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Synthesis and biological evaluation of semisynthetic analogs of glycyrrhetic acid is described.
Concise Article

3-(2,6-Dichloro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid, a semisynthetic derivative of glycyrrhetic acid: Synthesis, antiproliferative, apoptotic and anti-angiogenesis activity

Rajni Sharma,ab Santosh K. Guru,abcd Shreyans K. Jain,ab Anup Singh Pathania,c Ram A. Vishwakarma,abdef Shashi Bhushanb* and Sandip B. Bharatebd*

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Glycyrrhetic acid (2, 3ß-hydroxyl-11-oxo-olean-12-ene-29-oic acid), a pentacyclic triterpenoid isolated from Glycyrrhiza glabra is known to possess wide range of biological activities. Herein, we report synthesis and antiproliferative activity of 3-ß-ether derivatives of glycyrrhetic acid. The cytotoxicity of prepared derivatives was investigated in three cancer cell lines, including human pancreatic (MIAPaCa-2), prostate (PC-3) and human hepatocellular liver carcinoma (HepG-2). Amongst tested compounds, the 2,6-dichlorobenzyl 5b and 2,4-dichlorobenzyl derivative 5r displayed significant cytotoxicity in PC-3 cells with IC50 values of 6 and 18 µM, respectively. The dichlorobenzyl derivative 5b also displayed cytotoxicity in MIAPaCa-2 (IC50: 19 µM) and HepG-2 cells (IC50:19 µM). Further, the compound 5b was investigated for apoptosis-induction by cell cycle analysis, nuclear morphological changes and mitochondrial membrane potential loss in PC-3 cells. Compound 5b led to increase in sub-G1 population in PC-3 cells, which is indicative of its apoptotic property. Interestingly, compound 5b also arrested S-phase of the cell cycle. The nuclear morphology of PC-3 cells after treatment with compound 5b was also investigated which confirmed the formation of apoptotic bodies. The compound 5b induced apoptosis through both intrinsic and extrinsic apoptotic pathways in PC-3 cells, which was confirmed by mitochondrial membrane potential loss, inhibition of pro-caspase-3, 8 and 9 and cleavage of PARP-1. Furthermore, there was a significant decrease in Bcl-2/Bax ratio by compound 5b in PC-3 cells. Interestingly, compound 5b also inhibited the VEGF-induced PC-3 cell migration and decreased wound closure percentage from 100 to 12% at 30 µM. Similarly, compound 5b inhibited angiogenesis-dependent cell migration in HUVEC cells and decreased wound closure from 100 to 20% at 30 µM, indicating its anti-angiogenic activity.

Introduction

Glycyrrhiza glabra (Licorice) is a tall shrub of the Leguminosae family, widely cultivated throughout Europe, the Middle East and Asia. The ethnomedicinal use of G. glabra has been documented in several traditional systems of medicine. The rhizomes of licorice have been used worldwide as an herbal medicine and natural sweetener (30-50 times sweeter than sucrose). G. glabra and its active components are reported to possess wide range of biological activities, however the most active component which is responsible for its medicinal properties is a triterpene saponin glycyrrhizin (I, also called as glycyrrhizinic acid and glycyrrhizic acid). Glycyrrhizin also inhibits specific changes that occur in a cell under the action of the TPA (12-O-tetradecanoylphorbol-13-acetate; a tumor promotor), and also suppressed estrogen-related endometrial cancer by inhibiting COX-2, IL-1α and TNF-α. Glycyrrhetic acid (2, 3ß-hydroxyl-11-oxo-olean-12-ene-29-oic acid; also called as glycyrrhetinic acid), is a glycone of glycyrrhizin, and is also present in G. glabra. Glycyrrhetic acid belongs to the class of ursane-type pentacyclic triterpenoid and has wide range of biological activities including anti-inflammatory, anti-ulcer, analgesic, anti-type IV allergic, prevention of metabolic and vascular diseases and anticancer activity. The glycyrrhetic acid (2) is reported to possess cytotoxicity, and apoptosis-inducing activity in different hepatic, stomach, melanoma, breast and leukemia cancer cell lines. It showed cytotoxic activity in HL-60 cells with IC50 of 63.2 µM. Glycyrrhetic acid also showed protection against UV-induced skin cancer, and also increased natural killer cell activity in metastatic tumor. Another interesting property of glycyrrhetic acid is chemosensitizing effect with various clinical oncology drugs. It partly reversed multidrug resistance (MDR) in Pgp-expressing cells (KB-C2) by increasing the intracellular accumulation of antitumor drugs. Based on these findings, it is likely that glycyrrhetic acid derivatives elicit antitumor activity through multiple signaling pathways. In general, the cytotoxic or apoptotic activity of glycyrrhetic acid in most of the studies was

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*Natural Products Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India
b Academy of Scientific & Innovative Research (AcSIR), CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India
c Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India
d Medicinal Chemistry Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-180001, India
E-mail: sbhushan@iium.ac.in; sbbhushan@iium.ac.in; ram@iium.ac.in
IIM Publication number IIM/16702014
Fax: +91-191-2569333; Tel: +91-191-2569111
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moderate; thus research efforts were mainly focused on the identification of its derivatives with improved activity.\textsuperscript{11b, 15} The structure-activity relationship of glycyrrhetic acid has been reviewed recently.\textsuperscript{11b} The modifications on ring A were found to be more effective. A 2-trifluoromethyl derivative displayed potent cytotoxicity in 253JB-V (IC\textsubscript{50} = 0.67 µM), KU7 (IC\textsubscript{50} = 0.38 µM), PANC-1 (IC\textsubscript{50} = 0.82 µM) and PANC-28 (IC\textsubscript{50} = 1.14 µM) cell lines.\textsuperscript{16} Glycyrrhetic acid derivative with C-3 alkoxyimino group and C-30 carboxylic acid methyl ester showed improvement in cytotoxicity in HL-60 cells from IC\textsubscript{50} of 63 to 19 µM.\textsuperscript{11a} Fused heterocyclic rings at C-2 and C-3 positions of glycyrrhetic acid led to improvement in cytotoxicity by 20-fold.\textsuperscript{17} In general, the C-3 modifications were more successful, and led to identification of two antiulcer drugs carbenoxolone (3) and acetoxolone (4).\textsuperscript{18} Carbenoxolone has also been reported to possess chemopreventive activity.\textsuperscript{19} The literature precedence indicated that substitution of lipophilic groups at C-3 position is beneficial. Thus, herein we aimed to prepare new C-3 ether derivatives of glycyrrhetic acid and investigate their cytotoxicity in various cancer cell lines. Furthermore, the most promising cytotoxic compound was then mechanistically investigated in detail, in a panel of assays for apoptosis-inducing activity and anti-angiogenesis activity.

**Figure 1.** Chemical structures of glycyrrhizin (1), its aglycone glycyrrhetic acid (2) and known derivatives 3-4 of glycyrrhetic acid

### Results and discussion

Glycyrrhizin (1) was isolated from *G. glabra* using reported procedure.\textsuperscript{20} Aglycone 2 was then obtained by acid hydrolysis of glycyrrhizin (1). The C-3 etherification was achieved by adding different benzyl or alkyl chlorides in a solution of glycyrrhetic acid (2) in dry acetone, under alkaline conditions (Scheme 1).

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<td>85</td>
<td>5r</td>
<td>Cl</td>
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</table>

Scheme 1. Synthesis of derivatives 5a-r from glycyrrhizic acid (1). Reagents and conditions: (a) 5% Aq. HCl, MeOH, 90 °C, 20 h. (b) K\textsubscript{2}CO\textsubscript{3} (1.2 equiv), R-Cl (for R, see Table 1, 5a to 5r) (1 equiv), dry acetone, 0 °C, 2 h, yield: see Table 1.

A series of ethers were synthesized and characterized by NMR and MS analysis. The list of synthesized derivatives is shown in Table 1.
All compounds were evaluated for antiproliferative activity in three human cancer cell lines including pancreatic (MIAPaCa-2), prostate (PC-3) and hepatocellular carcinoma (HepG-2). Cytotoxicity results of most promising compounds are shown in Table 2. Results for all compounds are shown in ESI (Table S1).

Table 2. Cytotoxicity of most active compounds in different cancer cell lines<sup>a,b,c,d</sup>

<table>
<thead>
<tr>
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<th>MIAPaCa-2</th>
<th>HepG2</th>
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<td>5a</td>
<td>10 ± 1.0</td>
<td>11 ± 0.97</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5b</td>
<td>6.0 ± 0.46</td>
<td>7.0 ± 0.56</td>
<td>19 ± 1.18</td>
</tr>
<tr>
<td>5c</td>
<td>9.0 ± 0.49</td>
<td>12 ± 1.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5r</td>
<td>18 ± 1.11</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.012 ± 0.012</td>
<td>0.1 ± 0.003</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>1.2 ± 0.067</td>
<td>0.19 ± 0.008</td>
<td>0.2 ± 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were grown in 96-well culture plates and treated with various concentrations of each test compounds for 48 h. Thereafter, cells were incubated with MTT solution for 2 h and the optical density of formazan crystals was measured as described in the experimental section. <sup>b</sup>HepG2, hepatocellular carcinoma cells; MIAPaCa-2, pancreatic cancer cells; PC-3, prostate cancer cells. <sup>c</sup>Compounds 2, and 5d-5q were inactive against all cell lines used (IC<sub>50</sub> > 20 µM). <sup>d</sup>Data are Mean ± SD (n= 3).

All the tested compounds were found to be more sensitive against prostate cancer PC-3 cells in comparison to MIAPaCa-2 and HepG-2 cells. Compound 5b (2,6-dichloro-benzyl derivative) showed significant cytotoxicity in all three tested cell lines and it caused concentration and time-dependent inhibition of PC-3 cell proliferation. It showed varying cytotoxicity potential (IC<sub>50</sub>) at different time points as depicted in Figure 2. The main purpose of this experiment was to check the IC<sub>50</sub> value of compound 5b at 24 h time point, for the purpose of cell cycle, MMP loss, microscopy, wound scratch and western blot experiments.

Figure 2. Cytotoxicity of compound 5b in PC-3 cells at different time points. The cells were grown in 96-well culture plate and treated with different concentrations of compound 5b for indicated time intervals. Cells were incubated with MTT solution and optical density of formazan crystals was measured as described in Materials and Methods. Data are Mean ± SD (n= 8 wells), and representative of three similar experiments.

In order to address the cell death caused by compound 5b, the extent of apoptotic death in PC-3 cells was assessed using flow cytometry through determination of sub-G1 cell population by propidium iodide (PI) staining. As depicted in Figure 3, the regions marked with different colors represent % population at different phases of the cell cycle. PC-3 cells exposed to compound 5b for 24 h exhibited a dose-dependent increase in sub-G1 fraction (<2n DNA), which may comprise both apoptosis and debris fraction implying together the extent of cell death (Figure 3). The sub-G1 apoptotic population was found to be 8, 12 and 37% following 5, 10 and 20 µM of 5b treatment compared to control (untreated cells - 3%). Interestingly, compound 5b significantly arrested the S-phase of the cell cycle in a dose-dependent manner in PC-3 cells, which ultimately results in blockage of cell (DNA) division.

Figure 3. DNA cell cycle analyses in PC-3 cells exposed to compound 5b. PC-3 cells were treated with different concentrations (5, 10 and 20 µM) of compound 5b for 24 h and stained with Propidium Iodide, PI (10 µg/ml) to determine DNA fluorescence and cell cycle phase distribution as described in Materials and Methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. The fraction of cells from apoptosis (sub-G1/G0), G1, S and G2 phases analyzed from FL2- A vs. cell counts are shown in %. Data are representative of one of three similar experiments.

The apoptosis induction results obtained from cell cycle analysis were further corroborated by studying nuclear morphological changes of cells by fluorescence microscopy. After the treatment at 5, 10, and 20 µM of compound 5b, characteristic changes of apoptosis such as nuclear condensation, membrane blebbing and
formation of apoptotic bodies were observed in the morphology of treated cells in a concentration-dependent manner, whereas untreated cells nuclei were found to be of normal intact morphology. The results suggest that compound 5b was able to induce apoptotic cell morphology in PC-3 cells (Figure 4).

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Control
5b, 5 µM

5b, 10 µM
5b, 20 µM
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Figure 4. Effect of compound 5b on cellular and nuclear morphology of PC-3 cells. Cells were treated with indicated concentrations of compound 5b for 24 h time period and subsequently stained with Hoechst 33258 as described in experimental section and visualized for nuclear morphology and apoptotic bodies’ formation. Data are representative of one of three similar experiments and magnification of the pictures was 30X on Olympus IX 70 inverted microscopes.

Compound 5b induced apoptosis through both intrinsic and extrinsic apoptotic pathways, which was confirmed by mitochondrial membrane potential (MMP) loss. Mitochondrial damage to cells results in perturbation of MMP. The loss in MMP (∆ψm) of PC-3 cells by compound 5b was studied using rhodamine123 dye, which was reduced by healthy mitochondria into fluorescent probe whose fluorescence was measured by flow cytometer in FL-1 channel. In the untreated control cells, almost all cells were functionally active with high Rh-123 fluorescence. Mitochondrial damage results in decrease in Rh-123 Fluorescence. Compound 5b at 10 µM caused mitochondrial damage and hence led to increase in the mitochondrial membrane potential loss by about 15%, which was further increased to 38% at 20 µM (Figure 5). The loss of mitochondrial membrane potential (Δψm) is largely due to the opening of mitochondrial permeability transition pores (PTP), which conduit the leakage of proapoptotic proteins from mitochondria to cytosol.21

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(a). Control
(b). 5b, 5 µM
(c). 5b, 10 µM
(d). 5b, 20 µM
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Figure 5 Compound 5b induced mitochondrial membrane potential loss in prostate cancer PC-3 cells. Cells were treated with compound 5b at 5, 10, and 20 µM concentration for 24 h time period. Cells were stained with Rhodamine-123 (200 nM) dye for 30 min and analyzed in FL-1 vs. counts channels of flow cytometer. Data are representative of one of three similar experiments at different time period.

Next, the effect of compound 5b on key mitochondrial apoptotic proteins (pro-caspases and PARP-1) and Bcl-2/Bax ratio was investigated. The expression of anti-apoptotic protein Bcl-2 was significantly decreased by compound 5b at 10 and 20 µM concentrations in PC-3 cells (Figure 6a). The downregulation of anti-apoptotic Bcl-2 protein caused the structural deformation of mitochondria, which opens mitochondrial permeability transition pores and release pro-apoptotic proteins to the cytosol and translocation of Bax from cytosol to mitochondria. Activation of Bax leads to cleavage of pro-caspase-9, activation of pro-caspase-3 and finally cleavage of downstream target poly(ADP-ribose) polymerase-1 (PARP-1). Compound 5b was also found to inhibit the pro-caspase-8 expression in PC-3 cells. Hence, both intrinsic and extrinsic apoptotic pathways seemed to play a role in the
activation of executioner pro-caspase-3. The Bcl-2/Bax ratio in PC-3 cells was also determined. The compound 5b drastically reduced the ratio from 5 to 0.5 (Figure 6b), indicating significant apoptotic behavior of the compound 5b.

Figure 6. Influence of compound 5b on the expression of important proteins involved in the initiation of apoptosis. PC-3 cells were treated with 5-20 µM concentrations of compound 5b for 24h. Protein lysates were prepared and electrophoresis as described in Materials and Methods. β-actin was used as an internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of the indicated proteins in designated cell lysates. (a) Compound 5b induced differential activation of different caspases, mitochondrial apoptotic proteins PARP-1 in PC-3 cells. Western blot analyses of the indicated proteins were performed in the whole cell lysate. Data are representative of one of three similar experiments. (b) Influence of compound 5b on the Bcl-2/Bax ratio in PC-3 cells. Mitochondria and Bcl-2 family of proteins play a pivotal role in the induction of apoptosis. Bcl-2 associated proteins have both pro-apoptotic and anti-apoptotic effects in cancer cells. These proteins regulate mitochondrial outer membrane potential and control the release of many apoptotic factors originating in the mitochondria. Compound 5-b decreases the expression of anti-apoptotic protein Bcl-2 and increase the expression of pro-apoptotic protein Bax in a dose dependent manner. The relative density of each band was measured as arbitrary units by Quantity One software of Bio-RAD gel documentation system. Data expressed as mean ± SD of three independent experiments.

Angiogenesis is one of the common hallmark manifestations of all cancers and it is an elementary event in the development of tumor growth and malignancy. To appraise the anti-angiogenic property of compound 5b in vitro, the chemotactic motility of PC-3 cells was examined by wound-healing migration assay. Cell migration is necessary for tumor growth and metastasis. It was observed that compound 5b significantly inhibited VEGF-induced HUVEC migration and decrease wound closure percentage from 100% to 12% at 20 µM concentrations (Figure 7a-b). The effect of compound 5b was also investigated in angiogenesis dependent cell migration in HUVEC cells. Results are shown in Figure 7c-d, which indicated that compound 5b inhibited angiogenesis-dependent cell migration in HUVEC cells in a dose-dependent manner. The significant % wound closure was observed at 10 and 20 µM of 5b treatment.

Conclusion

In conclusion, the semisynthetic derivative 5b displayed significant antiproliferative activity in human prostate PC-3 cells. Mechanistic study revealed that, derivative 5b also possesses potent apoptotic and anti-angiogenesis potential in PC-3 cells. It induces apoptosis which is confirmed by apoptotic bodies’ formation, increase of sub-G1 population and induction of various pro-apoptotic proteins. Compound 5b caused disruption of mitochondrial membrane potential, rendered Bcl-2 inhibition, Bax translocation, decrease Bcl-2/Bax ratio, and released pro-apoptotic factors from the mitochondria. Mitochondria and Bcl-2 family of proteins play a pivotal role in the induction of apoptosis. The cell death regulated by Bcl-2 associated proteins have both pro-apoptotic and anti-apoptotic effects in cancer cells. These proteins regulate mitochondrial outer membrane potential and control the release of many apoptotic factors originating in the mitochondria. These events are accompanied by activation of pro-caspases-3, 8, -9, which cleave PARP-1 and finally induce apoptosis. Compound 5b also inhibit pro-caspase-8 level, therefore it induces apoptosis through both intrinsic and extrinsic apoptotic pathways. These results provide the basis for further in-depth drug targetted studies, while the pro-apoptotic feature of compound 5b raises its potential as a future anticancer therapeutics.

Experimental Section

General. All chemicals were obtained from Sigma-Aldrich Company and used as received. 1H, 13C and DEPT NMR spectra were recorded on Bruker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm, δ) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent (CDCl3, 7.26 ppm). Carbon nuclear magnetic resonance spectra (13C NMR) were recorded at 125 MHz or 100 MHz: chemical data for carbons are reported in parts per million (ppm, δ scale) downfield from tetramethylsilane and are referenced to the carbon resonance of the solvents (CDCl3, 77.36 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. IR spectra were recorded on Perkin-Elmer IR spectrophotometer. Melting points were recorded on digital melting point apparatus. HPLC analysis was done on Waters and Shimazu HPLC systems (detailed method provided in ESI).

Plant material. The dried Glycyrrhiza glabra roots were purchased from the local market of Jammu and it was authenticated by B. K. Kapahi, Biodiversity and Applied Botany Division of Indian Institute of Integrative Medicine (CSIR), Jammu. A specimen sample (accession number: 13485) was preserved in Janaki Ammal Herbarium at the CSIR-IIIM, Jammu, India.

Isolation of glycyrrhizin. G. glabra roots were extracted with water: ethanol (8: 2) under warm condition (55-60 °C). The crude extract was filtered and concentrated, which was washed with cold dichloromethane and cold acetone to remove other low molecular weight phenolics. By repeating this procedure glycyrrhizin-enriched fraction (80% glycyrrhizin) was obtained. This material was directly used for further hydrolysis.

Hydrolysis of glycyrrhizin. The glycyrrhizin was hydrolyzed by 5%aq. HCl to get glycyrrhetic acid, which was purified over
silica column in EtOAc: hexane – 25: 75. Pure glycyrrhetic acid was characterized by comparison of m.p. and spectral data with literature values. The glycyrrhetic acid was then used for modifications at C-3 position for the synthesis of O-alkylated/benzylated glycyrrhetic acid derivatives.

**General method for preparation of O-alkylated/benzylated glycyrrhetic acid (3β-hydroxy-11-oxoolean-12-en-30-olic acid).** To the solution of 3β-hydroxy-11-oxoolean-12-en-30-olic acid (2, 100 mg, 1 mmol) in dry acetone (5 mL) was added K₂CO₃ (1.2 mmol) followed by addition of different alkyl and benzyl halides (1 mmol). The mixture was stirred at room temperature for 8 h under inert atmosphere and concentrated under reduced pressure. The reaction mixture was diluted with chloroform. Water was added to the resultant mixture leading to formation of a white precipitate in the aqueous layer. The organic layer was decanted off and the remaining solid residue was washed 5-6 times with chloroform. Combined chloroform layer was evaporated under reduced pressure and the residue obtained was purified by silica gel (#100-200) column chromatography using hexane-ethyl acetate as an eluent to yield the different alkylated products 5a–r.

**Figure 7. (a-b).** Effect of compound 5b on VEGF arbitrated in vitro angiogenesis in PC-3 cells. Compound 5b inhibits PC-3 migration in the wound healing assay. PC-3 cells were scratched by a sterile micro tip and the areas were quantified in three random fields in the terms of wound closure percentage. (c-d). Effect of compound 5b on angiogenesis-dependent cell migration in HUVEC cells. Data were mean ± S.D. of three independent experiments.
3-(2,6-Dichloro-benzoyl)-11-oxo-olean-12-ene-29-oic acid (5b): white solid; HPLC: \( t_R = 26.7 \text{ min} \) (97% purity); yield: 95%; m.p. 226-228 °C; IR (CHCl\(_3\)): \( \nu_{\text{max}} \) 3435, 2923, 2852, 1729, 1656, 1579, 1513, 1462, 1386, 1327, 1260 cm\(^{-1}\).

1H NMR (400 MHz, CDCl\(_3\)); \( \delta \) (ppm) 7.39 (d, 1H, Ar-4´), 7.37 (d, 1H, Ar-5´), 7.36 (m, 2H, Ar-3´, 7´), 6.56 (m, 2H, Ar-6´, 12), 3.32 (dd, \( J = 4.0, 12.0 \text{ Hz} \)), 2.77 (t, 1H, CH-18), 2.01 (m, 2H, CH-2), 1.91 (m, 2H, CH-1), 1.54 (s, 3H, Me-27), 1.17 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 0.93 (s, 3H, Me-26), 0.73 (s, 3H, Me-28), 0.71 (s, 3H, Me-28), 0.69 (s, 1H, CH-5), 0.63 (d, 1H, CH-5). HR-ESIMS: m/z 613.3459 [M+H]\(^+\) calculated for C\(_{37}\)H\(_{50}\)Cl\(_2\)F\(_4\)O\(_2\)H \(^+\) (613.3454).

3-(3-Nitro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5e): cream solid; HPLC: \( t_R = 49.6 \text{ min} \) (90% purity); yield: 95%; m.p. 186-188 °C; IR (CHCl\(_3\)): \( \nu_{\text{max}} \) 3448, 3053, 2928, 2868, 2304, 1730, 1656, 1513, 1462, 1386, 1327, 1260, 1206, 1149, 1039 cm\(^{-1}\); 1H NMR (400 MHz, CDCl\(_3\)); \( \delta \) (ppm) 7.81 (d, 1H, Ar-4´), 7.33 (m, 2H, Ar-5´, 6´), 7.27 (m, 2H, Ar-6´), 7.20 (m, 2H, Ar-12), 3.22 (dd, \( J = 4.0, 12.0 \text{ Hz} \)), 2.77 (t, 1H, CH-18), 2.01 (m, 2H, CH-2), 1.91 (m, 2H, CH-1), 1.32 (s, 3H, Me-27), 1.17 (s, 3H, Me-25), 1.11 (s, 3H, Me-29), 0.99 (s, 3H, Me-26), 0.80 (s, 3H, Me-28), 0.73 (s, 3H, Me-28), 0.69 (d, 1H, CH-5), 0.63 (d, 1H, CH-5). HR-ESIMS: m/z 659.2917 [M+H]\(^+\) calculated for C\(_{37}\)H\(_{50}\)Cl\(_2\)N\(_2\)O\(_2\)H \(^+\) (659.2904).

3-(3-Methyl-but-2-enyloxy)-11-oxo-olean-12-ene-29-oic acid (5f): white solid; HPLC: \( t_R = 8.4 \text{ min} \) (96% purity); yield: 90%; m.p. 238-240 °C; IR (CHCl\(_3\)): \( \nu_{\text{max}} \) 3448, 3053, 2928, 2868, 2304, 1730, 1656, 1513, 1462, 1386, 1327, 1260, 1206, 1149, 1039 cm\(^{-1}\); 1H NMR (400 MHz, CDCl\(_3\)); \( \delta \) (ppm) 7.39 (d, 1H, Ar-4´), 7.37 (d, 1H, Ar-5´), 7.36 (m, 2H, Ar-3´, 7´), 3.22 (dd, \( J = 4.0, 12.0 \text{ Hz} \)), 2.77 (t, 1H, CH-18), 2.01 (m, 2H, CH-2), 1.91 (m, 2H, CH-1), 1.32 (s, 3H, Me-27), 1.17 (s, 3H, Me-25), 1.11 (s, 3H, Me-29), 0.99 (s, 3H, Me-26), 0.80 (s, 3H, Me-28), 0.73 (s, 3H, Me-28), 0.69 (d, 1H, CH-5), 0.63 (d, 1H, CH-5). HR-ESIMS: m/z 659.2917 [M+H]\(^+\) calculated for C\(_{37}\)H\(_{50}\)Cl\(_2\)N\(_2\)O\(_2\)H \(^+\) (659.2904).
3-(2,4-Bis-trifluoromethyl-benzoyloxy) - 11-oxo-olean-12-ene-29-oic acid (5h): white solid; HPLC: $t_R = 42.4$ min (98% purity); yield: 90%; m.p. 254-256 °C; IR (CHCl$_3$): $\nu_{\text{max}}$ 3448, 2928, 2860, 2928, 2868, 2304, 1730, 1706, 1656, 1565, 1585, 1462, 1386, 1237, 1313, 12679, 1262, 1210, 1149, 1039 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 7.95 (d, 1H, Ar-4'), 7.87 (d, 1H, J = 8 Hz, Ar-6'), 7.72 (s, 1H, J = 8 Hz, Ar-7), 5.60 (s, 1H, CH-12), 28.3 (m, 2H, CH-2), 3.23 (dd, $J = 8.0, 12.0$ Hz, 1H, CH-3), 2.80 (t, 1H, CH-18), 2.33 (s, 1H, CH-19) 1.35 (s, 3H, Me-27), 1.21 (s, 3H, Me-25), 1.13 (3H, Me-29) 0.81 (s, 3H, Me-21), 0.80 (s, 3H, Me-28), 0.71 (d, 1H, CH-5); $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 142.3 (C-2), 142.3 (C-5), 137.7 (C-23), 137.2 (C-10), 32.7 (C-2), 29.7 (C-21), 28.5 (C-29), 28.3 (C-28), 28.1 (C-27), 27.3 (C-26), 26.5 (C-15), 26.4 (C-16), 25.7 (C-5'), 23.3 (C-27), 18.1 (C-26), 17.5 (C-6), 16.6 (C-4'), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: $m/z$ 539.4016 [M+H]$^+$ calc. for C$_{35}$H$_{56}$O$_4$ + H$^+$ (539.4094).

3-Methoxy-11-oxo-olean-12-ene-29-oic acid (5i): white solid; HPLC: $t_R = 49.6$ min (95% yield); yield: 85%; m.p. 262-264 °C; IR (CHCl$_3$): $\nu_{\text{max}}$ 3290, 2931, 2869, 2872, 1772, 1657, 1618, 1466, 1368, 1358, 1323, 1246, 1189, 1066, 1040 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 5.59 (s, 1H, CH-12), 3.62 (s, 3H, Me-1), 3.16 (dd, $J = 8.0, 12.0$ Hz, 1H, CH-3), 2.74 (tt, 1H, CH-18), 2.27 (s, 1H, CH-9), 1.55 (m, 2H, CH-2), 1.18 (s, 3H, Me-27), 1.08 (s, 3H, Me-25), 1.08 (s, 3H, Me-29), 1.07 (s, 3H, Me-27), 1.06 (s, 3H, Me-23), 0.94 (s, 1H, Me-24), 0.93 (s, 3H, Me-28), 0.74 (d, 1H, CH-5); $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 200.3 (C-11), 177.0 (C-30), 169.3 (C-13), 128.5 (C-12), 78.8 (C-3), 54.9 (C-5), 51.8 (C-1), 48.4 (C-18), 45.4 (C-14), 44.0 (C-20), 43.2 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 28.5 (C-29), 28.3 (C-28), 28.1 (C-27), 27.3 (C-26), 26.4 (C-15), 26.6 (C-16), 25.7 (C-5'), 23.3 (C-27), 18.7 (C-26), 17.4 (C-6), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: $m/z$ 485.3616 [M+H]$^+$ calc. for C$_{35}$H$_{56}$O$_4$ + H$^+$ (485.3625).

3-(3,7-Dimethyl-octa-2,6-dienyloxy) - 11-oxo-olean-12-ene-29-oic acid (5j): yellow oil; HPLC: $t_R = 8.0$ min (100% purity); yield: 92%; IR (CHCl$_3$): $\nu_{\text{max}}$ 3442, 2925, 2854, 1725, 1660, 1620, 1545, 1385 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.57 (s, 1H, CH-12), 5.27 (t, 1H, CH-2), 5.01 (t, 1H, CH-6), 4.54 (m, 2H, CH-1), 3.16 (dd, $J = 8.0, 12.0$ Hz, 1H, CH-3), 2.74 (tt, 1H, CH-18), 2.26 (s, 1H, CH-9), 1.65 (s, 3H, Me-8), 1.60 (s, 3H, Me-9), 1.52 (s, 3H, Me-10), 1.27 (s, 3H, Me-26), 1.18 (s, 3H, Me-25), 0.70 (s, 3H, Me-29), 0.60 (s, 3H, Me-26), 0.73 (s, 1H, Me-24), 0.73 (s, 1H, Me-28), 0.71 (d, 1H, CH-5); $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ (ppm) 200.2 (C-11), 179.4 (C-30), 169.3 (C-13), 142.4 (C-7), 131.7 (C-3), 128.5 (C-12), 123.7 (C-6), 118.4 (C-2), 78.7 (C-3), 61.9 (C-8), 61.2 (C-1), 54.9 (C-5), 48.3 (C-18), 45.3 (C-14), 43.9 (C-20), 43.1 (C-8), 41.1 (C-19), 39.5 (C-4), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 29.7 (C-28) 28.3 (C-27), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 25.7 (C-10), 23.3 (C-23), 18.6 (C-26), 17.4 (C-6), 16.5 (C-8), 16.3 (C-25), 15.6 (C-24); HR-ESIMS: $m/z$ 607.4734 [M+H]$^+$ (607.4720).
3-(4-Bromo-benzylxy)-11-oxoolean-12-en-29-oic acid (5m): colorless oil; HPLC: \( t_R = 12.7 \) min (95% purity); yield: 95%; IR (CHCl\(_3\)): \( \nu_{\text{max}} = 3434, 2930, 2867, 1724, 1656, 1435, 1429, 1263, 1210, 1159, 1084, 1024 \) cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)); \( \delta (ppm) = 7.36-7.28 \) (m, 4H), Ar-3\(^{\circ}\) \(= 4.0\), 12.0 Hz 1H, CH-3), 2.78 (tt, 1H, CH-18), 2.32 (s, 1H, CH-9), 1.34 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.12 (s, 3H, Me-29), 0.81 (s, 1H, Me-24), 0.75 (dd, 1H, CH-5); \(^13\)C NMR (100 MHz, CDCl\(_3\)): \( \delta (ppm) = 145.5 \) (C-6), 142.3 (C-5), 142.2 (C-4), 141.7 (C-3), 143.5 (C-22), 138.7 (C-21), 131.2 (C-17), 131.1 (C-23), 129.8 (C-24), 129.7 (C-25), 129.5 (C-26), 129.4 (C-27), 122.8 (C-16), 111.8 (C-12), 78.5 (C-15), 65.4 (C-13), 65.1 (C-19), 48.2 (C-18), 37.0 (C-17), 31.7 (C-17), 31.6 (C-21), 28.4 (C-29), 28.1 (C-28), 27.3 (C-27), 26.4 (C-15), 26.3 (C-16), 23.3 (C-17), 18.6 (C-14), 16.4 (C-15), 16.3 (C-15), 15.5 (C-24); HR-ESIMS: \( m/z = 605.3835 \) [M+H\(^+\)] for calcd for C\(_{33}\)H\(_{35}\)BrO\(_2\)+H\(^+\) (605.3836).

3-(4-Methoxy-benzyloxy)-11-oxoolean-12-en-29-oic acid (5p): yellow oil; HPLC: \( t_R = 7.8 \) min (100% purity); yield: 90%; IR (CHCl\(_3\)): \( \nu_{\text{max}} = 3454, 2927, 2866, 1730, 1654, 1590, 1464, 1386, 1365, 1326, 1279, 1102, 1084, 1048 \) cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)); \( \delta (ppm) = 7.47-7.37 \) (m, 3H, Ar-4\(^{\circ}\)\(= 4.0\), 8.0 Hz 1H, CH-3), 2.80 (tt, 1H, CH-18), 2.32 (s, 1H, CH-9), 1.31 (s, 3H, Me-27), 1.11 (s, 3H, Me-25), 0.81 (s, 1H, Me-24), 0.80 (s, 3H, Me-28), 0.75 (d, 1H, CH-5); \(^13\)C NMR (100 MHz, CDCl\(_3\)): \( \delta (ppm) = 145.7 \) (C-6), 142.6 (C-5), 142.6 (C-4), 141.6 (C-3), 143.6 (C-22), 138.7 (C-21), 131.2 (C-17), 131.1 (C-23), 129.9 (C-24), 129.8 (C-25), 129.7 (C-26), 129.6 (C-27), 122.8 (C-16), 111.8 (C-12), 78.5 (C-15), 65.4 (C-13), 65.3 (C-19), 48.2 (C-18), 37.0 (C-17), 31.7 (C-17), 31.6 (C-21), 28.4 (C-29), 28.1 (C-28), 27.3 (C-27), 26.4 (C-15), 26.3 (C-16), 23.3 (C-17), 18.6 (C-14), 16.4 (C-15), 16.3 (C-15), 15.5 (C-24); HR-ESIMS: \( m/z = 621.4149 \) [M+H\(^+\)] for calcd for C\(_{33}\)H\(_{35}\)BrO\(_2\)+H\(^+\) (621.4149).

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Next day cells washed with fixing solution and dispersed in 50 µl in 400 µl cold acetic acid: methanol (1+3, v/v) overnight at 4 °C. Treatment cells were collected, washed with PBS twice and fixed with PBS, subjected to RNase digestion (400 µg/ml) at 37 °C for 24 h. Cells were collected, washed in PBS, fixed at 70% cold ethanol and placed at −20 °C overnight. Cells were again washed with the indicated doses of compound 5b for 24 h. Rhodamine-123 (5 µM) was added 1 h before the termination of the experiment and cells were collected, washed in PBS. The fluorescence intensity of 10,000 events was analyzed in FL-1 channel on a BD FACS Calibur (Becton Dickinson, USA) flow cytometer. The decrease in fluorescence intensity caused by loss of mitochondrial membrane potential was analyzed in FL-1 channel.

**Western blot analysis:** Cells were treated with different concentration of compound 5b for 24 h. Cells were collected at 400×g for 4 °C, washed in PBS twice and cell pellets were incubated with cold RIPA buffer (Sigma Aldrich, India) containing 50 mM NaF, 0.5 mM NaVO₄, 2 mM PMSF and 1% protease inhibitor cocktail for 40 min. Cells were centrifuged at 12000 × g for 10 min at 4 °C and the supernatant was collected as whole cell lysates for western blot analysis of various proteins. Protein was measured employing Bio-Rad protein assay kit using bovine serum albumin as standard. Proteins aliquots (30-70 µg) were resolved on SDS-PAGE and then electro transferred to PVDF membrane overnight at 4 °C at 30V. Nonspecific binding was blocked by incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary antibodies (purchased from Santacruz Biotech) for 2 h and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated mouse or rabbit secondary antibodies (purchased from Santacruz Biotech.) for 1 h, washed again three times with TBST and signals detected using ECL plus chemiluminescence’s kit on X-ray film.

**Wound healing migration assay:** The wound-healing migration assay was performed as described previously. Briefly, PC-3 cells were treated with mitomycin-C to inactivate cell proliferation, wounded by micro tip, washed with PBS, supplemented with fresh medium treated with SAD. Images of the cells were taken after ~0 to 24 h of incubation and the percentage of wound closure was expressed with respect to untreated cells consider 100%. The wound-healing migration assay in HUVEC cells was also done using similar protocol.

**Statistical analysis:** Data expressed as mean ± SD or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intra group using one way ANOVA with post Bonferroni test through GraphPad Prism.
5.0.0.288 statistical analysis software. *p*-values *< 0.001 were considered significant.

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**Notes and references**