Research paper

Synthesis and screening of ursolic acid-benzylidine derivatives as potential anti-cancer agents

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A B S T R A C T

Ursolic acid present abundantly in plant kingdom is a well-known compound with various promising biological activities including, anti-cancer, anti-inflammatory, hepatoprotective, antiallergic and anti-HIV properties. Herein, a library of ursolic acid-benzylidine derivatives have been designed and synthesized using Claisen Schmidt condensation of ursolic acid with various aromatic aldehydes in an attempt to develop potent antitumor agents. The compounds were evaluated against a panel of four human carcinoma cell lines including, A-549 (lung), MCF-7 (breast), HCT-116 (colon), THP-1 (leukemia) and a normal human epithelial cell line (FR-2). The results from MTT assay revealed that all the compounds displayed high level of antitumor activities compared with the triazole analogs (previously reported) and the parent ursolic acid. However, compound 3b, the most active derivative was subjected to mechanistic studies to understand the underlying mechanism. The results revealed that compound 3b induced apoptosis in HCT-116 cell lines, arrest cell cycle in the G1 phase, caused accumulation of cytochrome c in the cytosol and increased the expression levels of caspase-9 and caspase-3 proteins. Therefore, compound 3b induces apoptosis in HCT-116 cells through mitochondrial pathway.

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1. Introduction

Natural products play a dominant role in drug discovery with around 60% anticancer and 75% anti-infective approved drugs owing their origin from the natural resources [1]. Fine tuning of the biologically active natural products has led to the development of potentially important bioactive molecules, leads, and drugs [2]. Triterpenes, on account of their omnipresence, amenable functionality for transformation and promising biological activities have been the target of interest for chemists and biologists throughout the world. The pentacyclic triterpenic acids like betulinic, oleanolic and ursolic acid are found abundantly in plants especially in vegetable and fruit bearing plants [3]. These compounds exhibit significant anti-cancer [4a], anti-inflammatory [4b,c], hepatoprotective [4d,e,f], anti-allergic and anti-HIV properties [4g,h,i]. The key functionalities such as C3–OH, Δ12 and C7–COOH embodied in these highly useful natural scaffolds make them amenable for a variety of chemical transformations [5]. Ursolic acid 1, a pentacyclic triterpene isolated in abundance from the peels of Malus pumila Mill [6] exhibits anti-inflammatory, antiallergic, antibacterial, antiviral, antitumor and cytotoxic activity [7–11]. Japanese researchers have ranked ursolic acid as one of the most promising tumor preventive medication [12]. Mechanistic studies revealed that compound 1 arrests cell cycle in G1 phase and induces apoptosis [13].

Chalcones distributed widely in fruits, vegetables, spices, tea and soya based foodstuff exhibit remarkable pharmacological activities [14,15]. Chemically, they are open chain precursors of flavonoids and isoflavonoids in which two aromatic rings are linked by a three-carbon α, β-unsaturated carbonyl system. Chalcones have been reported to possess antibacterial [16,17], antimalarial [18–20], anti-fungal [21,22], antiviral [23,24] and anti-inflammatory [25–27] properties. The present study was performed to synthesize the benzylidine derivatives of ursolic acid with an aim to get more potent anticancer agents.

2. Results and discussion

The promising biological activity of ursolic acid has led to its extensive chemical modification for the development of more potent antitumor agents [28–32]. Improved anti-cancer activity of ursolic acid derivatives synthesized [28–32], encouraged us to...
design and synthesize a library of ursolic acid benzylidine derivatives. The benzylidine derivatives were synthesized using Claisen Schmidt condensation protocol at the position 2.

2.1. Synthesis of benzylidine derivatives

The synthetic protocol for the ursolic acid benzylidine derivative \(3\) from the parent ursolic acid \(1\) involves two steps, including Jones oxidation and Claisen Schmidt condensation (Scheme 1). The compound \(1\) in acetone at \(0\) °C was treated with Jones reagent to form the C-3 oxidized derivative \(2\) in almost quantitative yield. The benzylidine derivative \(3a\) was prepared by Claisen Schmidt condensation of compound \(2\) with benzaldehyde in the presence of ethanolic potassium hydroxide at room temperature in excellent yields (Scheme 1). A sharp IR peak at around 1675 cm\(^{-1}\), C-13 signal at \(\delta\) 207.9, 137.7 & 133.8 for \(\alpha\), \(\beta\) unsaturated ketone and \(^1\)H NMR signal at \(\delta\) 7.52 for olefinic proton confirmed the product formation of \(3a\).

The above set optimized condition was then used for the condensation of various aromatic aldehydes with compound \(2\) to prepare a library of ursolic acid benzylidine derivatives. All the condensation reactions of aromatic aldehydes with compound \(2\) worked smoothly at room temperature to deliver the desired product in excellent yields (Table 1).

2.2. Biological evaluation

2.2.1. In vitro cytotoxicity

MTT assay was used to study the cytotoxicity of all the synthesized compounds against A-549 (lung), MCF-7 (Breast), HCT-116 (colon) and THP-1 (leukemia) carcinoma cell lines and FR-2, 2.2.1. In vitro cytotoxicity

2.2.2. Effect of compounds \(3b\) on apoptosis in HCT-116 cells

Apoptosis is the programmed cellular process that takes place during physiological and pathological conditions. However, in carcinoma cells the process of apoptosis is disrupted resulting in the overgrowth and proliferation of malignant cells. Therefore, most of the cancer treatment strategies involve induction of apoptosis. The effect of compound \(3b\) on the induction of apoptosis in HCT-116 cells was investigated using fluorescence staining. The morphological alterations induced by compound \(3b\) in HCT-116 cells were studied under fluorescence microscopy using acridine orange (AO)/ethidium bromide (EB), Hoechst 33258, JC-1 mitochondrial membrane potential staining.

2.2.2.1. AO/EB staining. The results from AO/EB staining revealed that HCT-116 cells exposed to compound \(3b\) stained yellow green after 24 h. The morphological examination showed the appearance of pycnosis, membrane blebbing and cell budding. However, the control cells stained green with no alterations in the morphological appearance (Fig. 1). These findings indicate that the treatment of HCT-116 cells with compound \(3b\) induced apoptotic cell death.

2.2.2.2. Hoechst 33258 staining. HCT-116 cells treated with compound \(3b\) at a concentration of 20 μM for 24 h showed strong blue fluorescence on Hoechst 33258 staining and typical apoptotic morphology. However, the nuclei of the control HCT-116 cells stained light green on exposure to Hoechst 33258 stain. These observations further confirmed that compound \(3b\) induced apoptosis in HCT-116 cells (Fig. 2).

2.2.3. Mitochondrial membrane potential staining

The HCT-116 cells treated with 20 μM concentration of compound \(3b\) for 24 h stained with JC-1, however, no JC-1 staining was observed in the control HCT-116 cells (Fig. 3). The compound \(3b\) treated cells exhibited strong green fluorescence and showed typical apoptotic morphology after 24 h whereas the control cells were normally red. Therefore, the above findings confirm that the treatment of HCT-116 cells with compound \(3b\) for 24 h induces apoptosis.

2.2.4. Cell cycle analysis

Investigation of the effect of compound \(3b\) on cell cycle distribution in HCT-116 cells was performed by flow cytometric analysis using propidium iodide (PI). The results revealed that treatment of HCT-116 cells with compound \(3b\) caused a significant increase in the percentage of cells in G1 phase (65.58%) compared to the control cells (41.51%). However, the percentage of cells in the G2 phase was significantly decreased in compound \(3b\) treated cells (11.65%) compared to control cells (32.43%) compared with the control cells (Fig. 4).

2.2.5. Alterations in mitochondrial protein expression

The results from western blot analysis revealed that compound \(3b\) induced significant increase in the expression of Bax and decrease in expression of Bcl-2 compared to the control cells. It also caused accumulation of cytochrome c in the cytosol, most probably due to the release of mitochondrial cytochrome c (Fig. 5). Treatment of HCT-116 cells with compound \(3b\) caused a significant increase in the expression levels of caspase-9 and caspase-3 proteins.

![Scheme 1](image-url)
compared to the control. Therefore, mitochondrial pathway plays an important role in compound 3b induced apoptosis in HCT-116 cells.

3. Conclusion

Thus a library of ursolic acid benzylidine derivatives were synthesized and screened for activity against four human carcinoma cell lines including, A-549, MCF-7, HCT-116, THP-1 and a normal human epithelial cell line. All the compounds exhibited better cytotoxicity against the tested carcinoma cell lines, however, the compound 3b was found to be more active against HCT-116 cell line. The mechanistic studies demonstrated that compound 3b induces apoptosis in HCT-116 cell through mitochondrial pathway. Therefore, compound 3b can be of therapeutic importance for the treatment of colon carcinoma.

4. Experimental section

4.1. Chemistry

Ursolic acid used in the present study was isolated from apple peels in 10:90 ethyl acetate:hexane. The solvents used for reactions and purification of compounds were distilled before use according to the usual methods reported in the literature. The reactions were monitored using 0.2 mm-thick, aluminum-backed TLC plates and

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Table 1
Ursolic acid-benzylidine derivatives varying at aromatic ring.
visualized at 254 nm under UV light. NMR spectra were recorded on a Bruker AV-400 NMR spectrometer. Mass spectra were determined on a FTMS ESI spectrometer.

4.1.1. Synthesis

The synthesis of the ursolic acid benzylidine derivatives 3a-3p was achieved by Claisen Schmidt condensation of compound 2 with various aromatic aldehydes.

4.1.2. General procedure for the synthesis of 2

To a solution of compound 1 (400 mg, 0.88 mmol) in acetone (5 ml) at room temperature was added Jones reagent drop wise till yellow colour persists. The reaction was monitored by TLC till its completion in 2 h. After quenching the reaction with cold water, the reaction mixture was filtered to remove insoluble residue. The filtrate was extracted with ethyl acetate (3 x 20 ml). The combined organic layer was dried over sodium sulphate, concentrated under vacuum and purified by column chromatography to give pure

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**Table 2**

Percentage inhibition data and IC_{50} values of the compounds against four cancer cell lines & a normal cell line.a

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* All experiments were carried out in triplicates; 5-FU, 5-fluorouracil; ND, not determined.

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**Fig. 1.** Effect of compound 3b on the HCT-116 cells. Normal cells showed the presence of circular nucleus present in the centre of the cell. The cells treated with 10 μM of compound 3b showed early apoptotic changes evident by yellow-green fluorescence with acridine orange (AO) staining. Treatment with 20 μM concentration of compound 3b showed late apoptotic changes.

**Fig. 2.** Morphological changes in HCT-116 cells. The cells were left untreated or treated with 10 and 20 μM concentration of compound 3b followed by Hoechst 33258 staining and visualization under fluorescent microscopy.
compound 2 (365 mg, 91% yield). White solid; mp: 276–278 °C; \( ^{1}H \) NMR (400 MHz, CDCl\(_3\)): \( \delta \) 5.29 (1H, s), 3.20 (1H, d, \( J = 12.0 \) Hz), 2.28 (1H, t, \( J = 8.0 \) Hz), 1.97 (2H, m), 1.81 (4H, m), 1.52 (12H, m), 1.30 (4H, m), 1.04 (3H, s), 0.91 (3H, s), 0.85 (3H, s), 0.82 (3H, s), 0.80 (3H, s), 0.75 (3H, s), 0.68 (3H, s); \( ^{13}C \) NMR (100 MHz, CDCl\(_3\)): \( \delta \) 216.6, 181.5, 151.9, 123.2, 53.6, 52.2, 51.2, 50.6, 49.3, 47.8, 46.8, 41.7, 40.1, 39.8, 39.7, 39.4, 39.3, 39.1, 38.9, 38.5, 38.4, 36.6, 28.3, 23.3, 21.2, 17.1, 17.0, 16.1, 15.3; IR (KBr): 2930, 1718, 1657, 1456, 1378, 751 cm\(^{-1}\); ESI-MS: 455.46 calcd. for C\(_{30}\)H\(_{48}\)O\(_{3}\) [M+Na\(^{+}\)]

4.2. In vitro cytotoxicity

The carcinoma cell lines, A-549 (lung), MCF-7 (breast), HCT-116 (colon), THP-1 (leukemia) and a normal human epithelial cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum under humidified atmosphere of 5% CO\(_2\) at 37 °C onto the 96-well microtiter plates. The compounds and 5-FU dissolved in the culture medium with 1% DMSO were added to the wells. The control wells received only supplemented media with 1% DMSO. The microtiter plates were incubated for 24 h at 37 °C in humidified 5% CO\(_2\) atmosphere. Following incubation, MTT solution (10 ml, 5 mg/ml) was added to each well and the cultures were incubated again for 48. Dimethyl sulphoxide (150 ml) was added to each well for dissolving the formazan crystals. For the measurement of absorbance enzyme labeling instrument with 570/630 nm double wavelength was used. The final IC\(_{50}\) values were calculated using Bliss method (n = 5). All the experiments were performed at least three times independently.

4.2.1. AO/EB staining

HCT-116 cells were distributed at a density of 5 \( \times \) 10\(^5\) cells per ml in 2 ml volume on a sterile cover slip in six-well tissue culture plates and incubated for 24 h. After incubation, the medium was replaced with fresh medium supplemented with 10% fetal bovine serum and compound 3b (20 \( \mu \)M). Following incubation, the cover slip with monolayer cells was inverted on a glass slide with 20 \( \mu \)M of AO/EB stain (100 mg/ml). Nikon ECLIPSE TE2000-S fluorescence microscope (OLYMPUS Co., Japan) was used to read the fluorescence.

4.2.2. Hoechst 333258 staining

HCT-116 cells grown in six-well plates on a sterile cover slip were treated with the compounds for indicated time. After

**Fig. 3.** Compound 3b induced apoptotic changes in the morphology of HCT-116 cells. The cells after treatment with compound 3b at 10 and 20 \( \mu \)M concentration or untreated cells were subjected to JC-1 staining.

**Fig. 4.** Compound 3b induces cell cycle arrest in G1 phase in HCT-116 cells. The cells treated with 20 \( \mu \)M concentration of compound 3b or untreated were observed by flow cytometric analysis using propidium iodide (PI).

**Fig. 5.** Compound 3b changes the mitochondrial protein expression. HCT-116 cells were treated with 20 \( \mu \)M concentration of compound 3b and then subjected to western blot analysis.
incubation, the culture medium containing compounds was removed and the cells were fixed for 15 min in 4% paraformaldehyde. The cells were washed twice with PBS, stained with Hoechst 33258 (Beyotime, Haimen, China) for 15 min and then washed again twice with PBS. The cells were then observed under an Nikon ECLIPSETE2000-S fluorescence microscope using 350 nm excitation and 460 nm emission.

4.2.3. Mitochondrial membrane potential staining

The depolarization of mitochondrial membrane in HCT-116 cells was measured using JC-1 (Beyotime, Haimen, China) probe. For this purpose the cells cultured in six-well plates after treatment with compound 3b were incubated with JC-1 staining solution (5 μg/ml) at 37 °C for 30 min. The cells were rinsed twice with PBS followed by peroxidase-labeled secondary antibody followed by determination of the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using an Nikon ECLIPSETE2000-S fluorescence microscope. The enhancement in the green/red fluorescence intensity ratio indicates the mitochondrial membrane depolarization.

4.2.4. Apoptosis analysis by flow cytometry

Apoptosis in HCT-116 cells was determined using annexin V-FITC/propidium iodide test. For this purpose, the cells distributed at a density of 2 × 10^5 cells per well into 6-well plates in 10% FBS-DMEM were treated with compounds 3b for 24 h. After incubation, the cells were washed twice in PBS and resuspended in 1 × Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl2). 100 μl of the cell suspension was then transferred to 5 ml culture tubes and to each tube 5 μl of FITC Annexin V (BD, Pharmingen) and 5 μl propidium iodide (PI) were added. The tubes were incubated for 30 min under dark atmosphere followed by addition of 200 μl PBS. For the system software (Cell Quest; BD Biosciences) was used.

4.2.5. Cell cycle analysis

HCT-116 cells after incubation with compound 3b for 48 h were washed twice with ice-cold PBS, fixed and permeabilized in ice-cold 70% ethyl alcohol at −20 °C overnight. The cells were exposed to 100 μg/ml RNase A for 45 min at 37 °C washed with ice-cold PBS and then stained with 1 mg/ml propidium iodide (PI) under dark atmosphere at 4 °C for 30 min. Analysis was performed with the system software (Cell Quest; BD Biosciences).

4.2.6. Western blot

HCT-116 cells were cultured in 6-well plates at a density of 3 × 10^5 cells per well to attain 80–90% confluence. The medium was then replaced with 0.1% FCS RPMI 1640 and the cell cultures were incubated for 12 h. Compound 3b was added to each well of the plate and the plates were incubated for 24 h. After incubation, the cells were washed with ice-cold PBS followed by lysis in lysis buffer (50 mM HEPES buffer, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM vanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1% NP-40, and a cocktail of protease inhibitors). The cell debris were removed and the proteins separated by 15% SDS-PAGE followed by transfer to nitrocellulose membranes. The membranes were blocked with 5% non-fat-milk solution and then incubated with the primary antibody followed by peroxidase-labeled secondary antibody. For the visualization of bands enhanced chemiluminescence (ECL) detection technique (Amersham Corporation) was used.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.01.026

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