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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.5b00310 • Publication Date (Web): 01 Jul 2015

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New Indole Tubulin Assembly Inhibitors Cause Stable Arrest of Mitotic Progression, Enhanced Stimulation of Natural Killer Cell Cytotoxic Activity and Repression of Hedgehog-dependent Cancer

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32 In memory of Prof. Alberto Gulino,[°] an outstanding scientist and a dear colleague

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3 ABSTRACT
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6 We designed 39 new 2-phenylindole derivatives as potential anticancer agents bearing the 3,4,5-
7 trimethoxyphenyl moiety with a sulfur, ketone or methylene bridging group at position 3 of the indole
8 and with halogen or methoxy substituent(s) at positions 4-7. Compounds **33** and **44** strongly inhibited
9 the growth of the P-glycoprotein-overexpressing multidrug resistant cell lines NCI/ADR-RES and
10 Messa/Dx5. At 10 nM, **33** and **44** stimulated the cytotoxic activity of NK cells. At 20-50 nM, **33** and **44**
11 arrested >80% of HeLa cells in the G2/M phase of the cell cycle, with stable arrest of mitotic
12 progression. Cell cycle arrest was followed by cell death. Indoles **33**, **44** and **81** showed strong
13 inhibition of the SAG-induced Hedgehog signaling activation in NIH3T3 Shh-Light II cells with IC₅₀'s
14 of 19, 72 and 38 nM, respectively. Compounds of this class potently inhibited tubulin polymerization
15 and cancer cell growth, including stimulation of natural killer cell cytotoxic activity and repression of
16 Hedgehog-dependent cancer.
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INTRODUCTION

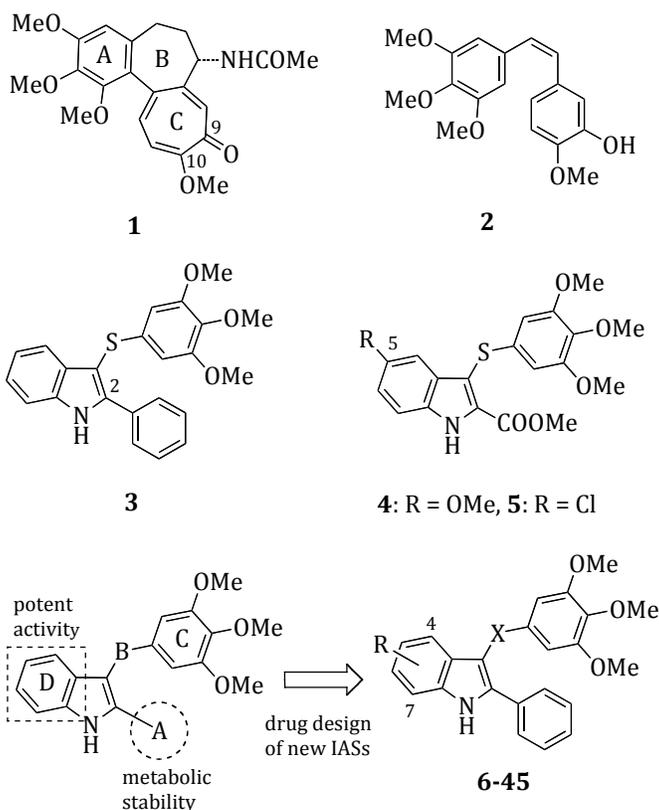
Microtubules (MTs) are cylindrical structures mainly composed of α,β -tubulin heterodimers. MT assembly is a highly dynamic process because of continuous transitions between polymerization and depolymerization. MTs are involved in a number of essential cellular functions, such as maintenance of cell shape, cell motility, intracellular transport and cell division. Interfering with the MT dynamic equilibrium, by either inhibiting tubulin polymerization or blocking MT disassembly, prevents proper MT function and ultimately leads to cell death. Interfering with these cellular processes has resulted in a productive strategy for the development of efficient anticancer agents.¹⁻⁴

Colchicine (**1**),^{5,6} combretastatin A-4 (CSA4, **2**)⁷ (Chart 1) and the *Catharanthus* alkaloids vincristine (VCR) and vinblastine (VBL) inhibit MT assembly by preventing tubulin polymerization, and this leads cell to death. In contrast, taxoids and epothilones bind at a luminal site on the β -subunit^{8,9} following entry into the MT through pores in its wall¹⁰ that are shaped by various tubulin subunits on the MT surface. Some evidence indicates a transient binding of MT stabilizing agents at a specific pore site. Paclitaxel (PTX) stimulates MT polymerization and stabilization at high concentrations, whereas lower concentrations of PTX inhibit MT dynamics with little effect on the proportion of tubulin in polymer.¹¹

The development of MT-targeting drugs with different mechanisms of action¹² has achieved substantial progress. However, drug resistance, toxicity and unwanted side effects still remain unsolved problems. Therefore, the quest for new MT inhibitors as components of improved anticancer treatments remains mandatory.¹³

Arylthioindole (ATI) derivatives are potent inhibitors of tubulin polymerization that bind to the colchicine site on β -tubulin. A number of ATIs proved to be more potent than **1**, **2**, VBL, and PTX and thus are potential new anticancer agents.^{14,15}

Chart 1. General Structures of Compounds 1–45.



ATI derivatives bearing an aromatic ring at position 2 of the indole (A region) inhibit tubulin polymerization and cancer cell growth. These compounds hamper mitotic progression, thus causing cells to undergo apoptosis (i.e., ATI 3: tubulin assembly $IC_{50} = 3.3 \mu\text{M}$; MCF-7 $IC_{50} = 52 \text{ nM}$)^{14,15} (Chart 1). In contrast, chemical modification of positions 4-7 of the indole (D region) were not exhaustively explored, although a few ATI derivatives bearing an ethoxycarbonyl functionality at position 2 and a halogen atom or a methoxy group at position 5 proved to be potent tubulin assembly inhibitors (i.e., ATI 4: tubulin assembly $IC_{50} = 2.0 \mu\text{M}$; MCF-7 $IC_{50} = 13 \text{ nM}$).¹⁶ It should also be noted that major differences in structure-activity relationships (SAR) were found in comparing ATIs with the 3-aryloindoles reported by Hsieh and co-workers.¹⁷ These compounds are characterized by the presence of a methoxy group at position 6 of the indole.

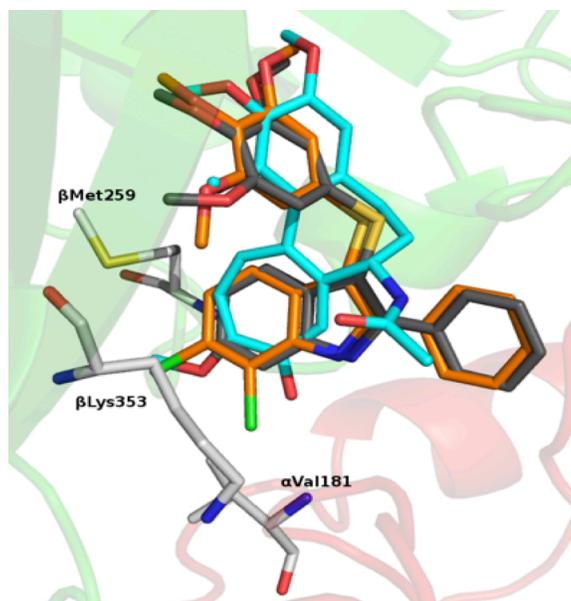
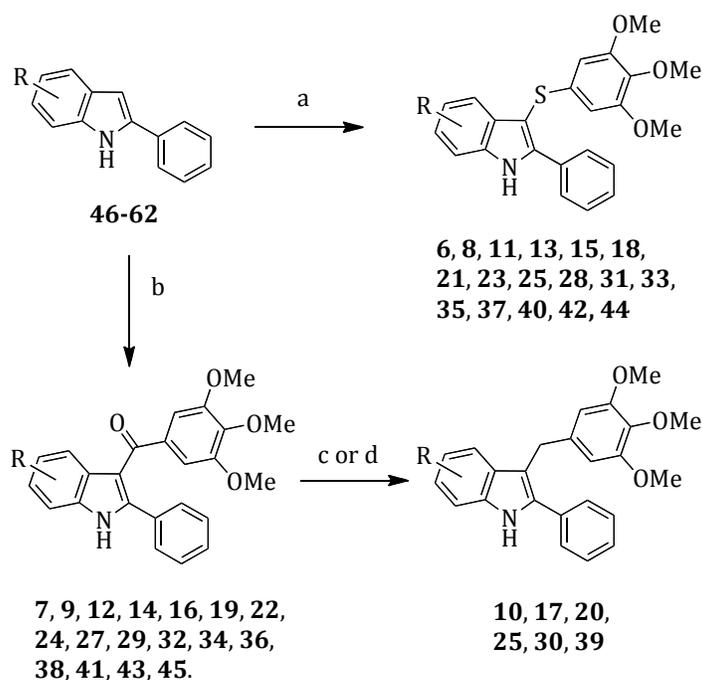


Figure 1. Proposed binding modes of **1** (cyan), **3** (gray) and **44** (orange). Tubulin is represented as a cartoon for the α - (red) and β - (green) subunits. Residues forming interactions with the ATI D region are depicted in white.

Docking studies of compounds **1** and **3** into the colchicine site of tubulin showed a good superimposition of the trimethoxyphenyl (TMP) moieties and of the tropolone ring C with the phenyl core of the indole^{14,15} (Figure 1). These findings suggested that appropriate substituents at positions 4-7 of the indole could resemble the methoxy and carbonyl groups at positions 10 and 9, respectively, of the tropolone ring. Docking simulations with PLANTS,¹⁸ using our methodology described previously,¹⁹ revealed binding poses consistent with the previous ATI series: (i) the TMP group formed polar interactions with β Cys241, β Met259 and β Leu255; (ii) the indole NH established an H-bond with α Thr179; (iii) the phenyl ring at position 2 of the indole set up hydrophobic interactions with the β Lys254 and β Leu248 side chains. In comparison with **3**, the chlorine atom(s) of **10**, **18**, **28**, **37** and **44** fitted into a new hydrophobic pocket formed by the β Lys353, β Asn258, β Met259 and α Val181 residue side chains (Figure 1S, Supporting Information). The 7-fluoro atom of **40**, the most potent tubulin polymerization inhibitor within the series, behaved as a H-bond acceptor with α Val181 (Figure 2S, Supporting Information). The methoxy group of **33** mimicked the corresponding group at position 10 of **1**, resembling its interaction with the ϵ N of β Lys353.

Scheme 1. Synthesis of Compounds 6-45.



6, 7, 46, R = 4-Br; 8-10, 47, R = 4-Cl; 11, 12, 48, R = 4-F; 13, 14, 49, R = 4-OMe; 15-17, 50, R = 5-Br; 18-20, 51, R = 5-Cl; 21, 22, 52, R = 5-F; 23-25, 53, R = 5-OMe; 26, 27, 54, R = 6-Br; 28-30, 55, R = 6-Cl; 31, 32, 56, R = 6-F; 33, 34, 57, R = 6-OMe; 35, 36, 58, R = 7-Br; 37-39, 59, R = 7-Cl; 40, 41, 60, R = 7-F; 42, 43, 61, R = 7-OMe; 44, 45, 62, R = 6,7-Cl₂.

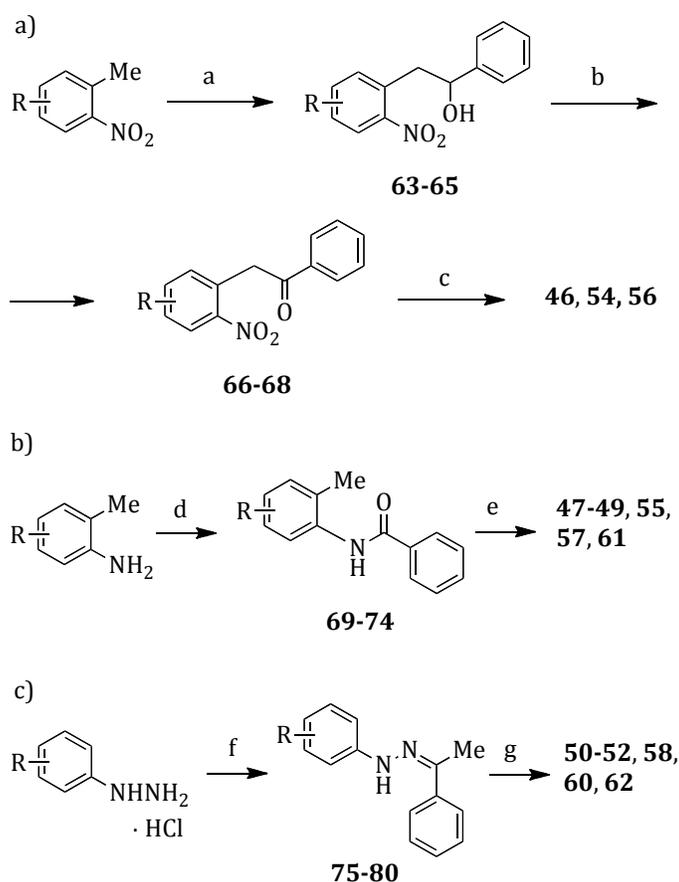
^aReagents and reaction conditions: (a) bis-(3,4,5-trimethoxyphenyl)disulfide, anhydrous DMF, closed vessel, 130 °C, 120 W, 2 min, venting-while-heating, 20-77%; (b) 3,4,5-trimethoxybenzoyl chloride, anhydrous AlCl₃, 1,2-dichloroethane, closed vessel, 110 °C, 150 W, 2 min, 20-78%; (c) (**10, 20, 25, 30** and **39**) borane tetrahydrofuran complex, acetonitrile/methanol, 50 °C, 1 h, 10-41%; (d) (**17**) triethylsilane, trifluoroacetic acid, 1,2-dichloroethane, 250 °C, 250 W, 20 min, 15%.

These observations prompted us to undertake SAR investigations at positions 4-7 of the indole (Chart 1 and Table 1). We planned the synthesis of 2-phenyl-1*H*-indole derivatives, keeping the 2-phenyl group fixed at position 2, because of the better metabolic profile compared to indole derivatives bearing an ester function at this position.¹⁵

CHEMISTRY

2-Phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1*H*-indoles **6**, **8**, **11**, **13**, **15**, **18**, **21**, **23**, **25**, **28**, **31**, **33**, **35**, **37**, **40**, **42** and **44** were prepared by reacting 2-phenylindoles **46-62** with bis-(3,4,5-trimethoxyphenyl)disulfide¹⁶ in the presence of sodium hydride at 130 °C (120 W) for 2 min according to our previously reported venting-while-heating microwave-assisted procedure²⁰ (Scheme 1). Microwave-assisted Friedel-Crafts reaction of the indoles **46-62** with 3,4,5-trimethoxybenzoyl chloride in the presence of AlCl₃ in 1,2-dichloroethane at 110 °C (150 W) for 2 min furnished the corresponding methanones **7**, **9**, **12**, **14**, **16**, **19**, **22**, **24**, **27**, **29**, **32**, **34**, **36**, **38**, **41**, **43** and **45**.²¹ Methylene derivatives **10**, **17**, **20**, **25**, **30** and **39** were prepared by reducing the appropriate ketones with borane tetrahydrofuran complex in acetonitrile/methanol at 50 °C for 1 h²² (**10**, **20**, **25**, **30** and **39**) or with triethylsilane/trifluoroacetic acid in 1,2-dichloroethane at 250 °C (250 W) for 20 min²³ (**17**).

Reaction of the appropriate 1-methyl-2-nitrobenzene with benzaldehyde in the presence of sodium ethoxide in anhydrous dimethyl sulfoxide at 25 °C for 12 h furnished the alcohols **63-65**. After oxidation of **63-65** to ketones **66-68** with pyridium chlorochromate in anhydrous dichloromethane at 25 °C for 1.5 h, the nitro group was reduced to amino with tin(II) chloride dihydrate/1 N HCl in acetic acid at reflux temperature for 12 h; the intermediate aminoketone underwent intramolecular cyclization to give the corresponding 2-phenylindoles **46**, **54** and **56** (Scheme 2a).²⁴ 2-Phenylindoles **47-49**, **55**, **57** and **61** were prepared by reaction of the *N*-(2-tolyl)benzamides **69-74** with *tert*-butyllithium in anhydrous tetrahydrofuran at -40 °C.²⁵ Amides **69-74** were obtained by treatment of an appropriate *ortho*-toluidine with benzoyl chloride in the presence of triethylamine in anhydrous tetrahydrofuran at reflux temperature for 2 h (Scheme 2b). Polyphosphoric acid-mediated cyclization at 110 °C for 1 h of phenylhydrazones **75-80**, prepared from the appropriate phenylhydrazine hydrochloride and acetophenone in the presence of sodium acetate in ethanol at 80 °C (250 W) for 5 min,²⁶ gave the corresponding 2-phenylindoles **50-52**, **58**, **60** and **62**²⁷ (Scheme 2c).

Scheme 2. Synthesis of Intermediates 46-62.^a

63, 66, R = 2-Br; **64, 67**, R = 4-Br; **65, 68**, R = 4-F; **69**, R = 3-Cl; **70**, R = 3-F; **71**, R = 3-OMe; **72**, R = 5-Cl; **73**, R = 5-OMe; **74**, R = 2-OMe; **75**, R = 4-Br; **76**, R = 4-Cl; **77**, R = 4-F; **78**, R = 2-Br; **79**, R = 2-F; **80**, R = 5,6-Cl₂.

^aReagents and reaction conditions: (a) benzaldehyde, sodium ethoxide, anhydrous DMSO, 25 °C, 12 h, 40-44%; (b) pyridium chlorochromate, anhydrous dichloromethane, 25 °C, 1.5 h, 16-40%; (c) tin(II) chloride dihydrate/1 N HCl, acetic acid, reflux temperature, 12 h, 19-24%; (d) benzoyl chloride, triethylamine, anhydrous THF, reflux temperature, 2 h, 49-88%; (e) (i) *tert*-butyllithium, anhydrous THF, 40 °C, Ar stream; (ii) 1 h, 0 °C; (iii) 25 °C, 12 h, 18-52%; (f) acetophenone, sodium acetate, ethanol, open vessel, 80 °C, 250 W, 5 min, cooling-while-heating, 25-95%; (g) polyphosphoric acid, 110 °C, 1 h, 25-82%.

RESULTS AND DISCUSSION

Inhibition of Tubulin Polymerization, the Binding of Colchicine to Tubulin and MCF-7 Breast Cancer Cell Growth. We synthesized compounds **6-45**, including ATI derivatives and some corresponding ketone and methylene compounds, to obtain SAR information on the substituent(s) introduced at positions 4-7 of the indole nucleus. The activities of compounds **6-45** as inhibitors of tubulin polymerization in vitro, the growth of MCF-7 human breast cancer cells and the binding of [³H]colchicine to tubulin are shown in Table 1. The majority of these new derivatives (23 compounds: **8, 11, 12, 15, 18, 21, 26-30, 33-38** and **40-45**) inhibited tubulin polymerization with IC₅₀ values in the 1.0–2.0 μM range, as compared with colchicine (**1**) (IC₅₀ = 3.2 μM) and CSA4 (**2**) (IC₅₀ = 1.0 μM).

Among the group of compounds **6-14**, bearing the substituent at position 4 of the indole, the 4-chloro derivative **8** inhibited tubulin assembly with an IC₅₀ of 1.6 μM and was 2.2-fold more active than corresponding ketone **9** (IC₅₀ = 3.6 μM). The thio/keto 4-fluoro derivatives **11** (IC₅₀ = 1.7 μM) and **12** (IC₅₀ = 1.6 μM) were almost equipotent as tubulin assembly inhibitors and showed the greatest inhibition of MCF-7 cell growth (**11**, IC₅₀ = 80 nM, and **12**, IC₅₀ = 65 nM). Neither the bromine atom nor the methoxy group were found among the most active compounds with position 4 substituents in both the thio and ketone series. In terms of effects on MCF-7 cell growth, there is no evident major difference between the thio and keto series with substituents at position 4, although the data are limited. Compounds **15-25** were characterized by substituents at position 5 of the indole nucleus. As tubulin polymerization inhibitors, the arylthioindoles **15** (IC₅₀ = 1.3 μM), **18** (IC₅₀ = 1.5 μM), **21** (IC₅₀ = 1.5 μM) and **23** (IC₅₀ = 2.1 μM) were all significantly more potent than the corresponding ketones **16, 19, 22** and **24** and slightly more active than the methylene compounds **17, 20** and **25**. The tubulin inhibitory activity of these compounds seemed to be weakly affected by the nature of the substituent. In contrast to the corresponding ester derivatives, all these compounds were weak inhibitors of MCF-7 cell growth with IC₅₀'s ≥100 nM (compare **4** (IC₅₀ = 13 nM) with **23**, and **5** (IC₅₀ = 42 nM) with **18**).

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3 Further, within the limitations of the data, there are no major differences in effects on MCF-7 cell
4 growth dependent on the bridging group (thio, ketone or methylene).
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8 Compounds **26-34**, bearing the substituent at position 6, inhibited tubulin polymerization with IC_{50} 's
9 $< 2.5 \mu\text{M}$, with the exception of the 6-fluoro derivative **32** ($IC_{50} = 10 \mu\text{M}$). As inhibitors of MCF-7 cell
10 growth, ketones were less potent than the corresponding thio derivatives. Among the thio derivatives,
11 the 6-bromo- (**26**, $IC_{50} = 9.0 \text{ nM}$) and 6-methoxy- (**33**, $IC_{50} = 1.3 \text{ nM}$) derivatives were the most potent
12 cell growth inhibitors. Compounds **35-43**, with the substituent at position 7 of the indole, were all
13 potent inhibitors of tubulin assembly, with IC_{50} 's $< 2.5 \mu\text{M}$. With the exception of **39** and **43**, these
14 compounds potently inhibited the growth of the MCF-7 cells, with compounds **36** ($IC_{50} = 4 \text{ nM}$) and **38**
15 ($IC_{50} = 9 \text{ nM}$) being the most active. In contrast to the findings with the 6-substituted indoles, in the 7-
16 substituted series, the bromo and chloro ketones tended to be more potent than the corresponding thio
17 counterparts as inhibitors of MCF-7 cell growth (compare **26**, **27** with **35**, **36**; and **28**, **29** with **37**, **38**).
18 Finally, we tried to join the features of **28**, **29** with those of **37**, **38** to further enhance activity. This
19 effort yielded the 6,7-dichloroindole derivatives **44** and **45**, both of which indeed were highly potent
20 inhibitors of tubulin polymerization ($IC_{50} = 1.2$ and $1.5 \mu\text{M}$, respectively) and MCF-7 cell growth (IC_{50}
21 $= 7$ and 15 nM , respectively). SAR summary of tubulin polymerization inhibition (TPI) and inhibition
22 of MCF-7 cell growth of ATI derivatives **6-45** is depicted in Figure 2.
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43 The results obtained from the docking simulations described above, provided us with a general
44 binding mode that was able to justify the biological activity of the compounds. However, there were
45 some exceptions that could not be fully rationalized (**7**, **13**, **14** and **16**) and we did not see any
46 correlation between docking scores and experimental data. This has prompted us to further investigate
47 the binding mode of ATIs with another set of docking simulations using a more recent tubulin structure
48 (pdb code 4O2A),²⁹ crystallized at higher resolution than the structure used previously (2.50\AA vs 3.58\AA
49 respectively).²⁹ Furthermore, we have performed the docking calculations with two other software that
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3 each rely on a different search algorithm: Glide³⁰ and Autodock.³¹
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5 The docking results obtained from these simulations were entirely consistent with the one obtained
6 using PLANTS. Indeed, we observed a virtually identical binding pose for the ATIs, regardless of the
7 tubulin structure or the algorithm used in the simulation (Figure 3S, Supporting Information). It should
8 be noted that we have not observed any correlation between the docking score and the experimental
9 data in any of the calculations (Table 1S, Supporting Information). This observation is not entirely
10 surprising considering this correlation is rarely observed.²⁸
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20 We analyzed the data from Table 1 comparing the microtubule assembly inhibitory concentrations
21 (mM) with % inhibition of colchicine binding since these data provide an indirect measure of the
22 affinity of the compounds for the colchicine site (Figure 3). The inhibition of tubulin polymerization
23 was in good agreement with inhibition of colchicine binding. The compounds that inhibited tubulin
24 assembly with IC₅₀ values in the 1.0-1.5 μM range, inhibited colchicine binding by 39-96% (mean
25 value of 79%); those that inhibited assembly with IC₅₀'s in the 1.6-2.0 μM range, inhibited colchicine
26 binding by 48-82% (mean value of 70%); and assembly inhibitors with IC₅₀'s in the 2.1-3.0 μM range,
27 inhibited colchicine binding by 33-74% (mean value of 53%) (CSA4: assembly IC₅₀ = 1.0 μM,
28 colchicine binding inhibition = 98%). We analyzed the MCF-7 cell growth inhibition (nM) of ATIs **6-**
29 **45** with the corresponding inhibitory concentration (μM) on tubulin assembly (Panel A, Figure 4S,
30 Supporting Information) and with % inhibition on the binding of [³H]colchicine to tubulin (Panel B,
31 Figure 4S, Supporting Information). In these plots we observed good correlation of the antiproliferative
32 data with the biochemical data, particularly with the ligand binding data (inhibition of colchicine
33 binding).
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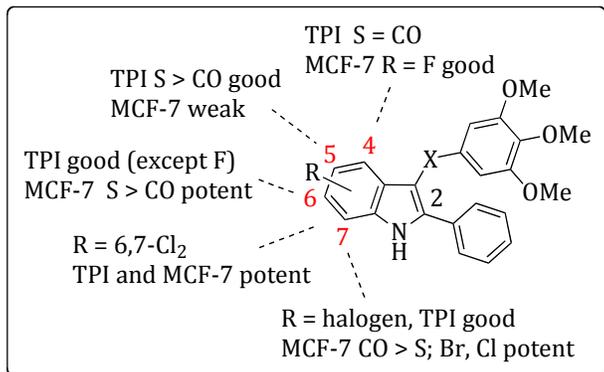


Figure 2. SAR summary of tubulin polymerization inhibition (TPI) and inhibition of MCF-7 cell growth of ATI derivatives **6-45**.

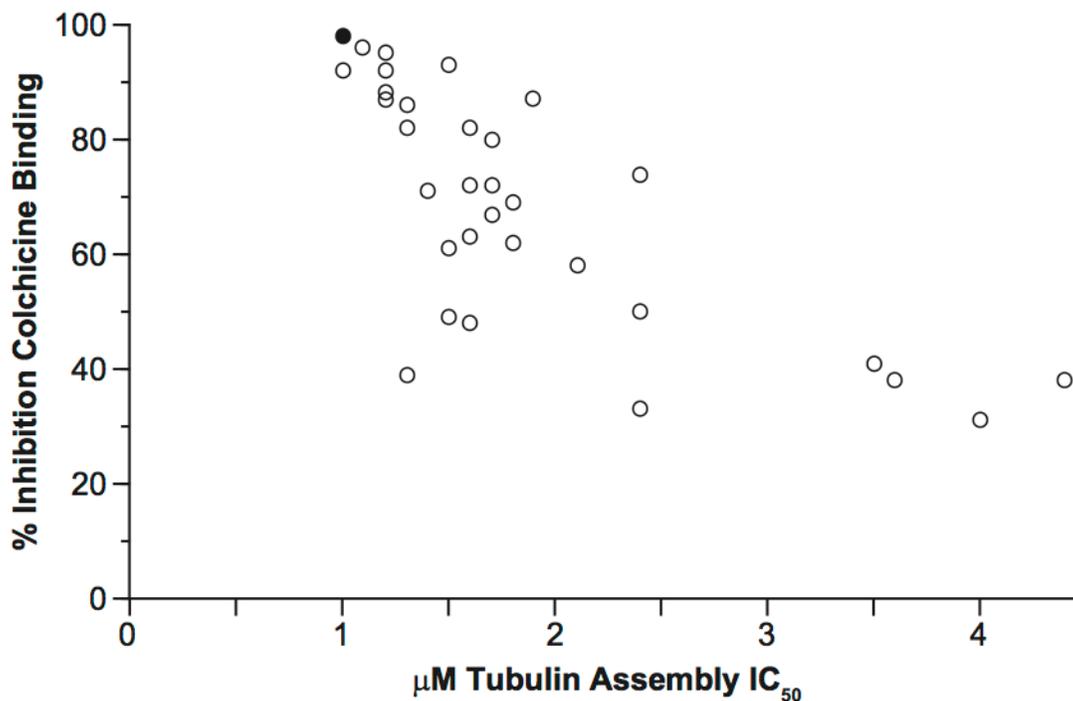
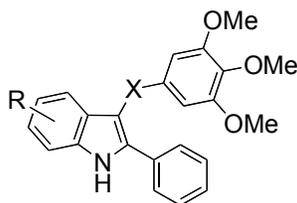


Figure 3. Correlation between tubulin assembly (IC_{50} values, μM) and inhibition of colchicine binding (% values). Data of ATI derivatives **6-45** are shown as open circles. Black circle represents CSA4 as reference compound.

Table 1. Inhibition of Tubulin Polymerization, Binding of Colchicine to Tubulin and Growth of MCF-7 Human Breast Carcinoma Cells by Compounds 6-45 and References 1-5.



6-45

compd	R	X	Tubulin Assembly ^a IC ₅₀ ± SD (μM)	MCF-7 ^{b,c} IC ₅₀ ± SD (nM)	Colchicine Binding ^d (% ± SD)
6	4-Br	S	2.4 ± 0.1	400 ± 70	33 ± 2
7	4-Br	C=O	>20 (partial activity) ^e	430 ± 100	nd
8	4-Cl	S	1.6 ± 0.07	290 ± 50	48 ± 5
9	4-Cl	C=O	3.6 ± 0.2	260 ± 50	38 ± 3
10	4-Cl	CH ₂	17 ± 0.2	>5000	nd ^f
11	4-F	S	1.7 ± 0.07	80 ± 30	80 ± 0.4
12	4-F	C=O	1.6 ± 0.08	65 ± 7	63 ± 1
13	4-MeO	S	>20 (partial activity) ^e	1400 ± 300	nd
14	4-MeO	C=O	>20 (no activity) ^g	>5000	nd
15	5-Br	S	1.3 ± 0.1	320 ± 100	39 ± 3
16	5-Br	C=O	>40	530 ± 100	nd
17	5-Br	CH ₂	4.0 ± 0.5	700 ± 300	31 ± 2
18	5-Cl	S	1.5 ± 0.2	280 ± 70	49 ± 5
19	5-Cl	C=O	>20	330 ± 100	nd
20	5-Cl	CH ₂	4.4 ± 0.8	310 ± 10	38 ± 3
21	5-F	S	1.5 ± 0.1	300 ± 0	61 ± 4
22	5-F	C=O	13 ± 0.4	240 ± 90	nd
23	5-OMe	S	2.1 ± 0.01	200 ± 80	58 ± 0.5
24	5-OMe	C=O	8.3 ± 1	170 ± 60	nd
25	5-OMe	CH ₂	3.5 ± 0.4	100 ± 0	41 ± 0.9

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4	26	6-Br	S	1.9 ± 0.2	9.0 ± 2	87 ± 0.6
5						
6	27	6-Br	C=O	1.3 ± 0.2	30 ± 2	86 ± 0.4
7						
8	28	6-Cl	S	1.2 ± 0.2	20 ± 10	88 ± 3
9						
10	29	6-Cl	C=O	1.4 ± 0.06	35 ± 7	71 ± 5
11						
12	30	6-Cl	CH ₂	1.6 ± 0.01	55 ± 20	72 ± 6
13						
14	31	6-F	S	2.4 ± 0.2	80 ± 20	74 ± 2
15						
16	32	6-F	C=O	10 ± 0.4	600 ± 0	nd
17						
18	33	6-MeO	S	1.1 ± 0.1	1.3 ± 0.6	96 ± 1
19						
20	34	6-MeO	C=O	1.2 ± 0.06	30 ± 10	95 ± 0.4
21						
22	35	7-Br	S	1.6 ± 0.2	25 ± 5	82 ± 1
23						
24	36	7-Br	C=O	1.7 ± 0.1	4.0 ± 0.5	72 ± 0.3
25						
26	37	7-Cl	S	1.3 ± 0.2	50 ± 10	82 ± 0.3
27						
28	38	7-Cl	C=O	1.8 ± 0.05	9.0 ± 1	69 ± 0.5
29						
30	39	7-Cl	CH ₂	2.4 ± 0.2	200 ± 0	50 ± 4
31						
32	40	7-F	S	1.0 ± 0.1	20 ± 9	92 ± 0.3
33						
34	41	7-F	C=O	1.7 ± 0.1	30 ± 0	67 ± 3
35						
36	42	7-OMe	S	1.2 ± 0.01	19 ± 10	87 ± 2
37						
38	43	7-OMe	C=O	1.8 ± 0.1	200 ± 0	62 ± 3
39						
40	44	6,7-Cl ₂	S	1.2 ± 0.1	7.0 ± 3	92 ± 2
41						
42	45	6,7-Cl ₂	C=O	1.5 ± 0.03	15 ± 5	93 ± 0.7
43						
44	1	—	—	3.2 ± 0.4	5.0 ± 1	nd
45						
46	2	—	—	1.0 ± 0.1	13 ± 3	98 ± 0.6
47						
48	3 ^h	—	—	3.3 ± 0.1	52 ± 7	nd
49						
50	4 ⁱ	—	—	2.0 ± 0.2	13 ± 3	93 ± 0.8
51						
52	5 ⁱ	—	—	2.5 ± 0.3	42 ± 10	76 ± 0.2

^aInhibition of tubulin polymerization. Tubulin was at 10 μM in the assembly assay. ^bInhibition of growth of MCF-7 human breast carcinoma cells. ^cCompounds that inhibited tubulin assembly with IC₅₀ ≤ 5 μM were tested in the cellular and colchicine binding assays. ^dInhibition of [³H]colchicine binding. Tubulin was at 1 μM. Both [³H]colchicine and inhibitor were at 5 μM. ^ePartial inhibition at 20 μM. ^fNo data. ^gLittle or no activity at 20 μM. ^hLit.¹⁵. ⁱLit.¹⁶

Table 2. Growth Inhibition of MDA-MB-468, MDA-MB-436, MDA-MB-231, A-549, MV4-11, NB4 and NCI-H1975 Cell Lines by Compounds 33 and 44 and Reference PTX.

compd	IC ₅₀ ± SD (nM)						
	MDA-MB-468 ^a	MDA-MB-436 ^a	MDA-MB-231 ^a	MV4-11 ^b (AML_M9)	NB4 ^b (AML_M3)	A-549 ^a	NCI-H1975 ^a
33	37 ± 0.5	62 ± 1	39 ± 1.2	2.5 ± 2.1	4 ± 1	28 ± 6	195 ± 158
44	33 ± 0.3	75 ± 1.1	47 ± 0.1	10.5 ± 0.7	10 ± 4	120 ± 10	305 ± 122
PTX	5 ± 1	8 ± 1.5	7 ± 2	nd ^b	2.3 ± 0.3	7 ± 2	2.5 ± 5

^aIncubation time was 72 h. ^bIncubation time was 48 h. ^bNo data.

Cell Growth Inhibition. ATIs **33** and **44** were assayed as growth inhibitors of a panel of cancer cell lines, including MDA-MB-468, MDA-MB-436, MDA-MB-231, A-549, MV4-11, NB4 and NCI-H1975, using PTX as reference drug. As a growth inhibitor of MDA-MB-468, MDA-MB-436, and MDA-MB-231 breast cancer cells, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, ATI **33** at 72 h showed IC₅₀ values of 37, 62 and 39 nM, respectively, with the corresponding values for **44** being 33, 75 and 47 nM, respectively (Table 2). Compounds **33** and **44** induced a dose and time-dependent growth inhibition of each treated cell line (Figures 5S-10S, Supporting Information). Compounds **33** and **44** inhibited the human acute myelocytic leukemia (AML) cell lines MV4-11 and NB4, showing at 48 h IC₅₀'s of 2.5 and 10.5, and 4 and 10 nM, respectively. As growth inhibitors of the A-549 and NCI-H1975 human lung adenocarcinoma cancer cells bearing the KRAS mutation and resistance to EGFR inhibitors, respectively, ATIs **33** and **44** yielded at 72 h IC₅₀ values in the nanomolar concentration range.

Table 3. Inhibition of Growth of the OVCAR-8 and NCI/ADR-RES, and Messa and Messa/Dx Cell Line Pairs by Compounds 33 and 44, and Reference Compounds 1, 2, VRB, VBL and PTX.^a

compd	IC ₅₀ ± SD (nM)			
	OVCAR-8	NCI/ADR-RES	Messa ^b	Messa/Dx5 ^b
33	4.3 ± 1	2.5 ± 1	20.7 ± 1.7	28.0 ± 1.0
44	14 ± 2	10 ± 4	3.5 ± 0.9	4.5 ± 1.9
1	30 ± 1	420 ± 100	11 ± 6	329 ± 166
2	2.8 ± 1	1.8 ± 1	2.7 ± 2	2.6 ± 1
VRB	300 ± 0	5000 ± 1000	nd ^c	nd
VBL	15 ± 7	200 ± 0	3 ± 2	144 ± 61
PTX	3.7 ± 1	6000 ± 500	4 ± 1	1764 ± 477

^aInhibition of growth of the indicated cell lines. ^bIncubation time was 72 h. ^cNo data.

Multidrug Resistant (MDR) Cell Lines. Compounds **33** and **44** were evaluated as inhibitors of the ovarian carcinoma cell lines OVCAR-8 and its cognate P-glycoprotein (Pgp) overexpressing line NCI/ADR-RES and of the human uterine sarcoma cell line Messa and its cognate MDR line Messa/Dx5, using **1**, **2**, vinorelbine (VRB), VBL and PTX as reference compounds (Table 3). The reference agents **1**, VRB, VBL and PTX weakly inhibited these MDR cell lines. Compounds **33** and **44** and the reference **2** were potent inhibitors of the MDR cell lines. ATI **33** (IC₅₀ = 2.5 ± 1 nM) was comparable to **2** (IC₅₀ = 1.8 ± 1 nM) as an inhibitor of the NCI/ADR-RES cell line, and **44** (IC₅₀ = 4.5 ± 1 nM) was comparable to **2** (IC₅₀ = 2.6 ± 1 nM) in the Messa/Dx5 cells.

Arrest of Mitotic Progression and Concomitant Cell Death Induction. To assess whether the growth suppressive effect of **33** and **44** reflected their antimitotic activity, we assessed their ability to induce mitotic arrest. We previously found that treatment with 20 nM VBL effectively arrested the cell

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3 cycle of HeLa cells in mitosis; at lower concentrations VBL did not fully prevent mitotic progression,
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5 such that cells assemble defective mitotic spindles, “slip” through the mitotic checkpoint and progress
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7 towards aberrant chromosome segregation.¹⁴ We examined HeLa cell cultures treated with 20, 50 and
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9 100 nM **33** or **44** in DMSO, 20 nM VBL or DMSO vehicle. Treatments were carried out for 24 h,
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11 allowing all cells to enter mitosis during the treatment. Cell cultures were then harvested, and their cell
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13 cycle profile was analyzed by flow cytometry after incubation with propidium iodide (PI) to reveal
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15 their genomic DNA content (linear scale). ATIs **33** and **44** at 20 nM induced >80% of HeLa cells to
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17 arrest with a G2/M genomic content, similar to VBL; very few cells progressed past the G2/M phase
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19 (Figure 4A). Thus, both **33** and **44** effectively arrested mitotic progression and prevented mitotic
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21 slippage, and hence the generation of hyperdiploid or polyploid cells. To assess whether G2/M-arrested
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23 cells underwent cell death over time, PI-stained cell samples were analyzed by plotting their DNA
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25 content on a logarithmic scale to resolve the sub-G1 region, in which hypodiploid cells represent the
26
27 terminal products of cell death. ATIs **33** and **44** at 20 nM induced both cell death and concomitant
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29 G2/M phase arrest and were comparable to VBL; these effects were even more substantial at 50 nM
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31 (Figure 4B). The induction of cell death by **33** seemed to be superior to **44** at all tested concentrations.
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33 Biparametric analysis after simultaneous incubation of non-permeabilized cells with annexin V, which
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35 reacts with phosphatidylserine residues on the outer cell membrane during early apoptosis, and PI, to
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37 which viable cells are not spontaneously permeable, discriminates early and late stages of apoptotic cell
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39 death (Figure 4C) from necrotic cells, which are permeable to PI but do not react with annexin V. We
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41 found that **33** and **44** induced cell death with apoptotic-like phenotypic features with comparable
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43 effectiveness and early-to-late kinetics as observed with VBL.
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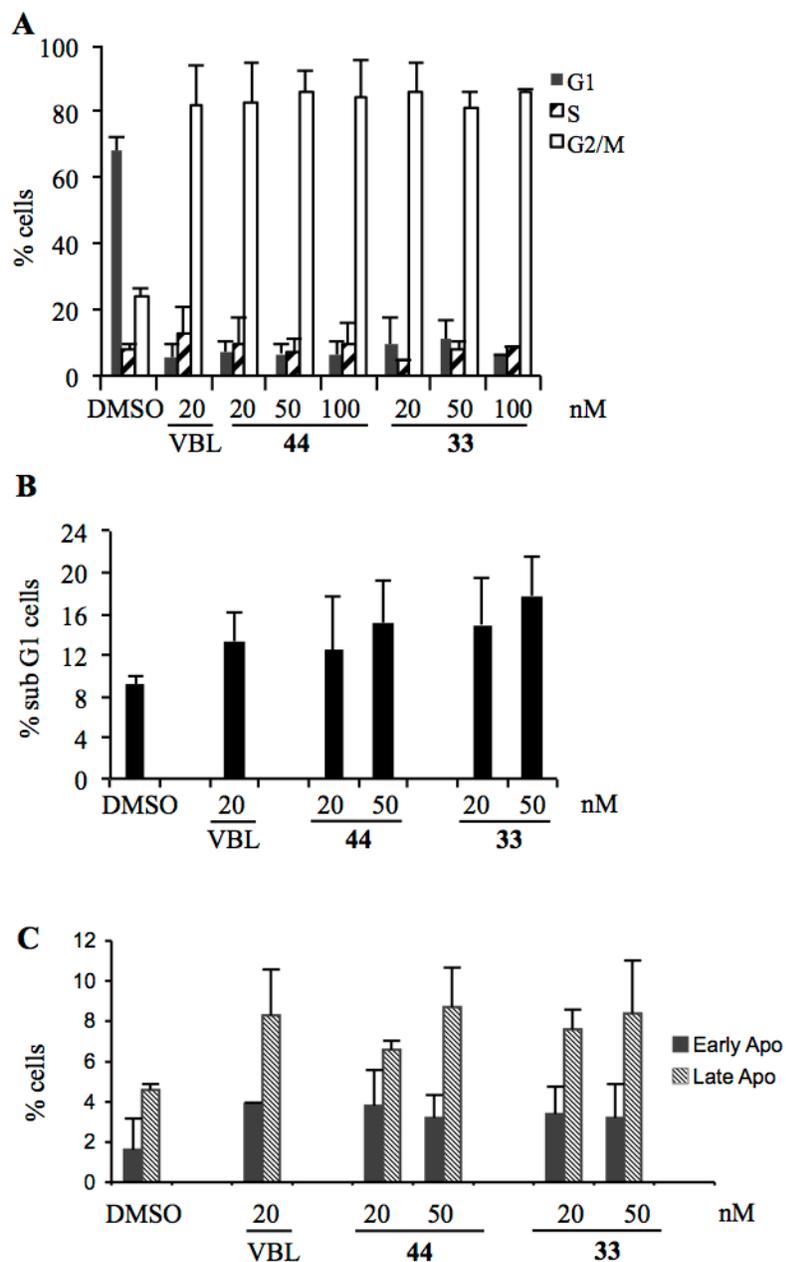


Figure 4. **A.** Cell cycle profiles of HeLa cell cultures exposed to **33**, **44** or VBL for 24 h at the indicated concentrations; the % of cells with 2C (G1 phase, black bars), 4C (G2/M phases, white bars), or between 2C and 4C (S phase, dashed bars) genomic DNA content is shown. Mean values were calculated from three independent experiments. **B.** Flow cytometry analysis of PI-stained cells with sub-G1 DNA content, representing terminal cell death, after 24 h. Mean values were calculated from four independent experiments. **C.** Distribution of cells simultaneously processed for annexin V reactivity and PI incorporation, representing early (reactive to annexin V, not permeable to PI) and late (reactive to both annexin V and PI) stages of the cell death process in cultures treated for 24 h. Mean values were calculated from three independent experiments.

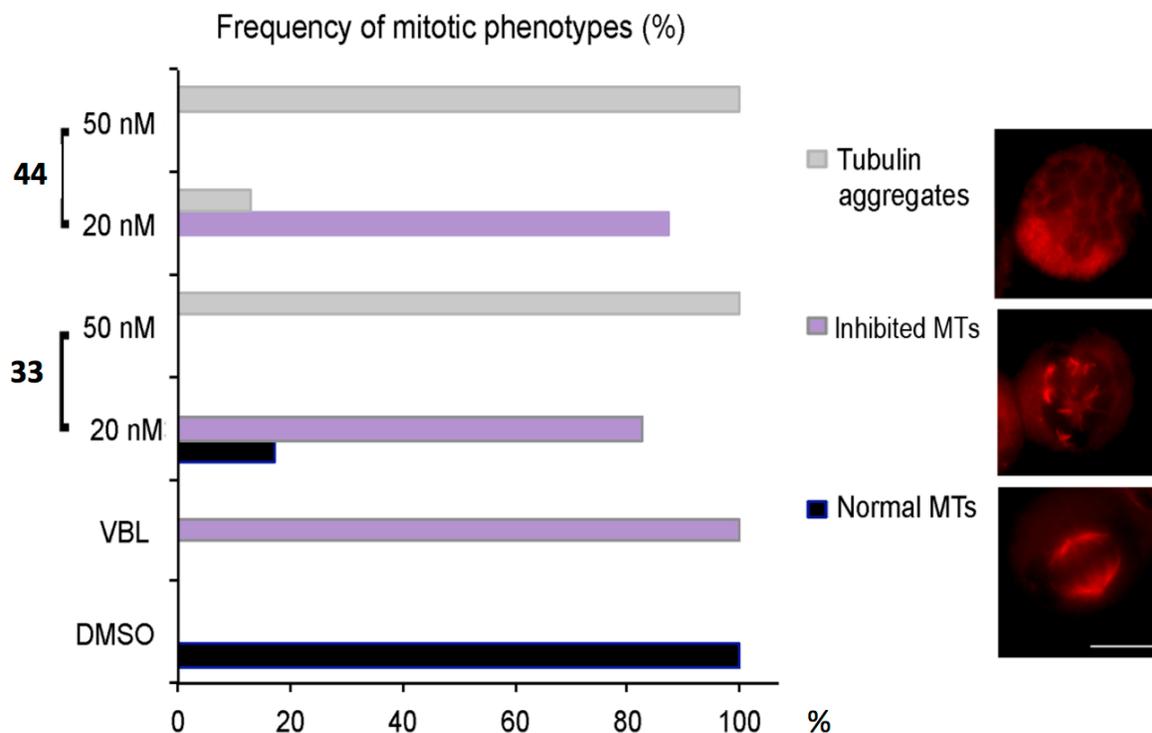


Figure 5. Mitotic phenotypes in HeLa cell cultures exposed to ATIs **33** and **44** for 24 h and processed for IF to tubulin (in red). The bar graph on the left indicates the statistical distribution of the cytological phenotypes depicted under the indicated conditions; the frequency of scored phenotypes is shown as % of all counted mitotic figures (330 to 400 counted mitotic figures per condition). Grey bars represent the frequency of mitotic cells with severe inhibition of tubulin polymerization, with no recognizable MTs and unstructured tubulin aggregates (exemplified in the top IF panel). Purple bars represent the frequency of mitotic cells with inhibited MT polymerization, yielding short MT stretches (middle panel). Black bars represent the frequency of normal mitoses with polymerized MT arrays forming a bipolar spindle (bottom panel).

ATIs 33 and 44 Strongly Inhibit Mitotic MT Assembly. To correlate cell cycle arrest and cell death induction with inhibition of MT dynamics in HeLa cells, we employed immunofluorescence (IF) to examine the effects of ATIs on tubulin and MTs. HeLa cells were treated with 20 or 50 nM **33** or **44** for 24 h, the same conditions that induced cell cycle arrest (Figure 4), then processed for tubulin IF and examined under fluorescence microscopy. We found that at both concentrations **33** or **44** arrested cells in mitosis with a prometaphase-like appearance. Consistent with the absence of hyperdiploid cells in flow cytometry analysis, both ATIs at 20 nM or higher effectively prevented mitotic slippage. At 20

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3 nM, the ATIs inhibited MT polymerization and left tubulin foci and/or small asters of short MT
4 fragments (an example is shown in Figure 5A, middle panel). The proportion of mitotic cells displaying
5 this inhibited MT phenotype was very similar to that observed with VBL (Figure 5B). Raising the ATI
6 concentrations to 50 nM yielded a phenotype with no recognizable mitotic MT remnants, but only
7 tubulin aggregates that formed an unstructured meshwork throughout the mitotic cells (Figure 5A, top
8 panel). In summary, the newly synthesized ATIs strongly affect cellular MT polymerization, resulting
9 in effective inhibition of formation of the mitotic apparatus, particularly when used at 50 nM, and this
10 phenotype was associated with a durable mitotic arrest and concomitant induction of cell death.
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22 **Inhibition of PC-3, RD, and HepG2 Cancer Cell Growth.** We determined the effects of ATIs **33**
23 and **44** as growth inhibitors of the human prostate cancer PC-3, rhabdomyosarcoma RD and human
24 liver hepatocellular carcinoma HepG2 cell lines, with VBL and PTX as reference compounds (Table 4).
25 ATIs **33** and **44** strongly inhibited these cell lines at nanomolar concentrations and were superior to the
26 references VBL and PTX. Compound **33** was generally more effective than **44** as an inhibitor of the
27 growth of these three cell lines.
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36 PC-3, RD, and HEPG2 cell cultures were treated for 24 h with increasing concentrations of ATIs **33**
37 and **44** and of PTX as reference drug at 500, 1000, and 2000 nM. After treatment, cells and vehicle
38 controls (0.1% DMSO) were incubated with PI to analyze their DNA content in flow cytometry assays.
39 Both ATIs arrested cell cycle progression in all three cell lines at low concentrations. ATIs **33** and **44**
40 induced an accumulation in the G2/M phase in PC-3 and RD cells, as did PTX, whereas in HepG2 cells
41 these compounds caused a stronger effect on cell cycle progression as compared with the reference
42 drug (Figure 11S, Supporting Information).
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Table 4. Growth Inhibition of PC-3, RD and HepG2 Cell Lines by Compounds **33 and **41** and References VBL and PTX.**

compd	IC ₅₀ ± SD (nM) ^a		
	PC3	RD	HepG2
33	0.3 ± 0.06	0.2 ± 0.04	0.1 ± 0.02
44	19 ± 1	16 ± 1.3	62 ± 2
VBL	766 ± 1000	53 ± 2.5	81 ± 2.4
PTX	4900 ± 1.3	>10000	2600 ± 1,5

^aIncubation time was 48 h.

After a 24 h exposure and subsequent incubation in drug-free medium for 24 h, the PC-3, RD, and HepG2 cell lines treated with **33** or **44** showed a strong accumulation of cells in G2/M (Figure 6). Moreover, **33** and **44** induced an irreversible cell cycle arrest at the concentrations of 500, 1000, and 2000 nM in the PC-3 and RD cells.

A correlation between cell cycle arrest and cell death was investigated by exposing the cells to a 500, 1000 or 2000 nM concentration of **33**, **44** or PTX, followed by incubation with fluorescently conjugated annexin V and PI (Figure 7). All the cell lines treated with **33** or **44** at 500 nM showed higher rates of cell death than the same populations treated with 500 nM PTX. A dose-response trend in cell death was observed in the RD cell line after a 48 h exposure with either **44** or **33** (Figure 7B) and in the PC3 and HepG2 cell lines following treatment with PTX (Figure 7A and 7C).

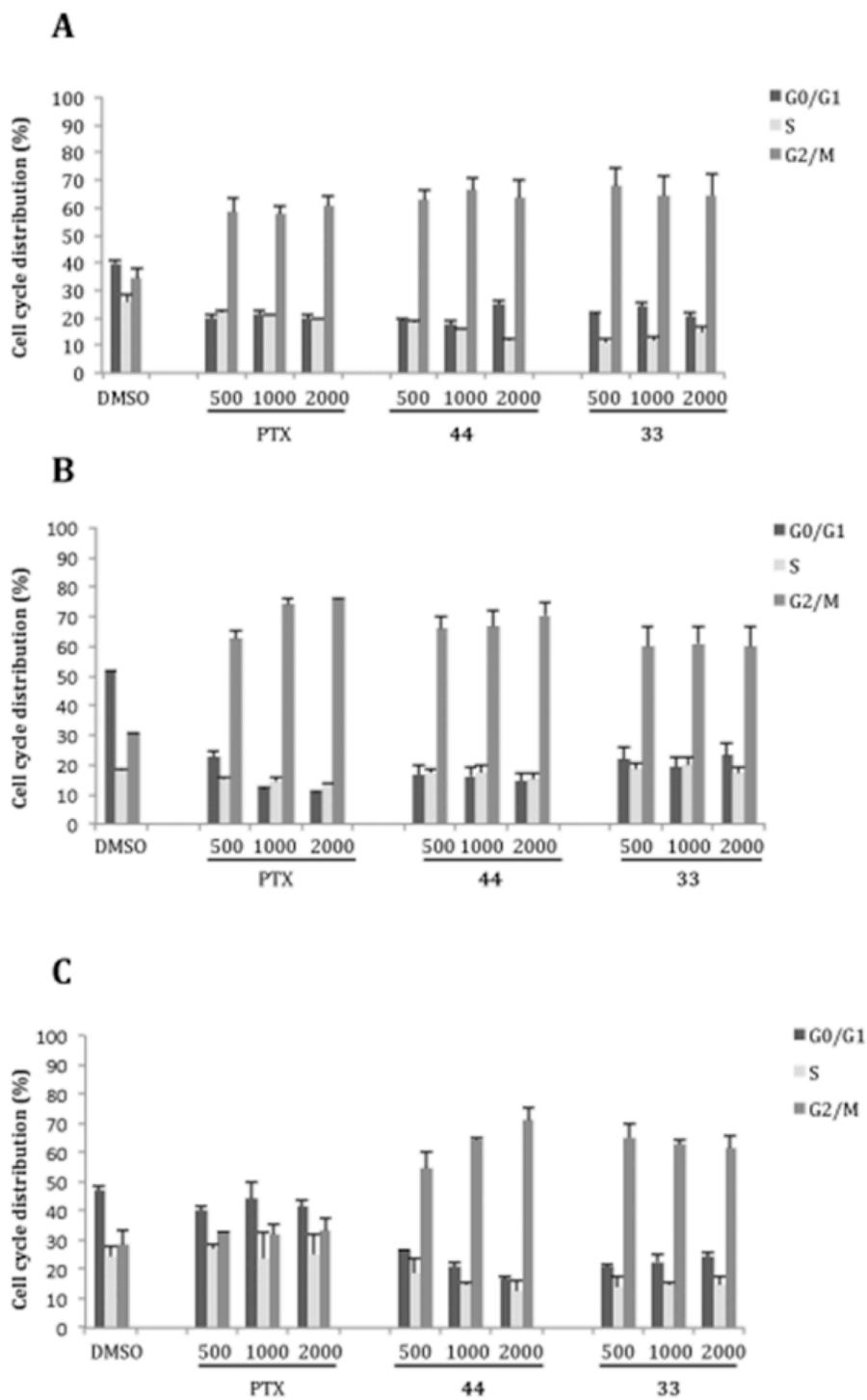


Figure 6. Cell cycle analysis of PC-3 (A), RD (B) and HepG2 (C) cells treated with 0.1% DMSO or 500, 1000 or 2000 nM **33**, **44** or PTX for 24 h, followed by a 24 h recovery in drug-free medium. Histograms represent % of cells with G0/G1, S and G2/M DNA content expressed as mean values \pm SD of three independent experiments.

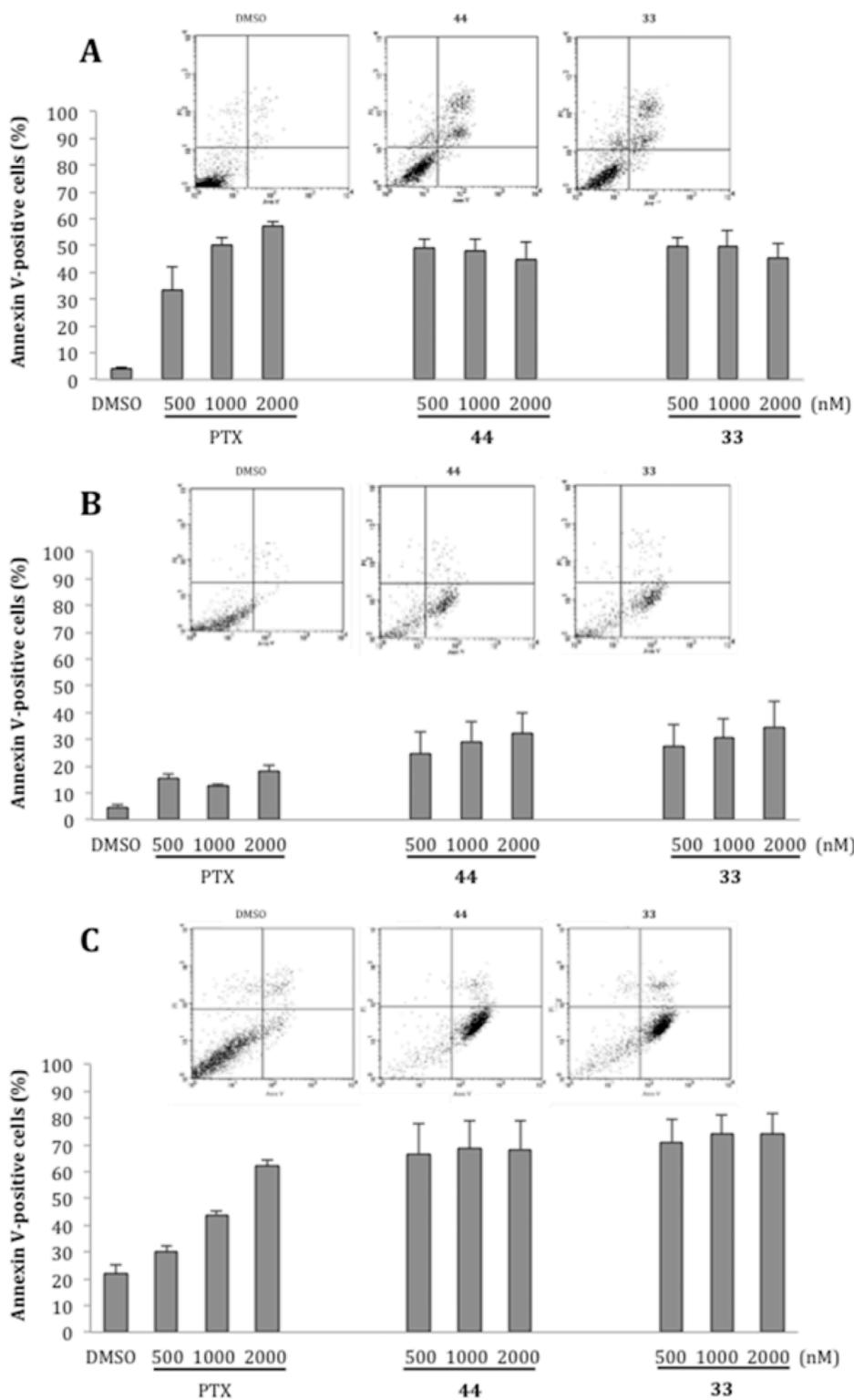


Figure 7. Cell death cytometric analysis of PC-3 (A), RD (B) and HepG2 (C) cells treated with 0.1% DMSO or 500, 1000 or 2000 nM **33**, **44** or PTX for 48 h. Flow cytometric profiles of cell populations following treatment with DMSO or 2000 nM **33** or **44** are at the top of each panel (annexin-V-FITC staining on the x axis and PI on the y axis). Histograms represent % of cells in early apoptosis (annexin V-FITC staining) and late apoptosis (annexin V-FITC and PI staining) expressed as mean values \pm SD calculated from three independent experiments.

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4 **Inhibition of T98G and U343MG Cancer Cell Growth.** Malignant gliomas develop from gradual
5 accumulation of multiple genetic alterations, resulting in either activation of oncogenes or inactivation
6 of tumor suppressor genes.³² Human glioblastoma multiforme T98G and U343MG cells show typical
7 hallmarks of glioblastoma multiforme tumors in patients. We evaluated the ability of compounds **33** and
8 **44** to inhibit the growth of T98G and U343MG cancer cells, which show different genetic profiles for
9 the expression of key cell survival proteins, such as p53, MDM2, EGFR, RB, cyclin D, and MMPs.³³
10 Treatment of T98G and U343MG cells with increasing concentrations of **33** or **44** for 24, 48 or 72 h
11 significantly inhibited cell growth in a dose- and time-dependent manner (Figures 12S and 13S,
12 Supporting Information). The IC₅₀ values were calculated taking into account the relative doubling time
13 (CDT).^{34,35} after 48 h for the T98G cells and after 72 h for the U343MG cells. As a cell growth inhibitor,
14 compound **33** yielded IC₅₀ values of 15.2 ± 1.6 nM in T98G cells, and 0.5 ± 0.05 nM in U343 cells; for
15 **44**, IC₅₀ values of 16.3 ± 1.5 nM nM in T98G cells and 0.6 ± 0.05 nM nM in U343 cells were obtained.

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32 **Expression of MICA and MICB ligands in HeLa Cells, Resulting in Enhanced Natural Killer**
33 **(NK) Cell Degranulation.** In previous studies,³⁶ treatment of HeLa and HepG2 tumor cell lines with
34 sodium butyrate, a potent repressor of histone deacetylases that causes spindle abnormalities and
35 mitotic arrest, resulted in up-regulation of the expression of NK cell receptor activating ligand MICA
36 and MICB at both the mRNA and protein levels and in enhanced susceptibility of both cell lines to NK
37 lysis. We evaluated the expression of NKG2D and DNAM-1 ligands in HeLa cells after treatment with
38 either ATI **33**, **37** or **44**, in particular whether the compounds could modulate their expression. We first
39 characterized HeLa cell growth inhibition by **33**, **37** or **44**, at a sub-lethal concentration after a 48 h
40 treatment (MTT assay). HeLa cells were more sensitive to **33** and **44** (IC₅₀ value = 10 nM) than to **37**
41 (IC₅₀ = 76 nM). After a 48 h treatment with 10 nM ATI, flow cytometric biparametric analysis of HeLa
42 cells by annexin V/PI staining showed only a weak increase of early apoptotic cells compared to
43 control cultures (Figure 14S, Supporting Information).
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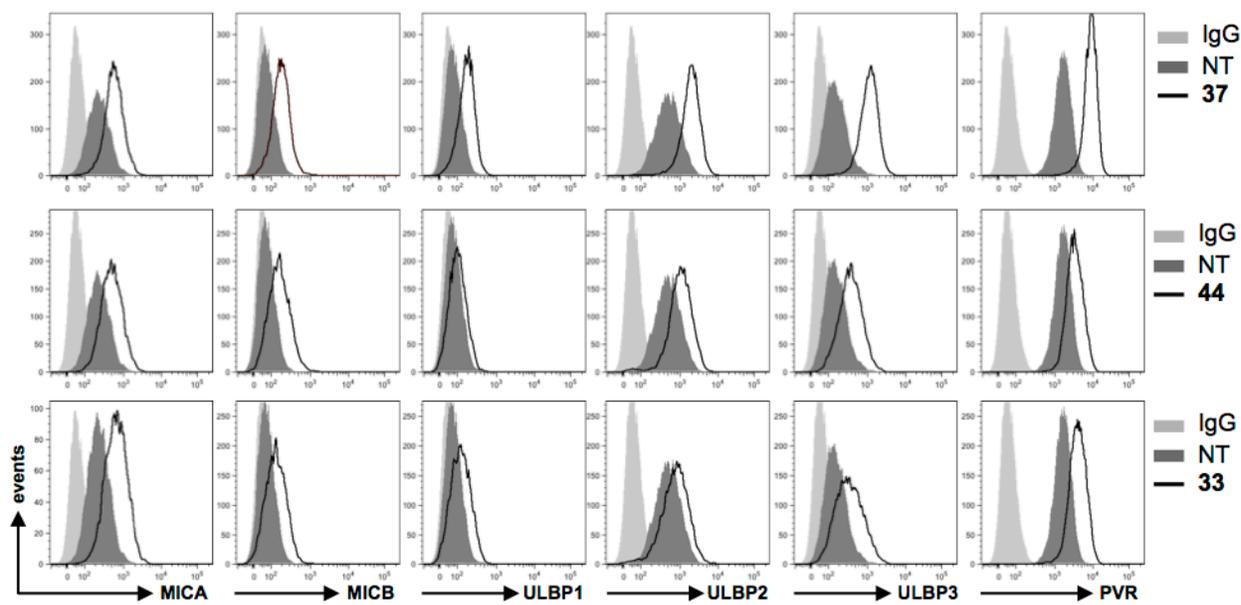


Figure 8. ATIs **33**, **37** and **44** up-regulate DNAM-1 and NKG2D ligands in HeLa cells. NKG2D and DNAM-1 ligand surface expression was analyzed by flow cytometry after a 48 h treatment with the indicated ATI compound. Data are representative of one out of three independent experiments.

NK cell receptor activating ligand analysis by combined IF and flow cytometry revealed a different modulation of NKG2D and DNAM-1 ligands in ATI-treated HeLa cells after a 48 h treatment with sub-lethal doses. ATI **33**, **37** or **44** behaved as strong enhancers of MICA, ULBP3 and PVR expression, while treatment with the compounds had weaker effects on MICB, ULBP1 and ULBP2 ligand expression (Figure 8) and no effect on the expression of the Nec-2 ligand (data not shown). Interestingly, (1-(3-aminophenyl)-1*H*-pyrrol-3-yl)(3,4,5-trimethoxyphenyl)methanone, a potent tubulin polymerization inhibitor belonging to the ARAP class,³⁷ was unable to induce the NKG2D and DNAM-1 ligands (data not shown). The expression of ligand surface on treated HeLa cells was not accompanied by a corresponding increase in mRNA levels, as indicated by real-time PCR data (Figure 15S, Supporting Information).

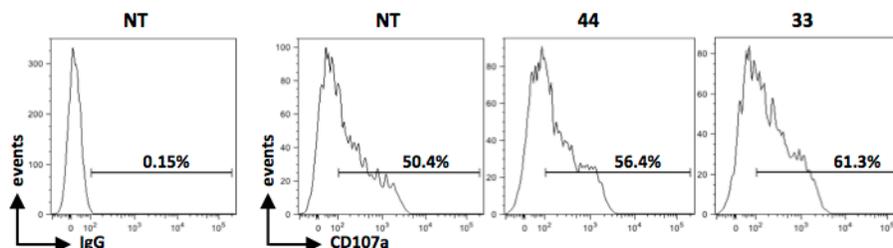


Figure 9. NK cell-mediated killing of HeLa cells increased after a 48 h treatment with ATI **33** or **44**. Results are expressed as % of CD107a⁺ cells after subtraction of the % of the control antibody and represent one of two independent experiments. The basal CD107a expression on NK cells was about 2%.

Based on these findings, we evaluated whether ATIs **33** and **44** would increase NK-cell degranulation towards HeLa cells. The expression of the lysosomal marker CD107a, which correlates with NK cell cytotoxicity,³⁸ was evaluated by IF and flow cytometry analysis by gating on NK cells upon their interaction with treated or untreated HeLa cells, used as targets. The up-regulation of NKG2D and DNAM-1 ligands was verified before the degranulation assay (data not shown). The expression of CD107a on NK cells contacting treated-HeLa target cells indicated that those cells were more susceptible to NK cell lysis (Figure 9).

Hedgehog Inhibiting Activity. Hedgehog (Hh) signaling pathway is deeply involved in tumorigenesis, and inhibitors of the Hh pathway have shown great potential as cancer therapeutics. In recent years, an ever growing interest in the Hh pathway has led to the development of a variety of small molecules targeting key Hh components, i.e., Smoothed (Smo), Sonic hedgehog protein (Shh), and Gli1.³⁹ Currently, some antagonists of Smo, the positive signaling transducer in Hh pathway, are undergoing clinical trials.

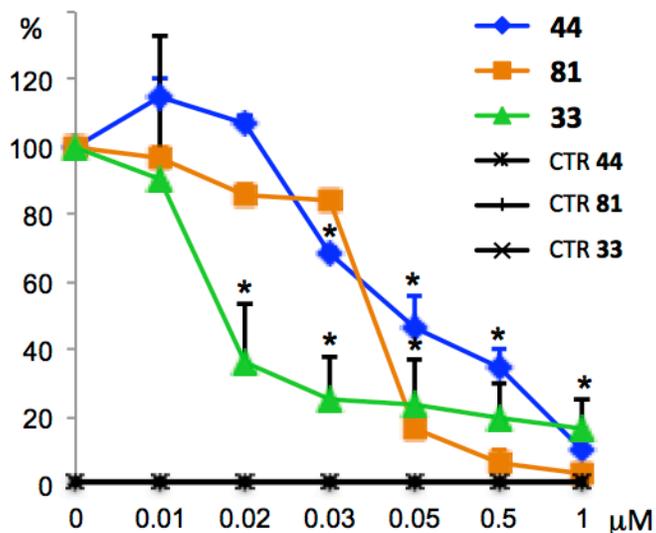


Figure 10. Inhibition of endogenous Hh signaling in Shh-L II cells by **33**, **44** and **81**. Dose-response curve of the indicated compounds in SAG-treated cells in comparison with untreated Shh-Light II cells as a control (CTR). Treatment time was 48 h, and normalization was against *Renilla* luciferase. Data from three independent experiments. Error bars indicate SD. **P*, 0.05 vs CTR.

We wished to investigate whether ATI compounds, like the structurally related ARAP derivatives,³⁷ could behave as inhibitors of Hh signaling. We selected three highly potent ATI derivatives, **33**, **44** and 2-(1H-imidazol-1-yl)-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (**81**),¹⁵ and their effects were evaluated by a luciferase assay performed in NIH3T3 Shh-Light II (Shh-LII) cells. In these cells, in which is stably incorporated an Hh-responsive (Gli-RE) reporter, the induction of the pathway occurs following treatment with the SMO agonist SAG. ATIs **33**, **44** and **81** showed a strong reduction of luciferase activity in cells treated with SAG in a dose-dependent manner (Figure 10), yielding IC₅₀'s of 19, 72 and 38 nM, respectively. The treatment did not decrease the control *Renilla* luciferase activity, preventing any cytotoxicity mediated effects on the inhibition of Hh signaling. To address the ability of these compounds to affect D283 medulloblastoma cell proliferation and survival, we performed a trypan blue count assay. The in vitro treatment with ATI **33**, **44** or **81** at 1 μM impaired cell growth and the percentage of cell death, as shown in Figure 16S, Supporting Information.

Table 5. Metabolic Stability with Human and Mouse Liver Microsomes^a and Aqueous Solubility of Compounds 33 and 44.

compd	Clearance ($\mu\text{L}/\text{min}/\text{mg protein}$) ^{b,c}		Solubility pH 7.4 ^d (μM)
	Human liver microsomes	Mouse liver microsomes	
33	15.2 \pm 8.0	43.1 \pm 7.9	0.99 \pm 0.13
44	<3	25.6 \pm 1.7	3.18 \pm 0.50
7-ethoxycoumarin	209 \pm 10.2	710.8 \pm 1.2	—
propranolol	19.1 \pm 2.2	235.1 \pm 24.1	—

^aMetabolic stability: <3, good; 3–60, medium; >60, low. ^bResults are expressed as mean \pm SD, n = 2. ^cThe standard compounds 7-ethoxycoumarin and propranolol showed metabolic stability in agreement with the literature and internal validation data REF.

^dHigh throughput screening solubility assay.

Hh inhibitors are attracting ever growing attention because of benefits displayed in the treatment of Hh-dependent cancers, such as medulloblastomas.⁴⁰ However, the problem of drug resistance to Smo mutations, arising during clinical treatment, has led to a quest for new Hh inhibitors. ATIs **33**, **44** and **81** showed potent inhibition of the Hh signaling pathway, suggesting that the ATI class has potential as Hh-dependent anticancer agents.

Metabolic stability. Compounds **33** and **44** were examined in a microsomal stability assay in comparison with 7-ethoxycoumarin and propranolol as control compounds, using both human and mouse liver microsomes, to estimate compound stability to phase I oxidative metabolism (Table 5). Compound **44** showed metabolic stability of 25.6 and <3 $\mu\text{L}/\text{min}/\text{mg protein}$ in mouse and human liver microsomes, respectively. Compound **33**, showed medium metabolic stability with human liver microsomes and low-medium metabolic stability with mouse liver microsomes (relative stabilities are defined in the Table 5 legend).

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3 **Aqueous solubility.** The solubility in aqueous pH 7.4 buffer of compounds **33** and **44** was measured
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5 in a high throughput screening solubility assay. The solubility of compound **33** was about 1 μM , while
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7 under the same conditions, compound **44** showed a solubility of 3.2 μM (Table 5).
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10 11 12 13 CONCLUSION

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16 We designed 39 new 2-phenylindole derivatives as potential anticancer agents bearing the 3,4,5-
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18 trimethoxyphenyl moiety linked through a sulfur, ketone or methylene bridging group at position 3 of
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20 the indole and with halogen or methoxy substituent(s) at positions 4-7. Twenty-three new derivatives
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22 inhibited tubulin polymerization with IC_{50} values of 1.0-2.0 μM and inhibited colchicine binding with a
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24 mean value $>70\%$; fifteen derivatives inhibited the growth of human MCF-7 cells with IC_{50} values ≤ 50
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30 Compounds **33** and **44**, representative members of this series, uniformly inhibited at nanomolar
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32 concentration a panel of cancer cells, including MDA-MB-468, MDA-MB-436, MDA MB-231, MV4-
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34 11, NB4, A-549, NCI-H1975, T98G and U343 cells. As inhibitors of PC-3, RD, and HepG2 cancer cell
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36 growth, **33** and **44** were superior to the references VBL and PTX. ATI **33** and **44** were comparable to **2**
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38 as inhibitors of the MDR NCI/ADR-RES and Messa/Dx5 cell lines. Besides the ability to inhibit
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40 tubulin polymerization, for which they were originally designed, ATIs **33** and **44** exhibited an
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42 unexpected stimulation of the cytotoxic activity of NK cells. Our findings demonstrated that **33** and **44**
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44 at doses of 10 nM, which did not severely affect cell viability, increased NKG2D and DNAM-1 ligand
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46 up-regulation of HeLa cells, resulting in an enhanced stimulation of NK cell cytotoxic activity. This
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48 novel effect of ATIs, elicited at sublethal doses, along with a stronger expression of NK cell receptor-
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50 activating ligand, led to an increased propensity of NK cells to degranulate against tumor cells. At
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52 higher concentrations of 20-50 nM, ATIs **33** and **44** induced $>80\%$ of HeLa cells to arrest with a G2/M
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54 DNA content, an effect similar to that observed with VBL, with very few cells progressing past the 4N
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3 G2/M phase. These compounds stably arrested mitotic progression, prevented mitotic slippage and the
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5 ensuing formation of aneuploid cells - a hallmark of aggressive cancers - and induced cell death. These
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7 findings suggest that the new ATIs **33** and **44** can arrest proliferation of cancer cells with effectiveness
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9 comparable or superior to that obtained with VBL. ATIs **44** and **81** showed strong inhibition of the Hh
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11 signaling pathway, inhibiting medulloblastoma D283 cells with IC₅₀s of 72 and 38 nM, respectively.
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15 In summary, these novel ATI compounds show potential to treat cancer via both MT-based and
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17 MT-independent pathways. Even at low concentrations where the ATIs do not fully prevent the
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19 assembly of the mitotic apparatus, and hence mitotic cell death, these agents effectively up-regulate NK
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21 ligands and trigger an alternative cytotoxic response via NK cells. Here we have described the broad
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23 potential of the new ATIs in an in-depth characterization of the cell response to increasing drug
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25 concentrations. We aim to assess whether specific MT-associated proteins (MAPs) can modulate cell
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27 sensitivity to these drugs and favor one pathway over the other. Such MAPs may serve as effective
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29 biomarkers to predict the prevalent response to ATI treatment. Compounds **33** and **44** represent novel
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31 lead compounds that will prompt further development of the ATI class to obtain new promising
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33 anticancer agents with enhanced stimulation of NK cell cytotoxic activity and repression of Hh-
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35 dependent cancers. Derivative **44** showed higher metabolic stability than **33** in human and mouse liver
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37 microsomes and greater water solubility. The present results highlight the therapeutic potential of the
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39 ATI class as anticancer agents and prompt further developmental studies.
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48 **Experimental Section**

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51 **Chemistry.** Microwave-assisted reactions were performed on a CEM Discover SP single mode
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53 reactor. The instrument settings were controlled with PC-running CEM Synergy 1.49 software. Closed
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55 vessel experiments were carried out in capped microwave-dedicated vials (10 mL) with cylindrical
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57 stirring bar (length 8 mm, diameter 3 mm). Open vessel experiments were carried out in 100 mL round-
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3 bottom flasks equipped with a Dimroth reflux condenser and a cylindrical stirring bar (length 20 mm,
4 diameter 6 mm). Stirring, temperature, irradiation power, maximum pressure (Pmax), PowerMAX
5 (simultaneous cooling-while-heating), ActiVent (simultaneous venting-while-heating), and ramp and
6 hold times were set as indicated. Temperature of the reaction was monitored by an external fiber optic
7 temperature sensor. After completion of the reaction, the mixture was cooled to 25 °C via air-jet
8 cooling. Melting points (mp) were determined on a Stuart Scientific SMP1 apparatus and are
9 uncorrected. Infrared (IR) spectra were run on a Perkin-Elmer SpectrumOne FT-ATR
10 spectrophotometer. Band position and absorption ranges are given in cm^{-1} . Proton nuclear magnetic
11 resonance (^1H NMR) spectra were acquired on a Bruker 400 MHz FT spectrometer in the indicated
12 solvent by TopSpin 2.1 software and the files processed by MestreLab Research S.L. MestreReNova
13 6.2.1-769 software. Chemical shifts are expressed in δ units (ppm) from tetramethylsilane.
14 Multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br
15 (broadened), broad s (broadened singlet), and coupling constants (J) are reported in Hertz (Hz). Flash
16 chromatography was carried out on Interchim Spot II Flash, using Merck SuperVarioFlash D26
17 cartridges packed with Merck Geduran 60 (0.040-0.063 mm) silica gel. Column chromatography was
18 performed on columns packed with alumina from Merck (70-230 mesh) or silica gel from Macherey-
19 Nagel (70-230 mesh). Aluminum oxide thin layer chromatography (TLC) cards from Fluka (aluminum
20 oxide precoated aluminum cards with fluorescent indicator visualizable at 254 nm) and silica gel TLC
21 cards from Macherey-Nagel (silica gel precoated aluminum cards with fluorescent indicator
22 visualizable at 254 nm) were used for TLC. Developed plates were visualized by a Spectroline ENF
23 260C/FE UV apparatus. Organic solutions were dried over anhydrous sodium sulfate. Evaporation of
24 solvents was carried out on a Büchi Rotavapor R-210 equipped with a Büchi V-850 vacuum controller
25 and a Büchi V-700 or V-710 vacuum pump. All commercially available reagents were used without
26 further purification. Elemental analyses of the compounds were found within $\pm 0.4\%$ of the theoretical
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3 values. Purity of tested compounds was determined by high pressure liquid chromatography (HPLC)
4 and was >95%. The HPLC system (Dionex UltiMate 3000, Thermo Fisher Scientific Inc.) was
5 equipped with a SR-3000 solvent rack, a LPG-3400SD quaternary analytical pump, a TCC-3000SD
6 column compartment, a DAD-3000 diode array detector and an analytical manual injection valve with
7 a 20 μ L loop. Compounds were dissolved in acetonitrile (10 mg/mL). HPLC analysis was performed
8 by using an Acclaim 120 C18 reversed-phase column (5 μ m, 4.6 x 250 mm, Thermo Fisher Scientific
9 Inc.) at a temperature of 30 ± 1 $^{\circ}$ C, an isocratic gradient (acetonitrile:water = 90:10), a flow rate of 1.0
10 mL/min and a detector signal of 254 and 365 nm. Chromatographic data were acquired and processed
11 by Chromeleon 6.80 software (Thermo Fisher Scientific Inc.).
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25 **1. Synthesis of Compounds 6-45. General Procedure for the Preparation of 6, 8, 11, 13, 15, 18,**
26 **21, 23, 26, 28, 31, 33, 35, 37, 40, 42 and 44.** *Example. 4-Bromo-2-phenyl-3-((3,4,5-*
27 *trimethoxyphenyl)thio)-1H-indole (6).* A mixture of 4-bromo-2-phenyl-1H-indole (**46**) (0.27 g, 1 mmol),
28 bis-(3,4,5-trimethoxyphenyl)disulfide (0.44 g, 1.1 mmol), and sodium hydride (0.053 g, 2.2 mmol;
29 60% in mineral oil) in anhydrous DMF (3 mL) was placed into the microwave cavity (closed vessel
30 mode, Pmax = 250 psi). Starting microwave irradiation of 120 W was used, the temperature being
31 ramped from 25 to 130 $^{\circ}$ C, while rapidly stirring and venting (pressure set point: 100 psi; times at set
32 point: 100; delta pressure: 20 psi). Once 130 $^{\circ}$ C was reached, taking about 1 min, the reaction mixture
33 was held at this temperature for 2 min. The mixture was diluted with water and extracted with ethyl
34 acetate. The organic layer was washed with brine and dried. Removal of the solvent gave a residue that
35 was purified by flash chromatography (silica gel, ethyl acetate/*n*-hexane as eluent) to furnish **6** (0.13 g,
36 27%), mp 202-205 $^{\circ}$ C (from ethanol). 1 H NMR (CDCl₃): δ 3.66 (s, 6H), 3.79 (s, 3H), 6.37 (s, 2H), 7.10
37 (t, J = 7.8 Hz, 1H), 7.38-7.41 (m, 5H), 7.71 (d, J = 6.7 Hz, 2H), 8.72 ppm (broad s, disappeared on
38 treatment with D₂O, 1H). IR: ν 3344 cm^{-1} . Anal. (C₂₃H₂₀BrNO₃S (470.38)) C, H, Br, N, S.
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4-Chloro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (8). Synthesized as **6**, starting from 4-chloro-2-phenyl-1H-indole (**47**). Yield 62%, mp 178-180 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.54 (s, 6H), 3.57 (s, 3H), 6.26 (s, 2H), 7.10-7.11 (m, 1H), 7.18-7.20 (m, 1H), 7.44-7.54 (m, 4H), 7.77-7.80 (m, 2H), 12.51 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3345 cm⁻¹. Anal. (C₂₃H₂₀ClNO₃S (425.93)) C, H, Cl, N, S.

4-Fluoro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (11). Synthesized as **6**, starting from 4-fluoro-2-phenyl-1H-indole (**48**). Yield 30%, mp 168-170 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.53 (s, 6H), 3.56 (s, 3H), 6.28 (s, 2H), 6.80-6.85 (m, 1H), 7.14-7.19 (m, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.44 (t, *J* = 7.1 Hz, 1H), 7.51 (t, *J* = 7.1 Hz, 2H), 7.80-7.82 (m, 2H), 12.30 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3307 cm⁻¹. Anal. (C₂₃H₂₀FNO₃S (409.47)) C, H, F, N, S.

4-Methoxy-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (13). Synthesized as **6**, starting from 4-methoxy-2-phenyl-1H-indole (**49**). Yield 41%, mp 133-136 °C (from toluene). ¹H NMR (CDCl₃): δ 3.66 (s, 6H), 3.78 (s, 6H), 6.44 (s, 2H), 6.57-6.59 (m, 1H), 7.05 (d, *J* = 8.2 Hz, 1H), 7.18 (t, *J* = 7.7 Hz, 1H), 7.38-7.48 (m, 3H), 7.73 (d, *J* = 7.2 Hz, 2H), 8.52 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3261 cm⁻¹. Anal. (C₂₄H₂₃NO₄S (421.51)) C, H, N, S.

5-Bromo-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (15). Synthesized as **6**, starting from 5-bromo-2-phenyl-1H-indole (**50**). Yield 18%, mp 152-155 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.53 (s, 6H), 3.56 (s, 3H), 6.27 (s, 2H), 7.32-7.35 (m, 1H), 7.44-7.58 (m, 5H), 7.86-7.88 (m, 2H), 12.29 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3316 cm⁻¹. Anal. (C₂₃H₂₀BrNO₃S (470.38)) C, H, Br, N, S.

5-Chloro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (18). Synthesized as **6**, starting from 5-chloro-2-phenyl-1H-indole (**51**). Yield 20%, mp 158-160 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.65 (s, 6H), 3.78 (s, 3H), 6.33 (s, 2H), 7.21-7.24 (m, 1H), 7.36-7.49 (m, 4H), 7.66-7.67 (m, 1H), 7.77-

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3 7.80 (m, 2H), 8.69 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3319 cm⁻¹. Anal.
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5 (C₂₃H₂₀ClNO₃S (425.93)) C, H, Cl, N, S.
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8 *5-Fluoro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (21)*. Synthesized as **6**, starting from
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10 5-fluoro-2-phenyl-1H-indole (**52**). Yield 26%, mp 158-160 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ
11 3.54 (s, 6H), 3.57 (s, 3H), 6.30 (s, 2H), 7.1 (t, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 9.2 Hz, 1H), 5.45 (t, *J* = 7.2
12 Hz, 1H), 7.50-7.55 (m, 3H), 7.88 (d, *J* = 7.4 Hz, 2H), 12.21 ppm (broad s, disappeared on treatment
13 with D₂O, 1H). IR: ν 3231 cm⁻¹. Anal. (C₂₃H₂₀FNO₃S (409.47)) C, H, F, N, S.
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18 *5-Methoxy-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (23)*. Synthesized as **6**, starting
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20 from 5-methoxy-2-phenyl-1H-indole (**53**). Yield 20% as an oil. ¹H NMR (CDCl₃): δ 3.63 (s, 6H), 3.77
21 (s, 3H), 3.83 (s, 3H), 6.35 (s, 2H), 6.90-6.93 (m, 1H), 7.10-7.11 (m, 1H), 7.32-7.46 (m, 4H), 7.76-7.77
22 (m, 2H), 8.58 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3337 cm⁻¹. Anal.
23 (C₂₄H₂₃NO₄S (421.51)) C, H, N, S.
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28 *6-Bromo-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (26)*. Synthesized as **6**, starting from
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30 6-bromo-2-phenyl-1H-indole (**54**). Yield 22%, mp 200-203 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ
31 3.57 (s, 3H), 3.58 (s, 6H), 6.78 (s, 2H), 7.23-7.27 (m, 3H), 7.35-7.41 (m, 3H), 7.48 (d, *J* = 8.3 Hz, 1H),
32 8.09 (s, 1H), 12.40 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3320 cm⁻¹. Anal.
33 (C₂₃H₂₀BrNO₃S (470.38)) C, H, Br, N, S.
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38 *6-Chloro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (28)*. Synthesized as **6**, starting from
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40 6-chloro-2-phenyl-1H-indole (**55**). Yield 25%, mp 207-210 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ
41 3.54 (s, 6H), 3.57 (s, 3H), 6.29 (s, 2H), 7.14 (dd, *J* = 1.1 and 7.7 Hz, 1H), 7.43-7.55 (m, 5H), 7.87 (d, *J*
42 = 7.9 Hz, 2H), 12.23 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3319 cm⁻¹. Anal.
43 (C₂₃H₂₀ClNO₃S (425.93)) C, H, Cl, N, S.
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6-Fluoro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (31). Synthesized as **6**, starting from 6-fluoro-2-phenyl-1H-indole (**56**). Yield 37% as an oil. ¹H NMR (CDCl₃): δ 3.64 (s, 6H), 3.78 (s, 3H), 6.35 (s, 2H), 6.96 (t, *J* = 8.8 Hz, 1H), 7.14 (d, *J* = 9.0 Hz, 1H), 7.41-7.50 (m, 3H), 7.58-7.59 (m, 1H), 7.78 (d, *J* = 8.0 Hz, 2H), 8.56 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3320 cm⁻¹. Anal. (C₂₃H₂₀FNO₃S (409.47)) C, H, F, N, S.

6-Methoxy-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (33). Synthesized as **6**, starting from 6-methoxy-2-phenyl-1H-indole (**57**). Yield 24%, mp 121-124 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.54 (s, 6H), 3.60 (s, 3H), 3.81 (s, 3H), 6.31 (s, 2H), 6.77 (dd, *J* = 2.2 and 8.6 Hz, 1H), 6.97 (d, *J* = 2.0 Hz, 1H), 7.34-7.42 (m, 2H), 7.50 (t, *J* = 7.4 Hz, 2H), 7.85 (d, *J* = 7.2 Hz, 2H), 11.88 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3322 cm⁻¹. Anal. (C₂₄H₂₃NO₄S (421.51)) C, H, N, S.

7-Bromo-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (35). Synthesized as **6**, starting from 7-bromo-2-phenyl-1H-indole (**58**). Yield 39%, mp 142-145 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.54 (s, 6H), 3.57 (s, 3H), 6.29 (s, 2H), 7.08 (t, *J* = 7.7 Hz, 1H), 6.97 (d, *J* = 7.7 Hz, 1H), 7.43-7.54 (m, 5H), 7.82 (d, *J* = 7.1 Hz, 1H), 12.11 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3335 cm⁻¹. Anal. (C₂₃H₂₀BrNO₃S (470.38)) C, H, Br, N, S.

7-Chloro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (37). Synthesized as **6**, starting from 7-chloro-2-phenyl-1H-indole (**59**). Yield 77%, mp 62-65 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.66 (s, 6H), 3.78 (s, 3H), 6.36 (s, 2H), 7.12-7.17 (m, 1H), 7.43-7.57 (m, 4H), 7.58-7.60 (m, 1H), 7.83-7.85 (m, 2H), 8.72 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3259 cm⁻¹. Anal. (C₂₃H₂₀ClNO₃S (425.93)) C, H, Cl, N, S.

7-Fluoro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (40). Synthesized as **6**, starting from 7-fluoro-2-phenyl-1H-indole (**60**). Yield 32%, mp 98-100 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ

3.54 (s, 6H), 3.57 (s, 3H), 6.30 (s, 2H), 7.05-7.10 (m, 2H), 7.31 (d, $J = 7.1$ Hz, 1H), 7.46 (t, $J = 7.2$ Hz, 1H), 7.53 (t, $J = 7.1$ Hz, 2H), 7.88 (d, $J = 7.2$ Hz, 2H), 12.46 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3247 cm⁻¹. Anal. (C₂₃H₂₀FNO₃S (409.47)) C, H, F, N, S.

7-Methoxy-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (42). Synthesized as **6**, starting from 7-methoxy-2-phenyl-1H-indole (**61**). Yield 57%, mp 158-160 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.53 (s, 6H), 3.57 (s, 3H), 3.97 (s, 3H), 6.29 (s, 2H), 6.79-6.81 (m, 1H), 7.04-7.08 (m, 2H), 7.40-7.49 (m, 3H), 7.83-7.86 (m, 2H), 12.09 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3300 cm⁻¹. Anal. (C₂₄H₂₃NO₄S (417.45)) C, H, N, S.

6,7-Dichloro-2-phenyl-3-((3,4,5-trimethoxyphenyl)thio)-1H-indole (44). Synthesized as **6**, starting from 6,7-dichloro-2-phenyl-1H-indole (**62**). Yield 57%, mp 158-160 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.55 (s, 6H), 3.57 (s, 3H), 6.29 (s, 2H), 7.32 (d, $J = 8.5$ Hz, 1H), 7.44-7.55 (m, 4H), 7.83-7.85 (m, 2H), 12.44 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1610, 3268 cm⁻¹. Anal. (C₂₃H₁₉Cl₂NO₃S (460.37)) C, H, Cl, N, S.

General Procedure for the Preparation of 7, 9, 12, 14, 16, 19, 22, 24, 27, 29, 32, 34, 36, 38, 41, 43 and 45. Example. *(4-Bromo-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (7)*. A mixture of **46** (0.27 g, 1 mmol), 3,4,5-trimethoxybenzoyl chloride (0.23 g, 1 mmol), and anhydrous aluminum chloride (0.13 g, 1 mmol) in anhydrous 1,2-dichloroethane (2 mL) was placed into the microwave cavity (closed vessel mode, Pmax = 250 psi). A starting microwave irradiation of 150 W was used, the temperature being ramped from 25 °C to 110 °C while stirring. Once 110 °C was reached, taking about 1 min, the reaction mixture was held at this temperature for 2 min. The reaction mixture was quenched on 1 M HCl/crushed ice and extracted with chloroform. The organic layer was washed with brine, dried, and filtered. Removal of the solvent gave a residue that was purified by flash chromatography (silica gel, ethyl acetate/*n*-hexane as eluent) to furnish **7** (0.09 g, 20%), mp 228-230 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.74 (s, 6H), 3.88 (s, 3H), 7.13-7.16 (m, 3H), 7.31-7.37 (m,

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3 4H), 7.42-7.46 (m, 3H), 8.58 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1618, 3344
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6 cm^{-1} . Anal. (C₂₄H₂₀BrNO₄ (466.32)) C, H, Br, N.

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8 *(4-Chloro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (9)*. Synthesized as **7**,
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10 starting from **47**. Yield 35%, mp 232-235 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.72 (s, 6H), 3.94 (s,
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12 3H), 7.17 (s, 2H), 7.19-7.23 (m, 2H), 7.30-7.33 (m, 3H), 7.38 (dd, J = 2.0 and 7.0 Hz, 1H), 7.45-7.48
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14 (m, 2H), 8.73 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1618, 3169 cm^{-1} . Anal.
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16 (C₂₄H₂₀ClNO₄ (421.87)) C, H, Cl, N.

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20 *(4-Fluoro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (12)*. Synthesized as **7**,
21
22 starting from **48**. Yield 25%, mp 212-215 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.62 (s, 6H), 3.64
23
24 (s, 3H), 6.84-6.89 (m, 1H), 6.99 (s, 2H), 7.18-7.22 (m, 1H), 7.30-7.35 (m, 4H), 7.42-7.44 (m, 2H),
25
26 12.34 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1618, 3205 cm^{-1} . Anal.
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28 (C₂₄H₂₀FNO₄ (405.42)) C, H, F, N.

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32 *(4-Methoxy-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (14)*. Synthesized as **7**,
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34 starting from **49**. Yield 51%, mp 144-146 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.53 (s, 3H), 3.64
35
36 (s, 6H), 3.69 (s, 3H), 6.55-6.57 (m, 1H), 7.02 (s, 2H), 7.09-7.16 (m, 2H), 7.29-7.39 (m, 3H), 7.49-7.51
37
38 (m, 2H), 11.95 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1622, 3220 cm^{-1} . Anal.
39
40 (C₂₅H₂₃NO₅ (417.45)) C, H, N.

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44 *(5-Bromo-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (16)*. Synthesized as **7**,
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46 starting from **50**. Yield 55%, mp 200-203 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.57 (s, 3H), 3.58
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48 (s, 6H), 6.78 (s, 2H), 7.23-7.27 (m, 3H), 7.35-7.41 (m, 3H), 7.48 (d, J = 8.3 Hz, 1H), 8.09 (s, 1H),
49
50 12.40 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1610, 3305 cm^{-1} . Anal.
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52 (C₂₄H₂₀BrNO₄ (466.32)) C, H, Br, N.
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(5-Chloro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**19**). Synthesized as **7**, starting from **51**. Yield 54%, mp 222-225 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.69 (s, 6H), 3.81 (s, 3H), 6.94 (s, 2H), 7.24-7.30 (m, 4H), 7.33-7.40 (m, 3H), 8.07-8.08 (m, 1H), 8.81 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1610, 3340 cm⁻¹. Anal. (C₂₄H₂₀ClNO₄ (421.87)) C, H, Cl, N.

(5-Fluoro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**22**). Synthesized as **7**, starting from **52**. Yield 66%, mp 198-200 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.58 (s, 3H), 3.59 (s, 6H), 6.78 (s, 2H), 7.10-7.15 (m, 1H), 7.24-7.27 (m, 3H), 7.34-7.37 (m, 2H), 7.51 (q, *J* = 4.6 Hz, 1H), 7.66 (dd, *J* = 2.5 and 10.1, 1H), 12.30 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1620, 3225 cm⁻¹. Anal. (C₂₄H₂₀FNO₄ (405.42)) C, H, F, N.

(5-Methoxy-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**24**). Synthesized as **7** starting from **53**. Yield 78%, mp 202-205 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.58 (s, 3H), 3.59 (s, 6H), 3.78 (s, 3H), 6.78 (s, 2H), 6.90 (dd, *J* = 2.6 and 8.8 Hz, 1H), 7.24-7.25 (m, 3H), 7.32-7.34 (m, 2H), 7.41 (d, *J* = 8.8 Hz, 1H), 7.48 (d, *J* = 2.4 Hz, 1H), 12.11 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1652, 3195 cm⁻¹. Anal. (C₂₅H₂₃NO₅ (417.45)) C, H, N.

(6-Bromo-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**27**). Synthesized as **7**, starting from **54**. Yield 40% as an oil. ¹H NMR (CDCl₃): δ 3.68 (s, 6H), 3.81 (s, 3H), 6.94 (s, 2H), 7.23-7.27 (m, 3H), 7.33-7.36 (m, 3H), 7.59 (s, 1H), 7.87 (d, *J* = 8.6 Hz, 1H), 8.84 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1610, 3304 cm⁻¹. Anal. (C₂₄H₂₀BrNO₄ (466.32)) C, H, Br, N.

(6-Chloro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**29**). Synthesized as **7**, starting from **55**. Yield 67%, mp 188-190 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.63 (s, 6H), 3.79 (s, 3H), 6.93 (s, 2H), 7.20-7.24 (m, 4H), 7.31-7.33 (m, 2H), 7.41-7.43 (m, 1H), 7.91 (d, *J* = 8.6 Hz, 1H),

8.84 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3319, 1618 cm⁻¹. Anal. (C₂₄H₂₀ClNO₄ (421.87)) C, H, Cl, N.

(6-Fluoro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**32**). Synthesized as **7**, starting from **56**. Yield 25% as an oil. ¹H NMR (CDCl₃): δ 3.68 (s, 6H), 3.80 (s, 3H), 6.94 (s, 2H), 7.05 (t, J = 8.9 Hz, 1H), 7.14 (d, J = 9.0 Hz, 1H), 7.26-7.33 (m, 5H), 7.99-8.02 (m, 1H), 8.58 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1621, 3265 cm⁻¹. Anal. (C₂₄H₂₀FNO₄ (405.42)) C, H, F, N.

(6-Methoxy-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**34**). Synthesized as **7**, starting from **57**. Yield 63%, mp 186-188 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 9H), 3.83 (s, 3H), 6.80 (s, 2H), 6.85 (dd, J = 2.3 and 8.8 Hz, 1H), 6.97 (d, J = 2.2 Hz, 1H), 7.24-7.26 (m, 3H), 7.32-7.34 (m, 2H), 7.79 (d, J = 8.8 Hz, 1H), 12.01 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1610, 3302 cm⁻¹. Anal. (C₂₅H₂₃NO₅ (417.45)) C, H, N.

(7-Bromo-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**36**). Synthesized as **7**, starting from **58**. Yield 58%, mp 188-190 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.71 (s, 6H), 3.82 (s, 3H), 6.97 (s, 2H), 7.18 (t, J = 7.8 Hz, 1H), 7.28-7.32 (m, 3H), 7.42-7.44 (m, 2H), 7.48 (d, J = 7.6 Hz, 1H), 7.99 (d, J = 8.0 Hz, 1H), 8.67 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1618, 3258 cm⁻¹. Anal. (C₂₄H₂₀BrNO₄ (466.32)) C, H, Br, N.

(7-Chloro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**38**). Synthesized as **7**, starting from **59**. Yield 64%, mp 148-150 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 3H), 3.61 (s, 6H), 6.80 (s, 2H), 7.20-7.29 (m, 4H), 7.34 (dd, J = 1.0 and 7.6 Hz, 1H), 7.38-7.41 (m, 2H), 7.88 (dd, J = 1.0 and 8.0 Hz, 1H), 12.41 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1607, 3215 cm⁻¹. Anal. (C₂₄H₂₀ClNO₄ (421.87)) C, H, Cl, N.

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(7-Fluoro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**41**). Synthesized as **7**, starting from **60**. Yield 65%, mp 172-175 °C (from toluene). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 3H), 3.61 (s, 6H), 6.81 (s, 2H), 7.07-7.18 (m, 2H), 7.26-7.27 (m, 3H), 7.38-7.40 (m, 2H), 7.72 (d, *J* = 7.8 Hz, 1H), 12.60 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1615, 3254 cm⁻¹. Anal. (C₂₄H₂₀FNO₄ (405.42)) C, H, F, N.

(7-Methoxy-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**43**). Synthesized as **7**, starting from **61**. Yield 20%, mp 185-188 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 3H), 3.60 (s, 6H), 3.98 (s, 3H), 6.79 (s, 2H), 6.83 (d, *J* = 7.4 Hz, 1H), 7.11 (t, *J* = 7.9 Hz, 1H), 7.20-7.23 (m, 3H), 7.35-7.38 (m, 2H), 7.47-7.49 (m, 1H), 12.24 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1606, 3270 cm⁻¹. Anal. (C₂₅H₂₃NO₅ (417.45)) C, H, N.

(6,7-Dichloro-2-phenyl-1H-indol-3-yl)(3,4,5-trimethoxyphenyl)methanone (**45**). Synthesized as **7**, starting from **62**. Yield 23%, mp 218-220 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 3H), 3.61 (s, 6H), 6.81 (s, 2H), 7.25-7.32 (m, 3H), 7.39-7.42 (m, 3H), 7.86 (d, *J* = 8.5 Hz, 1H), 12.58 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1607, 3271 cm⁻¹. Anal. (C₂₄H₁₉Cl₂NO₄S (456.32)) C, H, Cl, N, S.

General Procedure for the Preparation of 10, 20, 25, 30 and 39. *Example. 4-Chloro-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (10).* Borane tetrahydrofuran complex (1.0 mL, 1.0 M in tetrahydrofuran) was slowly added to a cold solution of **9** (0.1 g, 0.24 mmol) in anhydrous acetonitrile (1.4 mL) containing anhydrous methanol (0.02 mL) under an Ar stream. The reaction was stirred at 50 °C for 1 h. After cooling, the mixture was carefully diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried and filtered. Removal of the solvent gave a residue that was purified by flash chromatography (silica gel, ethyl acetate/*n*-hexane as eluent) to furnish **10** (0.01 g, 10%), mp 178-180 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.74 (s, 6H), 3.84 (s, 3H), 4.45 (s, 2H), 6.44 (s, 2H), 7.10-7.15 (m, 2H), 7.33 (dd, *J* = 2.2 and 6.8 Hz, 1H), 7.41-7.47 (m, 3H),

7.51-7.53 (m, 2H), 8.29 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3358 cm⁻¹. Anal. (C₂₄H₂₂ClNO₃ (407.89)) C, H, Cl, N.

5-Chloro-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (20). Synthesized as **10**, starting from **19**. Yield 41%, mp 172-175 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.76 (s, 6H), 3.82 (s, 3H), 4.16 (s, 2H), 6.41 (s, 2H), 7.15 (dd, $J = 2.0$ and 8.6 Hz, 1H), 7.31 (d, $J = 8.6$ Hz, 1H), 7.37-7.39 (m, 1H), 7.42-7.46 (m, 3H), 7.51-7.54 (m, 2H), 8.23 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3340 cm⁻¹. Anal. (C₂₄H₂₂ClNO₃ (407.89)) C, H, Cl, N.

5-Methoxy-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (25). Synthesized as **10**, starting from **24**. Yield 26%, mp 137-139 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 3.59 (s, 3H), 3.60 (s, 6H), 3.72 (s, 3H), 4.14 (s, 2H), 6.48 (s, 2H), 6.76 (dd, $J = 2.4$ and 8.9 Hz, 1H), 6.97 (d, $J = 2.4$ Hz, 1H), 7.27 (d, $J = 8.9$ Hz, 1H), 7.35-7.39 (m, 1H), 7.50 (t, $J = 7.4$ Hz, 2H), 7.61-7.64 (m, 2H), 11.12 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3361 cm⁻¹. Anal. (C₂₅H₂₅NO₄ (403.47)) C, H, N.

6-Chloro-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (30). Synthesized as **10**, starting from **29**. Yield 41%, mp 187-185 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.73 (s, 6H), 3.84 (s, 3H), 4.21 (s, 2H), 6.44 (s, 2H), 7.07 (dd, $J = 2.0$ and 8.4 Hz, 1H), 7.38-7.42 (m, 3H), 7.45-7.49 (m, 2H), 7.53-7.56 (m, 2H), 8.25 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3332 cm⁻¹. Anal. (C₂₄H₂₂ClNO₃ (407.89)) C, H, Cl, N.

7-Chloro-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (39). Synthesized as **10**, starting from **38**. Yield 41%, mp 156-158 °C (from ethanol). ¹H NMR (CDCl₃): δ 3.74 (s, 6H), 3.83 (s, 3H), 4.22 (s, 2H), 6.44 (s, 2H), 7.05 (t, $J = 7.8$ Hz, 1H), 7.22-7.24 (m, 1H), 7.39-7.45 (m, 2H), 7.48-7.51 (m, 2H), 7.59-7.61 (m, 2H), 8.34 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3347 cm⁻¹. Anal. (C₂₄H₂₂ClNO₃ (407.89)) C, H, Cl, N.

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5-Bromo-2-phenyl-3-(3,4,5-trimethoxybenzyl)-1H-indole (17). A mixture of **16** (0.25 g, 0.54 mmol), triethylsilane (0.14 g, 0.19 mL, 1.2 mmol) and trifluoroacetic acid (0.63 g, 0.41 mL, 5.5 mmol) in 1,2-dichloroethane (2.0 mL) was placed into the microwave cavity (closed vessel mode, Pmax = 250 psi). A starting microwave irradiation of 250 W was used, the temperature being ramped from 25 to 250 °C while rapidly stirring and cooling. Once 250 °C was reached, taking about 2 min, the reaction mixture was held at this temperature for 20 min. The reaction mixture was diluted with a saturated aqueous solution of NaHCO₃ and extracted with ethyl acetate. The organic layer was washed with brine, dried, and filtered. Removal of the solvent gave a residue that was purified by flash chromatography (silica gel, ethyl acetate/*n*-hexane as eluent) to furnish **17** (0.04 g, 15%) as a colorless oil. ¹H NMR (CDCl₃): δ 3.73 (s, 6H), 3.83 (s, 3H), 4.17 (s, 2H), 6.42 (s, 2H), 7.28 (s, 2H), 7.37-7.47 (m, 3H), 7.53-7.55 (m, 2H), 7.62 (s, 1H), 8.25 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3338 cm⁻¹. Anal. (C₂₄H₂₂BrNO₃ (452.34)) C, H, Br, N.

2. Synthesis of Compounds 46-62. General Procedure for the Preparation of 46, 54 and 56.

Example. *4-Bromo-2-phenyl-1H-indole (46)*. A mixture of **66** (0.1 g, 0.4 mmol), tin(II) chloride dihydrate (1.35 g, 6 mmol) and 1 N HCl (0.92 mL) in glacial acetic acid (4.80 mL) was heated at reflux temperature for 12 h. After cooling, the reaction mixture was diluted with a saturated aqueous solution of potassium carbonate and extracted with ethyl acetate. The organic layer was washed with brine, dried and filtered. Removal of the solvent gave a residue that was purified by column chromatography (silica gel, chloroform as eluent) to furnish **46** (0.02 g, 19%), mp 98-100 °C, Lit.⁴¹ mp 100-102 °C.

6-Bromo-2-phenyl-1H-indole (54). Synthesized as **46**, starting from **67**. Yield 46%, mp 188-190 °C (from ethanol), Lit.⁴² 187 °C.

6-Fluoro-2-phenyl-1H-indole (56). Synthesized as **46**, starting from **68**. Yield 24%, mp 170-171 °C (from ethanol), Lit.⁴³ 171-172 °C.

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3 **General Procedure for the Preparation of 47-49, 55, 57 and 61.** *Example. 4-Chloro-2-phenyl-1H-*
4 *indole (47).* A solution of **69** (2.0 g, 8 mmol) in anhydrous tetrahydrofuran (120 mL) was cooled at -
5
6 40 °C, and then a solution of *tert*-butyllithium (9.4 mL, 16 mmol, 7.7 M in pentane) was added
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8 dropwise under an Ar stream. The reaction mixture was stirred at 0 °C for 1 h and at 25 °C for 12 h,
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10 diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried and
11
12 filtered. Removal of the solvent gave a residue that was purified by column chromatography (silica gel,
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14 dichloromethane as eluent) to furnish **47** (0.56 g, 31%), mp 73-76 °C (from ethanol), Lit.⁴¹ 73-75 °C.
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20 *4-Fluoro-2-phenyl-1H-indole (48).* Synthesized as **47** starting from **70**. Yield 52%, mp 60-62 °C
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22 (from ethanol), Lit.⁴¹ 62-64 °C.
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25 *4-Methoxy-2-phenyl-1H-indole (49).* Synthesized as **47**, starting **71**. Yield 34%, mp 100-103 °C
26
27 (from ethanol), Lit.⁴⁴ 103-105 °C.
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30 *6-Chloro-2-phenyl-1H-indole (55).* Synthesized as **47**, starting from **72**. Yield 18%, mp 176-177 °C
31
32 (from ethanol), Lit.⁴⁵ 180-181 °C.
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35 *6-Methoxy-2-phenyl-1H-indole (57).* Synthesized as **47**, starting from **73**. Yield 31%, mp 170-
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37 173 °C (from ethanol), Lit.⁴⁶ 173-176 °C.
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40 *7-Methoxy-2-phenyl-1H-indole (61).* Synthesized as **47**, starting from **74**. Yield 28%, mp 83-87 °C
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42 (from ethanol), Lit.⁴⁷ 85-86 °C.
43

44 **General Procedure for the Preparation of 50-52, 58, 60 and 62.** *Example. 5-Bromo-2-phenyl-1H-*
45 *indole (50).* Compound **75** (0.80 g, 2.8 mmol) was added by portions to polyphosphoric acid (8.0 g)
46
47 preheated at 110 °C. The reaction mixture was stirred at the same temperature for 1 h and then
48
49 quenched on crushed ice. The solid was filtered and crystallized from ethanol to give **75** (0.65 g, 82%),
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51 mp 190-192 °C, Lit.⁴⁸ 193-196 °C.
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56 *5-Chloro-2-phenyl-1H-indole (51).* Synthesized as **50**, starting from **76**. Yield 37%, mp 198-200 °C
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58 (from ethanol), Lit.⁴⁹ 203-204 °C.
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3 *5-Fluoro-2-phenyl-1H-indole (52)*. Synthesized as **50**, starting from **77**. Yield 37%, mp 180-185 °C
4 (from ethanol), Lit.⁵⁰ 181-183 °C.
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8 *7-Bromo-2-phenyl-1H-indole (58)*. Synthesized as **50**, starting from **78**. Yield 14%, mp 115-117 °C
9 (from ethanol), Lit.⁵¹ 117-118 °C. ¹H NMR (DMSO-*d*₆): δ 6.97 (t, *J* = 7.7, 1H), 7.02 (s, 1H), 7.31-7.38
10 (m, 2H), 7.47 (t, *J* = 7.5 Hz, 2H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.98-8.00 (m, 2H), 11.36 ppm (broad s,
11 disappeared on treatment with D₂O, 1H). IR: ν 3436 cm⁻¹.
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18 *7-Fluoro-2-phenyl-1H-indole (60)*. Synthesized as **50**, starting from **79**. Yield 25%, mp 122-125 °C
19 (from ethanol). ¹H NMR (DMSO-*d*₆): δ 6.91-7.00 (m, 3H), 7.33-7.38 (m, 2H), 7.47 (t, *J* = 7.5 Hz, 2H),
20 7.95 (d, *J* = 7.4 Hz, 2H), 11.85 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3436 cm⁻¹.
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26 *6,7-Dichloro-2-phenyl-1H-indole (62)*. Synthesized as **50**, starting from **80**. Yield 27%, mp 105-
27 108 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 7.03 (s, 1H), 7.21 (t, *J* = 8.4 Hz, 1H), 7.37 (t, *J* = 7.4
28 Hz, 1H), 7.46-7.55 (m, 3H), 7.99 (d, *J* = 8.0 Hz, 2H), 11.71 ppm (broad s, disappeared on treatment
29 with D₂O, 1H). IR: ν 3248 cm⁻¹.
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35 *5-Methoxy-2-phenyl-1H-indole (53)* was synthesized according to a Lit.⁵² procedure.
36

37
38 *7-Chloro-2-phenyl-1H-indole (59)* was synthesized according to a Lit.⁵³ procedure.
39

40 **3. Synthesis of Compounds 63-80. General Procedure for the Preparation of 63-65. Example.**

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42 *2-(2-Bromo-6-nitrophenyl)-1-phenylethanol (63)*. To a solution of benzaldehyde (0.12 g, 0.1 mL, 1.1
43 mmol) and 2-bromo-6-nitrotoluene (0.25 g, 1.2 mmol) in anhydrous DMSO was added a solution of
44 sodium ethoxide in anhydrous ethanol (0.32 mL, 0.43 M). The reaction mixture was stirred at 25 °C for
45 12 h, carefully diluted with water and extracted with ethyl acetate. The organic layer was washed with
46 brine, dried and filtered. Removal of the solvent gave a residue that was purified by column
47 chromatography (silica gel, ethyl acetate:*n*-hexane = 1:3 as eluent) to furnish **63** (0.15 g, 40%) as an oil.
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56 ¹H NMR (DMSO-*d*₆): δ 3.21-3.24 (m, 2H), 4.64 (d, *J* = 7.4 Hz, 1H), 5.48 (broad s, disappeared on
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3 treatment with D₂O, 1H), 7.25-7.33 (m, 5H), 7.42 (t, *J* = 8.0 Hz, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.97
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5 ppm (d, *J* = 8.2 Hz, 1H). IR: ν 2961, 3030, 3063, 3325, 3560 cm⁻¹.
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8 *2-(4-Bromo-2-nitrophenyl)-1-phenylethanol (64)*. Synthesized as **63**, starting from 4-bromo-1-
9 methyl-2-nitrobenzene. Yield 44% as an oil. ¹H NMR (DMSO-*d*₆): δ 3.07-3.12 (m, 2H), 4.69-4.73 (m,
10 1H), 5.43 (d, *J* = 4.5 Hz, disappeared on treatment with D₂O, 1H), 7.21-7.25 (m, 1H), 7.28-7.33 (m,
11 4H), 7.39 (d, *J* = 8.3 Hz, 1H), 7.80 (dd, *J* = 1.7 and 8.2 Hz, 1H), 8.08-8.10 ppm (m, 1H). IR: ν 2934,
12 13 14 15 16 17 18 19 3030, 3065, 3418 cm⁻¹.

20 *2-(4-Fluoro-2-nitrophenyl)-1-phenylethanol (65)*. Synthesized as **63**, starting from 4-fluoro-1-
21 methyl-2-nitrobenzene. Yield 44% as an oil. ¹H NMR (DMSO-*d*₆): δ 3.12-3.14 (m, 2H), 4.68-4.73 (m,
22 1H), 5.40 (d, *J* = 4.4 Hz, disappeared on treatment with D₂O, 1H), 7.22-7.33 (m, 5H), 7.46-7.51 (m,
23 24 25 26 27 28 29 2H), 7.81 ppm (dd, *J* = 2.3 and 9.0 Hz, 1H). IR: ν 2932, 3032, 3087, 3404 cm⁻¹.

30 **General Procedure for the Preparation of 66-68.** *Example. 2-(2-Bromo-6-nitrophenyl)-1-*
31 *phenylethanone (66)*. A solution of **63** (0.1 g, 0.3 mmol) in anhydrous dichloromethane (1.0 mL) was
32 added to a suspension of pyridinium chlorochromate (0.10 g, 0.47 mmol) in the same solvent (2.0 mL).
33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 The reaction mixture was stirred at 25 °C for 1.5 h and diluted with water. The layers were separated,
and the organic phase was washed with brine, dried and filtered. Removal of the solvent gave a residue
that was purified by column chromatography (silica gel, chloroform:petroleum ether = 1:1 as eluent) to
furnish **66** (0.06 g, 40%), mp 113-115 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 4.89 (s, 2H), 7.52-
7.62 (m, 3H), 7.70 (t, *J* = 7.8 Hz, 1H), 8.05-8.10 ppm (m, 4H). IR: ν 1680 cm⁻¹.

2-(4-Bromo-2-nitrophenyl)-1-phenylethanone (67). Synthesized as **66**, starting from **64**. Yield 40%,
mp 114-116 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 4.87 (s, 2H), 7.53-7.60 (m, 3H), 7.70 (t, *J* = 7.1
Hz, 1H), 7.96-7.98 (m, 1H), 8.05 (d, *J* = 7.4 Hz, 2H), 8.28-8.30 ppm (m, 1H). IR: ν 1686 cm⁻¹.

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2-(4-Fluoro-2-nitrophenyl)-1-phenylethanone (68). Synthesized as **66**, starting from **65**. Yield 16%, mp 95-98 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 4.88 (s, 2H), 7.56-7.72 (m, 5H), 8.03-8.06 ppm (m, 3H). IR: ν 1680 cm⁻¹.

General Procedure for the Preparation of 69-74. *Example.* *N-(3-Chloro-2-methylphenyl)benzamide (69)*. A solution of benzoyl chloride (7.93 g, 6.55 mL, 56 mmol) in anhydrous tetrahydrofuran (20 mL) was added dropwise to a solution of 3-chloro-2-methylaniline (6.68 g, 5.64 mL, 47 mmol) and triethylamine (5.70 g, 7.85 mL, 56 mmol) at 0 °C in the same solvent (134 mL). The reaction was heated at reflux for 2 h. After cooling, the mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried and filtered. Removal of the solvent gave a residue that was triturated with diethyl ether to furnish **69** (5.66 g, 49%), mp 168-170 °C, Lit.⁵⁴ 170 °C. ¹H NMR (CDCl₃): δ 2.40 (s, 3H), 7.21 (t, *J* = 8.0 Hz, 1H), 7.26-7.29 (m, 1H), 7.51-7.55 (m, 2H), 7.58-7.62 (m, 1H), 7.75-7.80 (m, 2H; one proton disappeared after treatment with D₂O), 7.90-7.92 ppm (m, 2H). IR: ν 1647, 3244 cm⁻¹.

N-(3-Fluoro-2-methylphenyl)benzamide (70). Synthesized as **69**, starting from 3-fluoro-2-methylaniline. Yield 67%, mp 150-152 °C (from ethanol), Lit.⁵⁵ 157-158 °C. ¹H NMR (CDCl₃): δ 2.25 (s, 3H), 6.93 (t, *J* = 9.0 Hz, 1H), 7.20-7.27 (m, 1H), 7.52 (t, *J* = 7.7 Hz, 2H), 7.59 (t, *J* = 7.3 Hz, 1H), 7.71-7.75 (m, 2H; one proton disappeared after treatment with D₂O), 7.88-7.91 ppm (m, 2H). IR: ν 1670, 3230 cm⁻¹.

N-(3-Methoxy-2-methylphenyl)benzamide (71). Synthesized as **69**, starting from 3-methoxy-2-methylaniline. Yield 82%, mp 173-175 °C (from ethanol), Lit.⁵⁶ 177 °C. ¹H NMR (DMSO-*d*₆): δ 2.06 (s, 3H), 3.82 (s, 3H), 6.89 (d, *J* = 8.2 Hz, 1H), 6.94 (d, *J* = 7.8 Hz, 1H), 7.19 (t, *J* = 8.1 Hz, 1H), 7.53 (t, *J* = 7.6 Hz, 2H), 7.59 (t, *J* = 7.4 Hz, 1H), 7.98 (d, *J* = 7.2 Hz, 2H), 9.89 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 1649, 3229 cm⁻¹.

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3 *N*-(5-Chloro-2-methylphenyl)benzamide (**72**). Synthesized as **69**, starting from 5-chloro-2-
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methylamine. Yield 58%, mp 125-127 °C (from ethanol), Lit.⁵⁷ 121-123 °C.

N-(5-Methoxy-2-methylphenyl)benzamide (**73**). Synthesized as **69**, starting from 5-methoxy-2-
methylamine. Yield 88%, mp 113-115 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.17 (s, 3H), 3.74 (s,
3H), 6.77 (dd, *J* = 2.6 and 8.4 Hz, 1H), 6.98 (d, *J* = 2.6 Hz, 1H), 7.17 (d, *J* = 8.4 Hz, 1H), 7.53 (t, *J* =
7.6 HZ, 2H), 7.59 (t, *J* = 7.3 Hz, 1H), 7.98 (d, *J* = 7.1 Hz, 2H), 9.83 ppm (broad s, disappeared on
treatment with D₂O, 1H). IR: ν 1650, 3299 cm⁻¹.

N-(2-Methoxy-6-methylphenyl)benzamide (**74**). Synthesized as **69**, starting from 2-methoxy-6-
methylamine. Yield 82%, mp 127-130 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.50 (s, 3H), 3.74 (s,
3H), 6.87-6.93 (m, 2H), 7.20 (t, *J* = 8.0 Hz, 1H), 7.49-7.60 (m, 3H), 8.00 (d, *J* = 7.3 Hz, 2H), 9.57 ppm
(broad s, disappeared on treatment with D₂O, 1H). IR: ν 1647, 3360 cm⁻¹.

General Procedure for the Preparation of 75-80. *Example. 1-(4-Bromophenyl)-2-(1-phenylethylidene)hydrazine (75).* A mixture of 4-bromophenylhydrazine hydrochloride (3.35 g, 15 mmol), acetophenone (1.20 g, 1.16 mL, 10 mmol) and sodium acetate (1.23 g, 15 mmol) in ethanol (15 mL) was placed into the microwave cavity (open vessel mode). Microwave irradiation of 250 W was used, the temperature being ramped from 25 °C to 80 °C. Once 80 °C was reached, taking about 1 min, the reaction mixture was held at this temperature for 5 min, while stirring and cooling. The reaction mixture was cooled to 0 °C, filtered, washed with petroleum ether and dried to give **76** (0.90 g, 30%), mp 118-120 °C (from ethanol). ¹H NMR (CDCl₃): δ 2.25 (s, 3H), 7.08 (d, *J* = 8.9 Hz, 2H), 7.35-7.41 (m, 6H; one proton disappeared after treatment with D₂O), 7.83 ppm (d, *J* = 8.5 Hz, 2H). IR: ν 3353 cm⁻¹.

1-(4-Chlorophenyl)-2-(1-phenylethylidene)hydrazine (76). Synthesized as **75**, starting from 4-chlorophenyl hydrazine hydrochloride. Yield 25%, mp 105-107 °C (from ethanol), Lit.⁵⁸ 100-102 °C. ¹H NMR (CDCl₃): δ δ 2.25 (s, 3H), 7.12 (d, *J* = 8.8 Hz, 2H), 7.24 (d, *J* = 8.8 Hz, 2H), 7.31-7.35 (m,

2H; one proton disappeared after treatment with D₂O), 7.40 (t, $J = 7.0$ Hz, 2H), 7.79 ppm (d, $J = 7.1$ Hz, 2H). IR: ν 3352 cm⁻¹.

1-(4-Fluorophenyl)-2-(1-phenylethylidene)hydrazine (77). Synthesized as **75**, starting from 4-fluorophenyl hydrazine hydrochloride. Yield 95%, mp 110-112 °C (from ethanol), Lit.⁵⁹ 107 °C. ¹H NMR (DMSO-*d*₆): δ 2.25 (s, 3H), 7.00-7.10 (m, 2H), 7.22-7.29 (m, 3H), 7.38 (t, $J = 7.3$ Hz, 2H), 7.78 (d, $J = 7.2$ Hz, 2H), 9.31 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3380 cm⁻¹.

1-(2-Bromophenyl)-2-(1-phenylethylidene)hydrazine (78). Synthesized as **75**, starting from 2-bromophenyl hydrazine hydrochloride. Yield 86%, mp >300 °C (from ethanol). Spectral data were consistent with those reported in Lit.⁶⁰

1-(2-Fluorophenyl)-2-(1-phenylethylidene)hydrazine (79). Synthesized as **75**, starting from 2-fluorophenyl hydrazine hydrochloride. Yield 68%, mp >300 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 2.32 (s, 3H), 6.79-6.84 (m, 1H), 7.13-7.17 (m, 2H), 7.34-7.43 (m, 3H), 7.56-7.59 (m, 1H), 7.80-7.83 (m, 2H), 8.64 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3380 cm⁻¹.

1-(2,3-Dichlorophenyl)-2-(1-phenylethylidene)hydrazine (80). Synthesized as **75**, starting from 2,3-dichlorophenyl hydrazine hydrochloride. Yield 79%, mp 112-115 °C. ¹H NMR (DMSO-*d*₆): δ 2.36 (s, 3H), 7.09 (dd, $J = 1.4$ and 7.9 Hz, 1H), 7.31 (t, $J = 8.4$, 1H), 7.37-7.45 (m, 3H), 7.60 (dd, $J = 1.3$ and 8.4 Hz, 1H), 7.84-7.87 (m, 2H), 8.40 ppm (broad s, disappeared on treatment with D₂O, 1H). IR: ν 3270 cm⁻¹.

Molecular Modeling. All molecular modeling studies were performed on a MacPro dual 2.66GHz Xeon running Ubuntu 14. The tubulin structure was downloaded from the PDB data bank (<http://www.rcsb.org/>, PDB code: 1SA0).⁶ Ligand structures were built with MOE⁶¹ and minimized using the MMFF94x forcefield until a RMSD gradient of 0.05 kcal mol⁻¹ Å⁻¹ was reached. The docking simulations were performed using PLANTS¹⁸ on the 1SA0 crystal structure.

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3 **Biology. Tubulin Assembly.** The reaction mixtures contained 0.8 M monosodium glutamate (pH
4 6.6 with HCl in a 2 M stock solution), 10 μM tubulin, 4% (v/v) DMSO and varying concentrations of
5 drug. Following a 15 min preincubation at 30 $^{\circ}\text{C}$, samples were chilled on ice, GTP to 0.4 mM was
6 added, and turbidity development was followed at 350 nm in a temperature controlled recording
7 spectrophotometer for 20 min at 30 $^{\circ}\text{C}$. Extent of reaction was measured. Full experimental details
8 were previously reported.⁶²
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17 **[³H]Colchicine Binding Assay.** The reaction mixtures contained 1.0 μM tubulin, 5.0 μM
18 [³H]colchicine, and 5.0 μM inhibitor and were incubated 10 min at 37 $^{\circ}\text{C}$. Complete details were
19 described previously.⁶³
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25 **Cell Cultures.** Cell lines were obtained from the American Type Culture Collection (ATCC), unless
26 otherwise specified. MCF-7 breast carcinoma, OVCAR-8, and NCI/ADR-RES cells were obtained
27 from the National Cancer Institute drug screening laboratory, and NB4 cells and MV4-11 cells from the
28 Deutsche Sammlung von Mikroorganismen und Zellkulturen. All cell lines, except as indicated, were
29 grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum
30 (FBS), 20 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin and 1% L-glutamine; specific
31 requirements include the addition of sodium pyruvate (1-2% for RD rhabdomyosarcoma, HepG2
32 hepatoma and the three MDA breast carcinoma cell lines) and glucose (1 g/L for RD and HepG2, 4.5
33 g/L for PC3 prostate carcinoma). Cell lines were cultured at 37 $^{\circ}\text{C}$ in 5% CO_2 -95% air in a humidified
34 incubator. Treatments were initiated 24 h after cell seeding using ATI compound diluted in 0.1%
35 DMSO, or the indicated reference compound, or 0.1% DMSO vehicle, for 24-72 h as indicated.
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51 **Cell Viability Assays.** MCF-7 breast carcinoma and OVCAR-8 and NCI/ADR-RES ovary
52 carcinoma cells: methodology for evaluation of growth was previously described, except that cells were
53 grown for 96 h for IC_{50} determinations.⁶⁴
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3 MV4-11, NB4, A-549, NCI-H1975, Messa and Messa/Dx5: cells were seeded into 96-well plates
4 (Corning Inc., Costar) at a density of 2×10^3 cells/well in 50 μL of the appropriate medium. MDA-MB-
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6 468, MDA-MB-436 and MDA-MB-231 breast carcinoma cell lines: cells were plated in 100 μL
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8 medium in 96-well plates at a density of 3.5×10^3 per well for MDA-MB-468, 3×10^3 per well for
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10 MDA-MB-436 and 2×10^3 per well for MDA-MB-436. After 24 h, cells were treated with the inhibitor
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12 (100 nM to 0.39 nM) and then evaluated in MTT assays as described.⁶⁵ Statistical analysis was
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14 performed by analysis of variance (ANOVA) with Neumann-Keul's multiple comparison test or
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16 Kolmogorov-Smirnov where appropriate.
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22 PC-3, HepG2 and RD cell lines: cells were seeded in 24-well plates at a density of $95 \times 10^3/100 \mu\text{L}$
23 well (PC-3 and RD), or $120 \times 10^3/100 \mu\text{L}$ well (HepG2). After 24 h, the test compound was added (0.01-
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25 25 μM) for 48 h. After removal of the medium, MTT was added (500 μM final concentration in 500
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27 μL /well of phosphate-buffered saline; PBS) and incubation continued at 37 °C for 2 h in the dark. The
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29 formazan crystals were dissolved in isopropanol containing 0.04 N HCl (200 μL). A_{550} in the wells was
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31 determined using a Multiskan Spectrum Thermo Electron Corporation reader. IC_{50} values were
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33 calculated by nonlinear regression analysis (GraphPad Prism statistics software). Experiments were
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35 performed in triplicate. HeLa cells: 7×10^3 cell-aliqouts were seeded in a flat-bottom 96-well tissue
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37 culture plate and, after 24 h, were exposed to the inhibitor (10-100 nM) for 24 or 48 h. MTT (10 μL , 5
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39 mg/mL) (Sigma-Aldrich) was added to each well and cells were further incubated for 3 h at 37 °C.
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41 After solubilization of the crystals with isopropanol/0.04 N HCl, A_{570} measurements were made with
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43 an ELISA reader, and IC_{50} values were derived from dose-response curves.
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51 MV4-11, NB4 (AML), A-549 and NCI-H1975 (lung adenocarcinoma) cell lines: cell growth was
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53 measured using CellTiter-Fluor[®] (Promega), a nonlytic, single-reagent-addition fluorescence assay that
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55 detects the relative number of living cells in samples after experimental manipulation. The CellTiter-
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57 Fluor[™] Cell Viability Assay measures the conserved and constitutive protease activity within live cells
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3 and, therefore, acts as a marker for cell viability. NB4 and MV4-11 cells in exponential growth were
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5 incubated for 48 h with different concentrations of the inhibitors. After 48 h, CellTiter-Fluor[®] Reagent
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7 was added to the cell culture medium (1:1 vol/vol) and incubated for at least 90 min at 37 °C. A549 and
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9 NCI-H1975 cells were treated with the inhibitor for 72 h, then CellTiter-Fluor[®] Reagent was added to
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11 one fifth of the culture medium volume. Fluorescence was recorded (excitation wavelength, 360 nm;
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13 emission wavelength, 535 nm), and the IC₅₀ was calculated using GraphPad Software.
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18 Messa and Messa/Dx5 (resistant) sarcoma cell lines: the CellTiter-Glo luminescent cell viability
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20 assay was used (Promega, Madison, WI). Cells in exponential growth were incubated for 72 h with
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22 different concentrations of the inhibitor, then the same volume of CellTiter-Glo reagent was added. The
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24 solution was stirred for 2 min to induce cell lysis. Luminescence was recorded after an additional 10
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26 min. IC₅₀ values were calculated using nonlinear regression analysis (GraphPad Prism statistics
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28 software).
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32 T98G and U343MG cells: growth was measured by a colorimetric MTS conversion assay, as
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34 previously reported.¹⁵
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37 D283 medulloblastoma cells: 3x10⁵ D283 cells/well were plated in a 24-multiwell dish. After 24 h,
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39 ATI derivative **44** or **81** (1 μM) was added to the cells for the indicated time. Viability was evaluated
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41 with a trypan blue assay.
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44 **Antibodies and Immunostaining.** The following unconjugated monoclonal antibodies (mAbs)
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46 were used for immunostaining: anti-MICA (MAB159227), anti-MICB (MAB236511), anti-ULBP1
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48 (MAB170818), anti-ULBP2 (MAB165903) and anti-ULBP3 (MAB166510) from R&D Systems
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50 (Minneapolis, MN); anti-PVR (SKII.4) kindly provided by Prof M. Colonna (Washington University,
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52 St Louis, MO); anti-Nec-2 (R2.525) from BD Pharmingen (San Diego, CA); allophycocyanin (APC)-
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54 conjugated goat affinity purified F(ab')₂ fragment to mouse IgG (GAM) from Jackson
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56 ImmunoResearch Laboratories (West Grove, PA).
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3 **Flow Cytometry Analysis.** 3×10^5 HeLa cells were seeded in tissue culture dishes. After ATI
4 treatment, cell numbers were counted using a Z1 Coulter Particle Counter (Beckman Coulter). Cell
5 cycle phase distribution was analyzed in permeabilized cells incubated with PI (Sigma Aldrich P4170,
6 0.04 mg/ml). SS and FL-3 parameters were acquired in a linear amplification scale, FS and FL2, in a log
7 scale. Cell aggregates were gated out on the bi-parametric graph FL-3lin/Ratio. Apoptosis was
8 determined as the proportion of cells exhibiting a DNA content lower than 2N after gating out cell
9 debris on the bi-parametric graph FS/SS using the WinMDI software. Cell death was analyzed in
10 200,000 cell-aliquots in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2)
11 incubated with annexin V-FITC (Immunological Sciences, IK-11120) alone or annexin V-FITC in
12 combination with PI in the absence of permeabilizing agents. Cell samples were analyzed in a Coulter
13 Epics XL cytofluorimeter (Beckman Coulter) equipped with EXPO 32 ADC software. At least 10000
14 cells per sample were acquired.

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17 **IF and Image Analysis.** Cells were seeded on sterile polylysine-coated coverslips placed in tissue
18 culture plates. After treatment with ATI or VBL, as indicated, the cells were fixed with 3.7%
19 paraformaldehyde in PBS for 10 min at room temperature and then permeabilized in 0.1% Triton-X100
20 in PBS for 5 min. Blocking and antibody reactions were carried out in PBS/0.05% Tween 20
21 containing 3% BSA at room temperature using mouse anti-alpha-tubulin (1:2000, B-5-1-2, Sigma-
22 Aldrich) followed by FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).
23 Chromosomal DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, 0.1 $\mu\text{g}/\text{mL}$) and mounted
24 in Vectashield (Vector Laboratories). Images were analyzed using a Nikon Eclipse 90i microscope
25 equipped with a Qicam Fast 1394 CCD camera (Qimaging). To resolve MT remnants or unstructured
26 tubulin foci, some of the acquired images were deconvoluted and analyzed using the Extended Depth
27 of Focus on Z-serial optical sections using Nis-Elements AR 4.2 (Nikon).
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3 **IF and Flow Cytometry Ligand Analysis.** The expression of NKG2D and DNAM-1 ligand surface
4 expression on HeLa cells was analyzed by IF staining using anti-MICA, anti-MICB, anti-ULBP1/2/3,
5 anti-PVR or anti-Nec2 unconjugated mAbs, followed by secondary GAM/APC. Samples were
6 analyzed using a FACSCanto II (BD Biosciences, San Jose, CA). Flow cytometric analysis was
7 performed using the FlowJo software version 8.8.7 (TreeStar, Ashland, OR).
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11 **Degranulation Assay.** NK cell-mediated cytotoxicity was evaluated using the degranulation
12 lysosomal marker CD107a as previously described.⁶⁶ As a source of effector cells, we used human
13 peripheral blood mononuclear cells (PBMCs) isolated from healthy donors by Lymphoprep (Nycomed,
14 Oslo, Norway) gradient centrifugation and then co-cultured for 10 days with an irradiated (30 Gy)
15 Epstein-Barr virus (EBV)-transformed B-cell line. Cells were grown in RPMI 8866 at 37 °C in a
16 humidified 5% CO₂ atmosphere. On day 10, the cell population was routinely more than 90%
17 CD56⁺CD16⁺CD3⁻, as assessed by IF and flow cytometric analysis. After a 48 h treatment with **33** or
18 **44**, HeLa cells were incubated with activated NK cells at effector:target (E:T) ratios of 1:1 in a flat-
19 bottom 96-well tissue culture plate in complete medium (DMEM (Life Technologies, Gaithersburg,
20 MD) supplemented with 10% FCS). The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 2
21 h. Thereafter, cells were washed with PBS and incubated with anti-CD107a/APC (or cIgG/APC) for 45
22 min at 4 °C. Cells were also stained with anti-CD3/FITC and anti-CD56/PE to gate the CD3⁻CD56⁺
23 NK cell population.
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45 **Real-time PCR.** MICA, MICB, ULBP1, ULBP2, and ULBP3 and PVR mRNA expression was
46 analyzed by real-time PCR. Total RNA from HeLa cells was extracted using Trizol (Invitrogen) after a
47 maximum of 24 h of drug treatment. Total RNA (1 µg) was used for cDNA first-strand synthesis using
48 oligo-dT (Promega, Madison, WI) in a 25 µL reaction volume. To analyze ligand mRNA expression,
49 the cDNA was amplified in triplicate with the following primers: Hs00792952_m1 for MICA,
50 Hs00792952_m1 for MICB, Hs00197846_m1 for PVR, Hs00607609_m1 for ULBP2,
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3 Hs00225909_m1 for ULBP3, and Hs99999903_m1 for β -actin, all conjugated with fluorochrome FAM
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5 (Applied Biosystems). The level of ligand expression was measured using the Threshold Cycle value
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7 (Ct). The Δ Ct was obtained by subtracting the Ct value of the gene of interest (MICA, MICB or PVR)
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9 from the housekeeping gene (β -actin) Ct value. We used Δ Ct of NT sample as the calibrator. The fold
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11 change was calculated according to the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ was the difference between Δ Ct of
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13 the sample and that of the calibrator (according to the formula, the value of the calibrator in each run is
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20 **Hh-dependent Luciferase Reporter Assay.** The luciferase assay was performed in Shh-Light II
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22 (Shh-L II) cells, stably incorporating a Gli-responsive luciferase reporter and the pRL-TK *Renilla*
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24 (normalization control), treated for 48 h with SAG (200 nM) and the studied compounds. Luciferase
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26 and renilla activity were assayed with a dual-luciferase assay system according to the manufacturer's
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28 instructions (Promega, Madison, WI, USA). Results are expressed as luciferase/renilla ratios and
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30 represent the mean \pm SD of three experiments, each performed in triplicate.
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34 **LC-MS/MS Analytical method.** Samples were analyzed under the following conditions: UFLC
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36 (Shimadzu) AC20 coupled with a API 3200 Triple Quadrupole (ABSciex); eluents, phase A: 95%
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38 water, 5% acetonitrile + 0.1% HCOOH, phase B: 5% water, 95% acetonitrile + 0.1% HCOOH; flow
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40 rate, 0.3 mL/min; column, Gemini - Nx 5 μ C18 110A (50*2.00 mm) at 35 °C; injection volume, 5 μ L.
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42 LC-MS/MS analyses were carried out using an ESI(+) interface in multiple reaction monitoring mode.
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46 **Metabolic Stability.** Compounds **33** and **44** were dissolved in DMSO in duplicate at a final
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48 concentration of 1 μ M and preincubated for 10 min at 37 °C in potassium phosphate buffer pH 7.4, 3
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50 mM MgCl₂, with human or mouse liver microsomes (Xenotech) at a final concentration of 0.5 mg/mL.
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52 After the preincubation period, reactions were started by adding the cofactors mixture (NADP, Glc6P,
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54 G6P-DH). Samples were taken at times 0, 10, 20, 30 and 60 min. Acetonitrile was added to stop the
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56 reaction and centrifuged. Supernatants were analyzed and quantified by LC-MS/MS. A control sample
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3 without cofactors was always added in order to check the stability of test compounds in the reaction
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5 mixtures. The reference standards were 7-ethoxycoumarin and propranolol. A fixed concentration of
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7 verapamil was added in every sample as an internal standard for LC-MS/MS. The percent of the area of
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9 test compound remaining at the various incubation times were calculated with respect to the area of
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11 compound at time 0 min.
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15 The rate constant, k (min^{-1}) derived for the exponential decay equation (peak area/IS vs time) was
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17 used to calculate the rate of intrinsic clearance (CL) of the compound using the following equation:
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$$\text{CL } (\mu\text{L}/\text{min}/\text{mg protein}) = k/\text{microsomal conc.} \times 10^3$$

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25 **Aqueous Solubility.** The solubilities of compounds **33** and **44** were measured using a high
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27 throughput screening assay format. Samples prepared at the target concentration of 200 μM were
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29 placed in a 96-well filter plate and incubated at room temperature for 90 min. The plate was then
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31 filtered, and solutions were analyzed by LC-MS/MS. Final concentrations were evaluated by
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33 comparing the area under the curve of the MeOH stock solution with those of the test compound
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35 solutions.
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42 ASSOCIATED CONTENT

45 Supporting Information

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47 Additional chemical and biological material is available free of charge via the internet at
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49 <http://pubs.acs.org>.
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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by grants PRIN 2010-2011 (2010W7YRLZ_001 and 2010W7YRLZ_001006), PRIN 2012-2013 (2012C5YJSK002), AIRC IG 14534 (P. L.), AIRC IG 14723 (L. Di M.), Bando Futuro in Ricerca 2010 (RBFR10ZJQT), Progetti di Ricerca di Università, Sapienza Università di Roma (C26H135FL5 and C26A14TLFT), and Istituto Pasteur-Fondazione Cenci Bolognetti. We are grateful to Giulia Guarguaglini and Italia Anna Asteriti for microscopy analysis. We also thank Professor Claudia Piccoli and Dr. Matteo Landriscina, University of Foggia, Italy, for providing cell lines. We are grateful to Dr. Enrico Cundari for help with flow cytometric analysis.

ABBREVIATIONS USED

MT, microtubule; CSA4, combretastatin A-4; VLB, vinblastine; VCR, vincristine; VRB, vinorelbine; PTX, paclitaxel, ATI, arylthioindole; TMP, trimethoxyphenyl; Hh, Hedgehog; DMSO, dimethyl sulfoxide; SAR, structure-activity relationship; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDR, multidrug resistant; P-gp, P-glycoprotein; NK, natural killer; SAG, smoothened (Smo) agonist, 3-chloro-*N*-[*trans*-4-(methylamino)cyclohexyl]-*N*-[[3-(4-pyridinyl)-phenyl]methyl]-benzo[*b*]thiophene-2-carboxamide; DAPI, 4',6-diamidino-2-phenylindole; PBMC, peripheral blood mononuclear cell; PI, propidium iodide; DMEM, Dulbecco's modified Eagle medium;

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3 FBS, fetal bovine serum; PBS, phosphate-buffered saline; IF, immunofluorescence; APC,
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5 allophycocyanin; GAM, mouse IgG.
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