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Synthesis, proapoptotic screening, and structure–activity relationships of novel aza-lupane triterpenoids ☆

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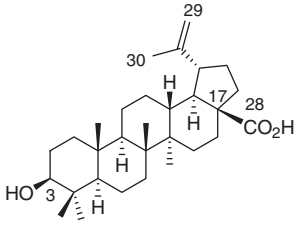
ABSTRACT

Apoptosis is a highly regulated process by which excessive cells are eliminated in order to maintain normal cell development and tissue homeostasis. Resistance to apoptosis often contributes to failure in cancer prevention and treatment. Apoptotic cell death regulators are considered important targets for discovery and development of new therapeutic agents in oncology research. A class of novel aza-lupane triterpenoids were designed, synthesized, and evaluated for antitumor activity against a panel of cancer cell lines of different histogenic origin and for ability to induce apoptosis. 3,30-Bis(aza) derivatives were identified not only to possess improved cytotoxicity compared to the natural product betulinic acid but also to affect cell death predominantly via apoptosis, whereas the mono(aza) derivatives apparently triggered cell death via different, non-apoptotic pathway(s).

Apoptosis is a highly regulated process by which excessive or harmful cells are eliminated in order to maintain normal cell development and tissue homeostasis.[1,2] Defects in the apoptotic cellular machinery and the apoptosis inducing pathway(s) often result in uncontrolled cell proliferation, tumor development, and other malignant diseases.[2] Resistance to apoptosis contributes greatly to the failure in cancer prevention and treatment.[3] Therefore, apoptotic cell death regulators are considered important targets for discovery and development of new therapeutic agents in oncology research. Betulinic acid, 3β-hydroxy-lup-20(29)-en-28-oic acid (**1**), is a phytochemical widely distributed in food, medicinal herbs, and other plants throughout the world and is identified to possess a wide range of biological properties including broad growth inhibitory activity and the ability to induce apoptosis in the most prevalent human cancer cells.[4] The betulinic acid-induced apoptosis is mediated by mitochondria.[5] The mitochondrial outer membrane is initially permeabilized, rendering the release of apoptogenic proteins, for example, cytochrome *c*, Smac, and apoptosis inducing factor (AIF), followed by the activation of caspase cascade.[6] Betulinic acid (**1**) has also been reported to induce cell death by selective proteosome-dependent inhibition of the specificity protein transcription factors which regulate vascular endothelial growth factor (VEGF) and survivin expression.[7]



1, Betulinic Acid

The analysis of cytotoxicity–structure relationships of betulinic acid (**1**) and its derivatives revealed that the carboxylic acid group attached at C17 position is essential for its cytotoxicity.[4] We have recently reported, however, that the C17-carboxylic acid group in the 2-cyano-1-en-3-one system of lupane triterpenoid was not critical to inhibit cancer cell proliferation and induce apoptosis[8] and that elongation of the chain length attached at the C17 position of **1**, while maintaining the carboxylic acid group at the chain end, was detrimental to the cytotoxicity.[9] In our continuing efforts to identify potential molecules capable of modulating the process of intrinsic mitochondrial pathway of apoptosis, we have embarked on the design and synthesis of lupane triterpenoid-based libraries that are anticipated to possess increased ability to induce controlled cancer cell death. Our approach involves the screening of new molecules for in vitro cytotoxicity, apoptosis induction and caspase activation and cytochrome *c* release in cell death signaling. Herein, we wish to report our initial investigation of a novel class of aza-lupane triterpenoids against cancer cell lines and their ability to induce apoptosis.

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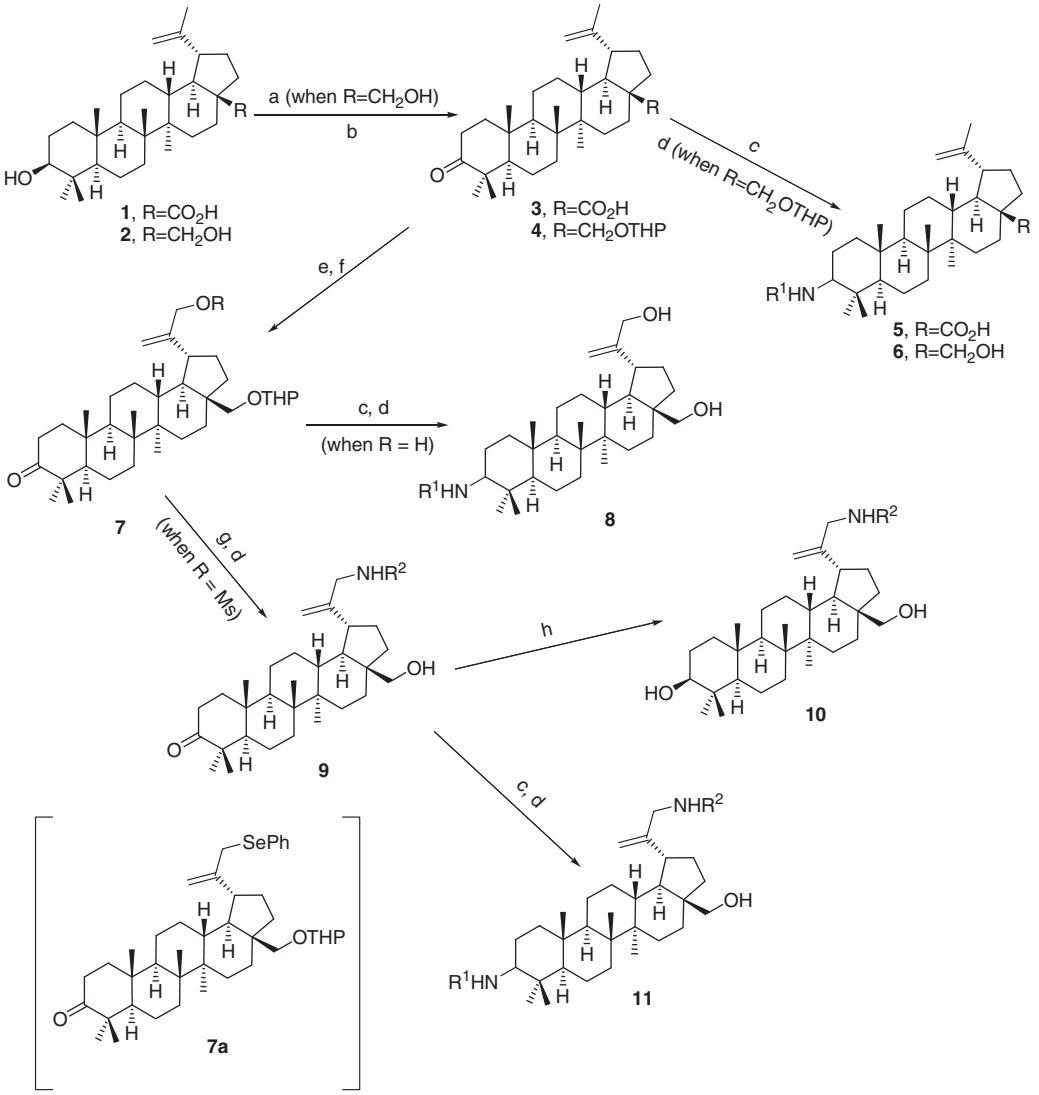
The synthesis of various aza-lupane triterpenoids is shown in Scheme 1. The 3-aza derivatives (**5** and **6**) were readily obtained from reductive amination of betulonic acid (**3**)[10] and the THP-protected betulone (**4**),[8] which were, respectively, prepared from the corresponding betulinic acid (**1**) and betulin (**2**) according to the published procedures. Hydroxylation of **4** via selenylation[11] followed by oxidation with H_2O_2 furnished the 30-hydroxy ketone **7**. Without the oxidation step, the 30-phenylselenium intermediate **7a** was isolated. Reductive amination of **7** led to the formation, after removal of the THP protecting group, of 3-amino-30-hydroxy analogues **8**. Mesylation of **7** followed by nucleophilic substitution with amines provided 30-aza-3-oxo derivatives **9**, subsequent to the removal of THP group. The 3-oxo group of **9** was reduced by $NaBH_4$ to generate 30-aza-3-OH compounds **10**. Reductive amination of **9** afforded 3,30-bis(amino) compounds **11**.

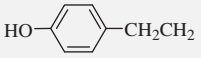
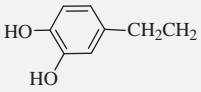
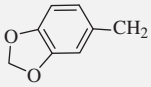
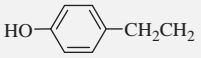
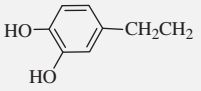
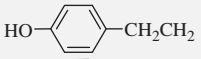
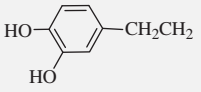
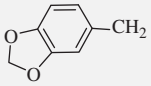
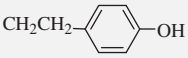
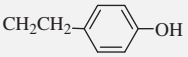
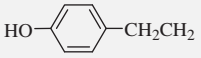
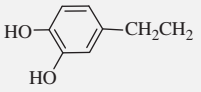
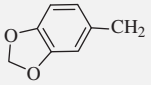
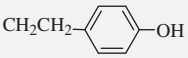
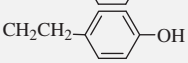
All the synthesized aza-lupane triterpenoids[12] were evaluated for their antiproliferative activity using the MTS assay[13] against a panel of cancer cell lines of different histogenic origin, including SK-MEL-2 and A-375 of malignant melanoma, Daoy and LN-229 of cerebellar medulloblastoma, OVCAR-3 of ovarian, HT-29 of colon and MCF-7 of breast adenocarcenoma and the results are shown in Table 1. Based on this small set of compounds, a few observations of structure–activity relationships can be made: (1) the aza derivatives were more cytotoxic than betulinic acid (**1**); (2) in contrast to the conventional betulinic acid analogues previously reported, the aza-compounds with C17-CH_2OH (**6**) tended to be more consistently cytotoxic than the compounds with C17-CO_2H (**5**); (3) the 30-OH group appeared not to confer a significant effect on the inhibition of cell proliferation (**8** vs **6**); (4) compared to the 3-aza derivatives, the 30-aza substitution seemed to afford less inhibitory compounds (**9** and **10** vs **6**); (5) 3,30-bis(aza) triterpenoids (**11**) were among the most active compounds generated and their cytotoxicities were consistent across the panel cell lines tested; and (6) the corresponding THP-protected intermediates were as cytotoxic as, and, in a few cases, were even more inhibitory than, those with the THP group removed (data not shown).

Compounds able to inhibit cancer cell proliferation in the MTS assay were further investigated for apoptosis inducing activity using the Annexin-V assay.[14] The examples of mono(aza) compounds are shown in Figure 1, where SK-MEL-2 cells were treated with 7.5 μM of the test compounds for 16 h. The results indicated that the 3-aza derivatives **5** and **6** did not significantly improve the apoptosis induction activity when compared to betulinic acid (**1**), even though **5** and **6** were generally more cytotoxic (lower IC_{50}) than **1** (Table 1). The increased cytotoxicity might be due to an uncontrolled necrotic approach. The mono(azo)derivatives **8** and



Scheme 1. Reagents: (a) DHP, PPTS, MeOH; (b) CrO_3, pyridine; (c) R^1NH_2, $NaCNBH_3$; (d) PPTS, MeOH; (e) PhSeCl, pyridine, 30% H_2O_2; (f) $MeSO_2Cl$, Et_3N; (g) R^2NH_2; (h) $NaBH_4$.

Table 1
Cytotoxicity of aza-lupane triterpenoids measured as IC_{50} (μM)[a]

Compound	R^1	R^2 (or R)	SK-MEL-2	A-375	Daoy	LN-229	OVCAR-3	HT-29	MCF-7
1 (betulinic acid)	—	—	7 (3.7)[b]	22	11 (2.3)[b]	29	40	31	30
5a	H	CO_2H	8	nt	7	nt	14	nt	nt
5b	$HOCH_2CH_2$	CO_2H	36	14	<2	nt	28	16	35
5c		CO_2H	6 (0.6)[b]	5	6 (0.6)[b]	5	6	4	6
5d		CO_2H	19	nt	30	29	22	65	37
5e		CO_2H	29	>75	38	>75	>75	>75	>75
6a	H	CH_2OH	9	2	19	<2	5	2	5
6b	$HOCH_2CH_2$	CH_2OH	<2 (0)[b]	2	6 (5.8)[b]	4	5	2	5
6c		CH_2OH	2 (0)[b]	4	<2 (0)[b]	8	5	2	5
6d		CH_2OH	17	6	18	9	4	6	17
6e	$MeOCH_2CH_2$	CH_2OH	7	7	8	nt	22	19	8
8a	H	—	34 (4.9)[b]	6	31 (6.3)[b]	17	14	17	15
8b	$HOCH_2CH_2$	—	34	16	29	37	39	65	41
8c		—	38	6	32	17	23	21	17
8d		—	24	6	34	17	18	37	31
8e		—	17	>75	16	73	20	>75	75
8f	$MeOCH_2CH_2$	—	38	18	34	38	40	47	40
9a	—	CH_2CH_2OH	45	10	47	16	67	45	47
9b	—		16	6	17	8	nt	4	6
10a	—	CH_2CH_2OH	15	nt	35	nt	nt	nt	nt
10b	—		16	nt	16	nt	nt	nt	nt
11a	H	CH_2CH_2OH	6 (3.2)[b]	5	5 (2.9)[b]	12	nt	<2	2
11b	$HOCH_2CH_2$	CH_2CH_2OH	7	6	13	16	nt	2	6
11c		CH_2CH_2OH	5	2	<2	14	40	<2	4
11d		CH_2CH_2OH	5	7	5	32	nt	4	4
11e		CH_2CH_2OH	4	5	13	32	64	5	13
11f	$MeOCH_2CH_2$	CH_2CH_2OH	8	4	5	28	nt	2	11
11g	H		3 (1.0)[b]	<2	<2 (0)[b]	<2	nt	2	5
11h	$HOCH_2CH_2$		6	5	2	6	nt	5	5

nt: not tested.
[a] The IC_{50} values were an average of four replicate wells at each concentration tested from a single experiment, unless otherwise stated. See Ref. 13 for experimental details.
[b] The IC_{50} values were an average of three separate experiments with each concentration replicated in four wells at each experiment. The standard deviations are shown in parentheses.

9 appeared to enhance the apoptosis-mediated cell death; once again, however, the necrotic population far exceeded the apoptotic population (Fig. 1). In stark contrast, the apoptosis assay results of the 3,30-bis(aza) triterpenoids (**11**) (Fig. 2) revealed not only that all the bis(aza) compounds induce apoptosis but, more significantly, that apoptosis appeared to be the primary route of cell death. The apoptotic population induced by **11a**, **11c**, **11g**, and **11h** accounted for more than 60% of the cell population whereas the necrotic population accounted for less than 15%. Similar results were obtained when Annexin assays were performed in Daoy cells (data not shown).

In summary, a novel class of 3,30-bis(aza) lupane triterpenoids (**11**) was identified not only to possess improved cytotoxicity compared to the natural product betulinic acid (**1**) but also to affect cell

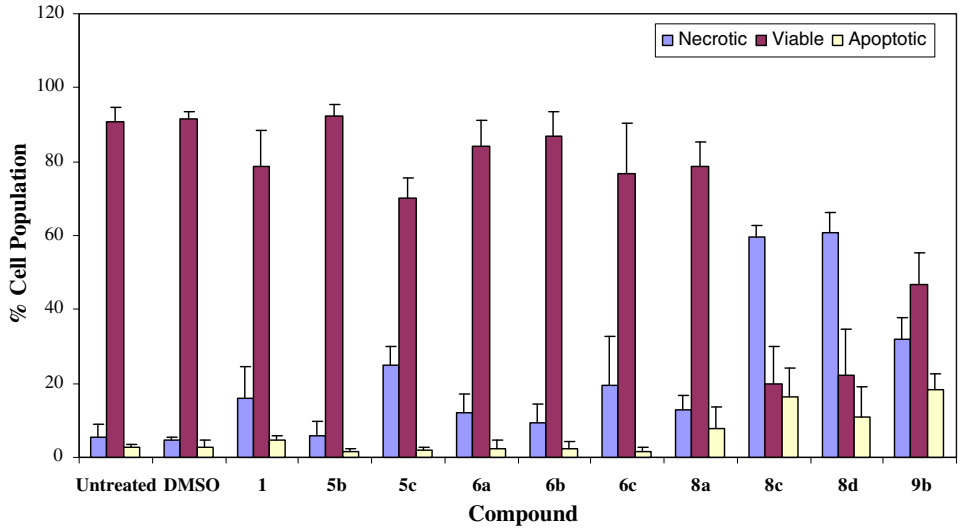


Figure 1. Annexin assay: SK-MEL-2 cells were treated with 7.5 μM of test compounds for 16 h (error bars representing standard deviations of three replicates).

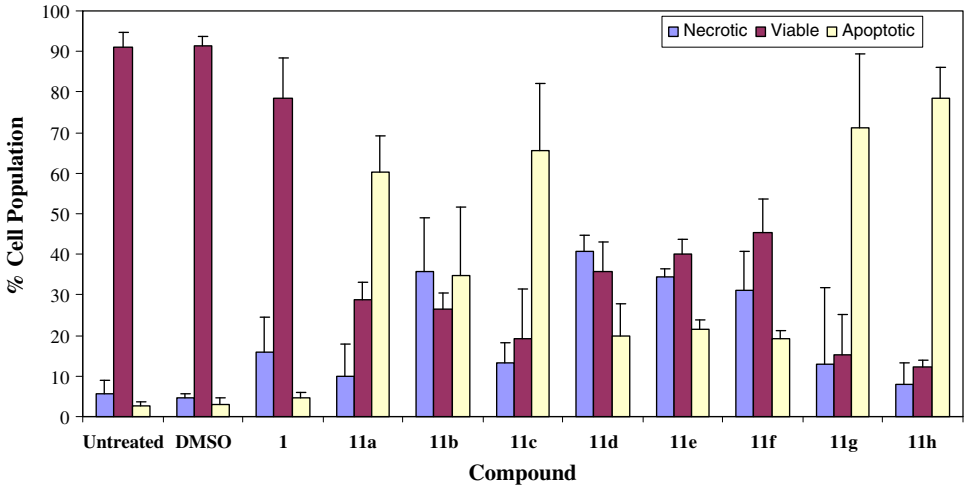


Figure 2. Annexin assay: SK-MEL-2 cells were treated with 7.5 μM of test compounds for 16 h (error bars representing standard deviations of three replicates).

death predominantly via apoptosis. The mono(aza) derivatives (**5**, **6**, **8**, **9**, and **10**) apparently triggered cell death via different, non-apoptotic pathway(s). Further studies to elucidate the mechanism of action by these aza-lupane triterpenoids are currently underway and will be reported in due course.

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12. The newly synthesized compounds provided satisfactory MS and NMR (500 MHz, DMSO-d_6) spectra without exhibiting any discernible impurities. For the 3-amino compound (**5**, **6**, **8** and **11**), the stereochemistry at C3 is yet to be determined. The selected analytical data of representative compounds are shown as follows. Compound **5c**: mp 204–210 °C. ^{1}H NMR δ 0.60 (s, 3H), 0.66 (d, J = 10.4 Hz, 1H), 0.78–0.98 (m, 2H), 0.75 (s, 3H), 0.86 (s, 6H), 0.93 (s, 3H), 1.06–1.54 (m, 15H), 1.56–1.68 (m, 3H), 1.64 (s, 3H), 1.75–1.90 (m, 3H), 2.11 (d, J = 8.4 Hz, 1H), 2.22 (t, J = 9.2 Hz, 1H), 2.55 (m, 2H), 2.80–3.00 (m, 2H), 4.56 (s, 1H), 4.68 (s, 1H), 6.64 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 9.10 (br s, 1H). MS (APCI+) m/e 577 (M+H); Compound **6a**: mp 156–163 °C. ^{1}H NMR δ 0.62 (s, 3H), 0.75 (s, 3H), 0.78–1.08 (m, 5H), 0.86 (s, 3H), 0.93 (s, 3H), 0.98 (s, 3H), 1.10–1.38 (m, 10H), 1.45–1.68 (m, 6H), 1.63 (s, 3H), 1.82–1.92 (m, 4H), 2.12 (dd, J = 5.3, 13.1 Hz, 1H), 2.38 (m, 1H), 3.08, 3.52 (d-AB Type, J = 4.4 Hz, J_{AB} = 10.6 Hz, 2H), 4.21 (t, J = 5.0 Hz, 1H), 4.54 (d, J = 1.0 Hz, 1H), 4.66 (d, J = 2.0 Hz, 1H). MS (ESI+) m/e 442 (M+H). Compound **8c**: mp 240–247 °C. ^{1}H NMR δ 0.60 (s, 3H), 0.66 (d, J = 11.0 Hz, 1H), 0.75 (s, 3H), 0.79–1.01 (m, 4H), 0.87 (s, 3H), 0.92 (s, 3H), 0.98 (s, 3H), 1.02–1.39 (m, 11H), 1.46 (d, J = 8.5 Hz, 1H), 1.54–1.70 (m, 5H), 1.80–2.01 (m, 4H), 2.22 (m, 1H), 2.50–2.60 (m, 2H), 2.87 (m, 1H), 3.05, 3.50 (d-AB Type, J = 5.4 Hz, J_{AB} = 10.4 Hz, 2H), 3.88 (br s, 2H), 4.20 (t, J = 5.3 Hz, 1H), 4.71 (t, J = 5.5 Hz, 1H), 4.74 (s, 1H), 4.84 (s, 1H), 6.64 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 8.5 Hz, 2H), 9.09 (s, 1H). MS (APCI+) m/e 578 (M+H). Compound **9b**: mp 173–182 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.82–1.12 (m, 3H), 0.85 (s, 3H), 0.93 (s, 3H), 0.94 (s, 3H), 0.99 (s, 3H), 1.01 (s, 3H), 1.14–1.28 (m, 3H), 1.30–1.46 (m, 9H), 1.56–1.72 (m, 4H), 1.76–2.00 (m, 4H), 2.26–2.46 (m, 3H), 2.58 (m, 2H), 2.64 (m, 2H), 3.07 (m, 3H), 3.52 (dd, J = 4.7, 10.2 Hz, 1H), 4.20 (t, J = 5.0 Hz, 1H), 4.75 (s, 2H), 6.65 (dd, J = 2.0, 6.5 Hz, 2H), 6.97 (d, J = 8.5 Hz, 2H), 9.10 (s, 1H). MS (APCI+) m/e 576 (M^+). Compound **11a**: mp 190–

203 °C. ^{1}H NMR δ 0.62 (s, 3H), 0.75 (s, 3H), 0.83–1.16 (m, 5H), 0.86 (s, 3H), 0.93 (s, 3H), 0.98 (s, 3H), 1.19–1.39 (m, 11H), 1.44–1.59 (m, 6H), 1.67 (dt, J = 3.5, 12.5 Hz, 1H), 1.82–2.00 (m, 4H), 2.13 (dd, J = 4.2, 11.2 Hz, 1H), 2.29 (br s, 1H), 2.54 (q, J = 6.0 Hz, 2H), 3.07 (m, 2H), 3.08, 3.51 (AB Type, J_{AB} = 10.5 Hz, 2H), 3.44 (t, J = 5.7 Hz, 2H), 4.20 (br s, 1H), 4.44 (br s, 1H), 4.76 (s, 1H), 4.80 (s, 1H). MS (APCI+) m/e 501 (M^+). Compound **11g**: mp 195–200 °C. ^{1}H NMR δ 0.66 (s, 3H), 0.76 (s, 3H), 0.68 (d, J = 11.0 Hz, 1H), 0.78–1.03 (m, 4H), 0.89 (s, 3H), 0.92 (s, 3H), 0.98 (s, 3H), 1.02–1.27 (m, 5H), 1.31–1.50 (m, 8H), 1.57 (d, J = 10.0 Hz, 3H), 1.67 (t, J = 11.5 Hz, 1H), 1.81–1.98 (m, 3H), 2.32 (m, 3H), 2.58 (q, J = 6.5 Hz, 2H), 2.64 (q, J = 6.5 Hz, 2H), 3.08 (m, 3H), 3.51 (d, J = 10.5 Hz, 1H), 4.19 (br s, 1H), 4.74 (s, 2H), 6.65 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 8.5 Hz, 2H), 9.12 (br s, 1H). MS (ESI+) m/e 577 (M+H).

13. *MTS assay*: The anti-proliferative activities of the molecules were measured by using the CellTiter 96 Aqueous Non-radioactive cell proliferation assay. Cells (1×10^4 per well) were plated onto a 96-well plate the evening before treatment in triplicate. Test compounds were initially dissolved in DMSO at 10 mM concentration and serially diluted in growth media to various concentrations. Each compound was tested at five different concentrations, 2, 10, 25, 50 and 75 μM, and each concentration was replicated in four wells. The negative control (0 μM of test compound) contained the same amount of DMSO as that of the highest test concentration. Upon treatment with the test compound solutions in growth media, they were incubated for 72 h for all the cell lines with exception for OVCAR which was treated for 120 h. Next, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium, inner salt) reagent was added and incubation was continued for 1.5–4 h at 37 °C. During the incubation, MTS is bioreduced to formazan by viable cells. The inhibitory effects were obtained by measuring the absorbance of the formazan at 490 nm on a Wallac Victor II plate reader and calculated by subtracting the absorbance measured at the same wavelength from DMSO-treated cells.
14. *Annexin-V assay*: The Guava Nexin Assay was used to measure the percentage of cells undergoing apoptosis by treatment with the test compounds. Cells (8.75×10^5 per well) were plated in a 60 mm dish the evening before treatment. Upon treatment with 7.5 μM test compound solutions in growth media without FBS, the cells were incubated at 37 °C for 16 h. Next 10% FCS was added to prevent cell damage and improve the efficiency of cell pelleting during subsequent centrifugation steps. After trypsinization and cell pelleting, the Nexin staining solution (including Annexin V and 7-ADD) was added and incubation was continued on ice for 20 min. Apoptosis was measured on a Guava instrument by setting the gates as described in the manufacturer's protocol to establish quadrants and to measure the population of viable, mid apoptotic and late apoptotic/necrotic cells in each quadrant.