyl-indole-2-carboxylic acid benzylidene-hydrazides (**9a**) were prepared as shown in Scheme 2. Treatment of ethyl α-acetyl-β-phenyl propionate with *p*-methylbenzenediazonium chloride,[13] which was prepared from *p*-methylaniline and sodium nitrite, generated ethyl 5-methyl-3-phenyl-indole-2-carboxylate (**7**). Refluxing of **7** with aqueous hydrazine produced 5-methyl-3-phenyl-indole-2-carboxylic acid hydrazide (**8**), which was condensed with an aromatic aldehyde to yield compound **9a**.

2.2. HTS assays

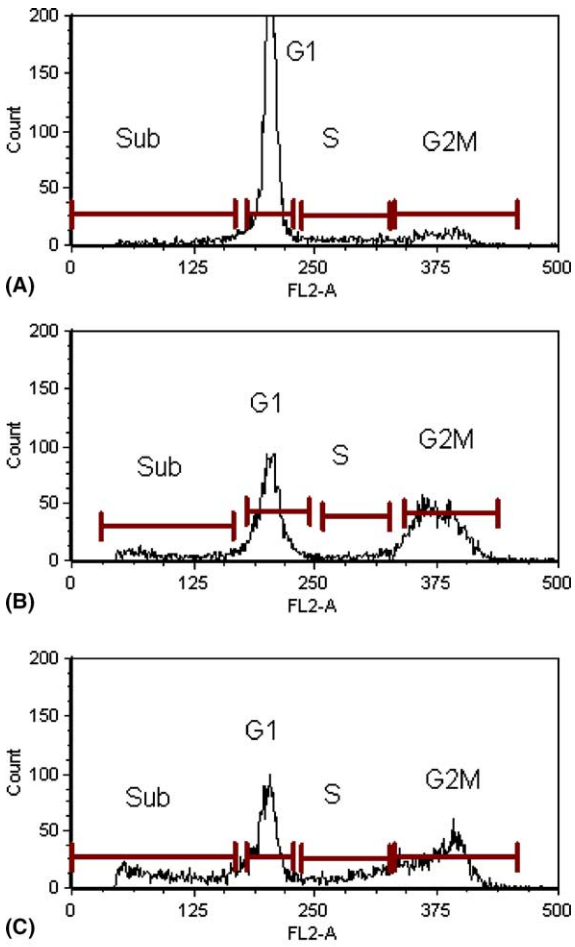
5-Chloro-3-methyl-indole-2-carboxylic acid (4-nitro-benzylidene)-hydrazide (**3a**) was identified as an inducer of apoptosis in T47D breast cancer cells from commercial compound library using our cell-based caspase-3 HTS assay. Briefly, human breast cancer T47D cells, in a 96-well microtiter plate containing 10 μM of testing compound, were incubated for 24 h for the induction of apoptosis. To the treated cells in the well was then added the fluorogenic caspase-3 substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110[9] in a caspase buffer and the sample incubated at room temperature for 3 h. Using a fluorescent plate reader, employing excitation at 485 nm and emission at 530 nm, the fluorescence of cleaved ethoxycarbonyl-R110 was measured, thereby determining the level of caspase activation. Compounds that induce apoptosis and activate the caspases produced a fluorescent signal that is higher than the background (signal/background ratio). Compounds found to give a ratio of >3, are considered active and retested in triplet for confirmation. Compounds confirmed to be active are then tested at several concentrations to give a dose–response and the caspase activation activity EC_{50} is calculated. Compound **3a** has an EC_{50} of 2.2 μM with a maximal ratio of approximately 7 versus untreated cells. This level of caspase activation is similar to that of 6-methyl-*N*-(4-ethoxy-2-nitro-phenyl)-pyridine-3-carboxamide (**1**), which has a ratio of 6, and is less than that of gambogic acid (**2**), which has a ratio of around 14.

2.3. Flow cytometric assay

The apoptosis-inducing activities of compound **3a** were also characterized by the flow cytometric analysis assay. Nuclear fragmentation is one of the hallmarks of caspase-mediated apoptosis.[14] T47D cells were treated with compound **3a** for 24 or 48 h at 37 °C, then labeled by propidium iodide and analyzed on a flow cytometer. An increase in G_2/M DNA content (M4) in cells treated for 24 h with compound **3a** was observed as shown in Figure 1B. At 48 h the sub-diploid DNA content of cells increased from 5% to 20% with compound treatment, indicating apoptotic cells, which have undergone DNA degradation or fragmentation (Fig. 1C). The results show that treatment of T47D cells by compound **3a** for 48 h results in mitotic arrest in the G_2/M phase, as well as induction of apoptosis (M1, apoptotic sub-G_1 area).



Scheme 2.



Entry	Sub	G1	S	G_2M
1A	5.4	70.7	8.3	12.1
1B	9.5	38.1	4.8	41.8
1C	19.5	28.9	13.1	33.5

Figure 1. (A)–(C) depict drug-induced apoptosis in T47D cells as measured by flow cytometric analysis. The *x*-axis is the fluorescence intensity and the *y*-axis is the number of cells with that fluorescence intensity. (A) Control cells showing most of the cells in G1. (B) Cells treated with 5 μM of compound **3a** for 24 h showing most of the cells have arrested in G2/M (M4). (C) Cells treated for 48 h showing a progression from G2/M to in cells with sub-diploid DNA content.

This is supported by the finding that compounds **3a**, as well as more potent analogs, inhibit microtubule assembly.

2.4. Structure–activity relationship (SAR) studies

The cell-based caspase HTS assay was also used for the testing of analogs of compound **3a** for SAR studies. The caspase activation activity (EC_{50}) of compound **3a** and its analogs in three cell lines, T47D breast cancer cells, H-1299 non-small cell lung cancer cells and DLD-1 colon cancer cells are summarized in Table 1.

Table 1 shows that compound **3a** has an EC_{50} of 2.2, 1.4, and 2.0 μM in T47D, H-1299, and DLD-1 cell lines in the caspase activation assay, respectively. For SAR studies, we first explored substitution in the benzene ring. The non-substituted analog **3b** was about 2-times less active than **3a**, suggesting that substitution in the *para*-position might be important for activity. Indeed, the *para*-position of the benzene ring was found to tolerate a variety of groups, from electron-withdrawing groups (NO_2 and Cl) to electron-donating groups (Me, OMe, NMe_2). The methyl substituted analog **6a** was 2-fold more potent than compound **3a**, suggesting that an electron-donating group might be slightly preferred. Interestingly, the *o*-Me-substituted analog **6g** and *m*-Me-substituted analogs **6f** were both not active up to 10 μM, a reduction of potency by at least 10-fold compared to *p*-Me **6a**, suggesting that there might be a size limited pocket around the *ortho*- and *meta*-positions. Similarly, the *m*-NO_2-substituted analog **6e** also was not active up to 10 μM.

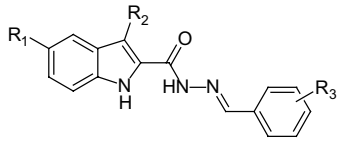
Next, the SAR of substitution in the 3-position of the indole ring was explored. Analog **6h** was >2-times less active than **3a**, suggesting that a substitution at the 3-position of indole is important for apoptotic activity. Analog **9b**, with a 3-phenyl group, was >20-fold more potent than **3a**, indicating that there is a bulky hydrophobic pocket in the 3-position. Analog **9c** was >10-fold more potent than **6c**, confirming that a bulky group in the 3-position is preferred.

We then explored the SAR of substitution at the 5-position of indole, by replacing the chloro group with a nitro and methyl group, respectively. Compound **6i** was about as active as **6b**, indicating that the nitro group is a good replacement for the Cl group. Compound **6j** was 4- to 5-fold more potent than **6b** and **6i** in the T47D cell line, suggesting that an electron-donating group might be preferred in the 5-position. Compound **9a**, with a 5-Me group and a 3-phenyl group, was among the most potent compounds in this series with an EC_{50} value of 0.1 μM in T47D cells.

Selective compounds were also tested by the traditional inhibition of cell proliferation (GI_{50}) assay to confirm that the active compounds can inhibit the growth of tumor cells, as well as to find whether there is a correlation between the activity from the caspase activation assay and the growth inhibition assay. Table 2 summarizes the GI_{50} values in comparison with the caspase activation activity (EC_{50}).

Table 2 shows that compounds **6c** and **9b** both inhibited the growth of T47D and DLD-1 cells, while the inactive **6e** also does not inhibit growth up to 10 μM. Compound **9b** had a GI_{50} value of 0.9 and 0.4 μM in T47D and DLD-1 cancer cells, respectively. In general, compounds more active in the apoptosis induction assay, as measured by caspase activation, also are more potent in the growth inhibition assay. As have been observed with other series of apoptosis inducers,[10,11] these data indicate that the cell-based caspase activation HTS assay is not only useful for the identification of inducers of

Table 1. Caspase activation activity of substituted indole-2-carboxylic acid benzylidene-hydrazides



Entry	R_1	R_2	R_3	EC_{50} (μM)[a]		
				T47D	H-1299	DLD
3a	Cl	Me	*p*-NO_2	2.2 ± 0.2	1.4 ± 0.14	2.0 ± 0.3
3b	Cl	Me	H	4.0 ± 0.4	3.5 ± 0.3	4.3 ± 0.5
6a	Cl	Me	*p*-Me	1.04 ± 0.1	0.7 ± 0.1	0.7 ± 0.4
6b	Cl	Me	*p*-Cl	2.5 ± 0.07	2.0 ± 0.4	1.5 ± 0.07
6c	Cl	Me	*p*-OMe	2.4 ± 0.3	0.98 ± 0.3	2.7 ± 0.09
6d	Cl	Me	*p*-NMe_2	1.3 ± 0.1	2.8 ± 0.4	1.6 ± 0.2
6e	Cl	Me	*m*-NO_2	>10	>10	>10
6f	Cl	Me	*m*-Me	>10	>10	>10
6g	Cl	Me	*o*-Me	>10	>10	>10
6h	Cl	H	*p*-NO_2	5.8 ± 0.4	4.9 ± 0.4	>10
6i	NO_2	Me	*p*-Cl	2.1 ± 0.4	2.6 ± 0.07	2.7 ± 0.1
6j	Me	Me	*p*-Cl	0.5 ± 0.08	1.5 ± 0.1	2.2 ± 0.5
9a	Me	Ph	*p*-Me	0.1 ± 0.03	0.5 ± 0.07	0.4 ± 0.1
9b	Cl	Ph	*p*-NO_2	0.1 ± 0.06	0.2 ± 0.07	0.6 ± 0.1
9c	Cl	Ph	*p*-OMe	0.2 ± 0.03	0.2 ± 0.03	0.4 ± 0.07

[a] Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

Table 2. Comparison of caspase activation activity and inhibition of cell proliferation activity of substituted indole-2-carboxylic acid benzylidene-hydrazides

Compound	EC_{50} (μM)		GI_{50} (μM)	
	T47D	DLD-1	T47D	DLD-1
6c	2.4 ± 0.3	2.7 ± 0.09	3.1 ± 0.7	4.8 ± 2.3
6e	>10	>10	>10	>10
9b	0.1 ± 0.06	0.6 ± 0.1	0.9 ± 0.2	0.4 ± 0.05

Data are the mean of three or more experiments and are reported as mean ± standard error of the mean (SEM).

apoptosis, but also is useful for subsequent optimization and SAR study of the hit found from the screening.

Since compound **3a** was found to be a G_2/M blocker from the flow cytometric assay, we suspected that these series of compounds might be tubulin inhibitors. We therefore tested selective compounds in an in vitro tubulin polymerization assay. Compound **3a**, with an EC_{50} value of 2 μM in the caspase activation assay, inhibited tubulin polymerization with an IC_{50} value of 10 μM. Compounds **9a**, **9b**, and **9c**, which are all with an EC_{50} value of less than 0.2 μM and at least 10-fold more potent than **3a** in the caspase assay, all inhibited tubulin polymerization with IC_{50}s at least 10-fold more active than compound **3a**, at or below the level of sensitivity for the assay of 1 μM. Therefore the caspase activation potency is correlated with inhibition of tubulin polymerization potency for the indole-2-carboxylic acid benzylidene-hydrazide series of apoptosis inducers, suggesting that inhibition of microtubule assembly most probably is the primary mechanism of action of this class of compounds. The interference with microtubule function represents an important mechanistic principle in anticancer drug discovery that underlies the antitumor activity of a variety of clinical validated anticancer agents,[15] such as taxanes, a class of microtubule stabilizer and vincas, a class of microtubule depolymerizer.

3. Conclusion

In conclusion, by applying a cell-based caspase HTS assay, a series of indole-2-carboxylic acid benzylidene-hydrazides has been identified as a new class of potent inducers of apoptosis. SAR studies indicate that the substitution at 3-position of the indole ring was important for apoptotic activity. A 20-fold increase of apoptotic activity was achieved from screening hit 5-chloro-3-methyl-indole-2-carboxylic acid (4-nitrobenzylidene)-hydrazide (**3a**) to compounds **9a** and **9b** with an EC_{50} of 0.1 μM in the caspase activation assay in T47D breast cancer cells. Compounds that are more potent in the caspase activation also were found to be more potent in the cell proliferation assay. Compound **3a** arrested T47D cells in the G_2/M phase, and induced apoptosis as measured by the flow cytometric analysis. Compound **3a** and its analogs were found to inhibit tubulin polymerization, and the more potent compounds **9a**–**c** were also more potent inhibitors of tubulin polymerization,

suggesting that inhibition of tubulin polymerization might be the primary mechanism of action for these compounds as inducers of apoptosis.

4. Experimental section

4.1. General methods and materials

The ^{1}H NMR spectra were recorded at Varian 300 MHz. Chemical shifts are reported in ppm (δ) and J coupling constants are reported in Hz. Elemental analyses were performed by Numega Resonance Labs, Inc. (San Diego, CA). Reagent grade solvents were used without further purification unless otherwise specified. Compounds **3a**, **3b**, **6c**, **6i**, **6j**, and **9c** were purchased from ChemDiv (San Diego, CA). Compound **9b** was purchased from Chemstar (Moscow, Russia), and their structures were confirmed by ^{1}H NMR and MS. Human breast cancer cell T47D, human non-small cell lung cancer cells H-1299 and human colon cancer cells DLD-1 were obtained from American Type Culture Collection (Manassas, VA).

4.1.1. 5-Chloro-3-methyl-indole-2-carboxylic acid (4-methylbenzylidene)-hydrazide (6a). A solution of 5-chloro-3-methyl-indole-2-carboxylic acid hydrazide (13 mg, 0.054 mmol), *p*-tolualdehyde (6.5 mg, 0.054 mmol) in ethanol (5 mL) was refluxed for 8 h. It was evaporated in vacuo and the residue was purified by column chromatography (silica gel, EtOAc/hexane = 4:1) to give 7.8 mg (42%) of **6a**. ^{1}H NMR (CD_3OD): 8.26 (s, 1H), 7.72 (d, $J = 8.1$, 2H), 7.62 (s, 1H), 7.39 (d, $J = 8.7$, 1H), 7.27 (d, $J = 8.1$, 2H), 7.25 (m, 1H), 2.58 (s, 3H), 2.39 (s, 3H). Anal. Calcd for $C_{18}H_{16}ClN_3O$: C, 66.36; H, 4.95; N, 12.90. Found: C, 66.02; H, 4.98; N, 12.88.

Compounds **6b**, **6d**–**h** were prepared by a method similar to that described for the preparation of compound **6a** from 5-chloro-3-methyl-indole-2-carboxylic acid hydrazide and the corresponding substituted benzaldehyde.

4.1.2. 5-Chloro-3-methyl-indole-2-carboxylic acid (4-chlorobenzylidene)-hydrazide (6b). (22%). ^{1}H NMR (CD_3OD): 8.90 (s, 1H), 8.18 (br s, 1H), 7.70 (d, $J = 7.80$, 2H), 7.50 (s, 1H), 7.32 (d, $J = 7.80$, 2H), 7.27 (m, 1H), 7.10 (m, 1H), 2.45 (s, 3H). Anal. Calcd for $C_{17}H_{13}Cl_2N_3O$: C, 58.98; H, 3.78; N, 12.14. Found: C, 58.15; H, 3.44; N, 12.27.

4.1.3. 5-Chloro-3-methyl-indole-2-carboxylic acid (4-dimethylaminobenzylidene)-hydrazide (6d). (21%). ^{1}H NMR (CD_3OD): 10.9 (br s, 1H), 9.07 (br s, 1H), 8.2–6.6 (m, 6H), 3.06 (s, 6H), 2.70 (s, 3H). Anal. Calcd for $C_{19}H_{19}ClN_4O$: C, 75.73; H, 6.10; N, 14.10. Found: C, 75.35; H, 6.28; N, 14.11.

4.1.4. 5-Chloro-3-methyl-indole-2-carboxylic acid (3-nitrobenzylidene)-hydrazide (6e). (44%). ^{1}H NMR (DMSO-d_6): 11.8 (br s, 1H), 11.6 (br s, 1H), 8.57 (s, 1H), 8.47 (br s, 1H), 8.27 (d, $J = 7.5$, 1H), 8.17 (d, $J = 7.5$, 1H), 7.80 (t, $J = 8.4$, 1H), 7.72 (s, 1H), 7.46 (d, $J = 8.4$, 1H), 7.24 (m, 1H), 2.51 (s, 3H). Anal. Calcd for $C_{17}H_{13}ClN_4O_3{\cdot}2H_2O$: C, 51.98; H, 4.36; N, 14.26 Found: C, 51.92; H, 4.41; N, 14.03.

4.1.5. 5-Chloro-3-methyl-indole-2-carboxylic acid (3-methylbenzylidene)-hydrazide (6f). (20%). ^{1}H NMR (DMSO-d_6): 11.5 (br s, 2H), 8.65 (br s, 1H), 7.84 (br s, 1H), 7.71 (br s, 1H), 7.46 (s, 1H), 7.44 (d, $J = 8.7$Hz, 1H), 7.34–7.20 (m, 3H), 2.50 (s, 3H), 2.45 (s, 3H). Anal. Calcd for $C_{18}H_{16}ClN_3O{\cdot}0.2H_2O$: C, 65.63; H, 5.00; N, 12.76. Found: C, 65.62; H, 4.68; N, 12.40.

4.1.6. 5-Chloro-3-methyl-indole-2-carboxylic acid (2-methylbenzylidene)-hydrazide (6g). (34%). ^{1}H NMR (DMSO-d_6): 11.46 (br s, 2H), 8.65 (br s, 1H), 7.88 (br s, 1H), 7.71(s, 1H), 7.42 (d, $J = 8.7$, 1H), 7.34–7.22 (m, 4H), 2.50 (s, 3H), 2.45 (s, 3H). Anal. Calcd for $C_{18}H_{16}ClN_3O$: C, 66.36; H, 4.95; N, 12.90. Found: C, 65.99; H, 4.82; N, 12.53.

4.1.7. 5-Chloro-indole-2-carboxylic acid (4-nitrobenzylidene)-hydrazide (6h). (60%). ^{1}H NMR (DMSO-d_6): 12.3 (br s, 1H), 12.1 (br s, 1H), 8.55 (s, 1H), 8.32 (d, $J = 8.7$, 2H), 8.03 (d, $J = 8.7$, 2H), 7.82 (s, 1H), 7.48 (d, $J = 8.4$, 1H), 7.36 (s, 1H), 7.25 (d, $J = 8.4$, 1H), 7.24 (m, 1H), 2.51 (s, 3H). Anal. Calcd. for $C_{16}H_{11}ClN_4O_3$: C, 56.07; H, 3.23; N, 16.35. Found: C, 55.97; H, 3.51; N, 16.46.

4.1.8. Ethyl 5-methyl-3-phenyl-indole-2-carboxylate (7). A solution of sodium hydroxide (6 g) in water (15 mL) was added to a solution of ethyl α-acetyl-β-phenyl propionate (11 g, 0.05 mmol) in ethanol (80 mL), followed immediately by addition of a solution of *p*-methylbenzenediazonium chloride (prepared from 4.65 g of *p*-methylaniline and 3.45 g of sodium nitrite in 25 mL of concentrated HCl), resulted in the precipitation of brown oil. The mixture was diluted by water (100 mL) and it was extracted with ethyl acetate (3 × 50 mL). The extracts were dried and evaporated to give a crude intermediate hydrazone. A solution of the crude hydrazone in hydrogen chloride saturated methanol (100 mL) was refluxed for 4 h. It was concentrated and the residue was purified by column chromatography ($EtOAc/CH_2Cl_2 = 4:1$) to give 3.2 g (24%) of **7**. ^{1}H NMR ($CDCl_3$): 8.90 (br s, 1H), 7.60–7.20 (m, 8H), 4.28 (q, 2H), 2.41 (s, 3H), 1.23 (t, $J = 7.2$, 3H).

4.1.9. 5-Methyl-3-phenyl-indole-2-carboxylic acid hydrazide (8). A suspension of **7** in H_2NNH_2/H_2O (4:1, 15 mL) was refluxed for 7 h. It was cooled to room temperature and concentrated. The residue was diluted with water (50 mL) and it was extracted with ethyl acetate (3 × 20 mL). The extracts were dried and evaporated,

and the crude product was washed with hexane, and dried to give 1.4 g (99%) of **8**. ^{1}H NMR ($CDCl_3$): 9.20 (br s, 1H), 7.60–7.05 (m, 8H), 2.39 (br s, 2H), 2.39 (s, 3H).

4.1.10. 5-Methyl-3-phenyl-indole-2-carboxylic acid (4-methylbenzylidene)-hydrazide (9a). Compound **9a** was prepared by a method similar to that described for compound **6a** from **8** and *p*-tolualdehyde (44%). ^{1}H NMR ($CDCl_3$): 9.42 (br s, 1H), 8.85 (s, 1H), 7.60–7.57 (m, 7H), 7.40 (d, $J = 8.4$, 1H), 7.26–7.15 (m, 4H), 2.41(s, 3H), 2.36 (s, 3H). Anal. Calcd for $C_{24}H_{21}N_3O{\cdot}0.5H_2O$: C, 76.57; H, 6.20; N, 10.80. Found: C, 76.80; H, 5.89; N, 11.16.

4.2. Caspase activation assay (EC_{50})

The potency of indole-2-carboxylic acid benzylidene-hydrazides analogs as inducers of apoptosis was measured by our caspase-based cell assay as previously described.[10] Briefly, human breast cancer cell lines T47D, human non-small cell lung cancer cells H-1299, and human colon cancer cell line DLD-1 were treated with various concentrations of test compound and incubated at 37 °C for 24 h. The samples were then treated with the fluorogenic substrate *N*-(Ac-DEVD)-*N*′-ethoxycarbonyl-R110, and incubated for 3 h. The fluorescent signal was measured using a fluorescent plate reader (Model Spectrafluor Plus Tecan). The EC_{50} (μM) was determined by a sigmoidal dose–response calculation (XLFit3, IDBS), as the concentration of compound that produces the 50% maximum response. The caspase activation activity (EC_{50}) in three cancer cell lines, T47D, H-1299 and DLD, are summarized in Table 1.

4.3. Flow cytometric analysis assay

Cell cycle analysis was performed as previously described.[10] Briefly, T47D cells were treated with 10 μM of compound **3a** for 24–48 h at 37 °C. Control cells were treated with the solvent (DMSO) for 48 h. After the incubation, cells were treated with propidium iodide and RNAse A, and analyzed on a flow cytometer. All flow cytometry analyses were performed on FACScalibur (Becton Dickinson) using Cell Quest analysis software. On the *x*-axis is plotted the fluorescence intensity and on the *y*-axis is plotted the number of cells with that fluorescence intensity.

4.4. Cell growth inhibition assays (GI_{50})

The potency of indole-2-carboxylic acid benzylidene-hydrazides analogs as inhibitors of cell proliferation was measured as previously described.[10] Briefly, T47D and DLD-1 cells were treated with various concentrations of test compound at 37 °C for 48 h, then treated with CellTiter-Glo™ reagent (Promega). The samples were mixed by agitation and incubated at room temperature for 10–15 min. Plates were then read using a luminescent plate reader (Model Spectrafluor Plus Tecan Instrument). GI_{50} values were calculated from dose–response curves using XLFit3 (IDBS) software. The GI_{50} (μM) are summarized in Table 2 in comparison with the caspase activation activity (EC_{50}).

4.5. Tubulin depolymerization assay (IC_{50})

Lyophilized tubulin (Cytoskeleton #ML113, 1 mg, MAP-rich) was assayed for the effect of the test compound on tubulin polymerization as measured by change in fluorescence for 4′,6-diamidino-2-phenylindole (DAPI).[16] One microliter of serial dilutions of each experimental compound (from 100× DMSO stock) was added in 96 well plate format and preincubated for 30 min with 94 μL of the non-GTP supplemented tubulin supernatant. Five microliters of DAPI/GTP solution was added to initiate polymerization and incubated for 30 min at 37 °C. Fluorescence was read with excitation 350 nm, emission wavelength 485 nm on a Tecan Spectraflour Plus. Polymerized tubulin (sample treated with DMSO and sample treated with the tubulin stabilizer taxol) gives a higher DAPI fluorescence as compared to non-polymerized tubulin (vinblastine and colchicine were used to determine baseline). The IC_{50} for tubulin inhibition was the concentration found to decrease the fluorescence of DAPI by 50% as calculated with Prism 3.0.

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