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Research paper

Design, synthesis and antitumor activity of triterpenoid pyrazine derivatives from 23-hydroxybetulinic acid

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ABSTRACT

Pyrazine-fused 23-hydroxybetulinic acid was synthesized by introducing a pyrazine ring between C-2 and C-3 position and further modifications were carried out by substitution of C-28 carboxyl group by ester and amide linkage to enhance the antitumor activity. The biological screening results showed that all of the derivatives exhibited more significant antiproliferative activity than the parent compound. In particular compound **12a** exhibited the most potent activity with IC_{50} values of 3.53 μM, 4.42 μM and 5.13 μM against cell lines SF-763, B16 and Hela, respectively. In the preliminary mechanism study, **12a** caused cell arrest in G1 phase and significantly induced apoptosis of B16 cells in a dose-dependent manner. Furthermore, the *in vivo* antitumor activity of **12a** was validated (tumor inhibitory ratio of 55.6% and 62.7%, respectively) in mice with H22 liver cancer and B16 melanoma.

1. Introduction

Natural products have been the single most productive source of leads for the development of drugs for the treatment of cancer. In fact, the majority of antitumor agents are of natural origin [1]. Betulinic acid (3-Hydroxy-lup-20(29)-en-28-oic acid, Fig. 1) is a pentacyclic lupane-type triterpene that is widely distributed throughout the plant kingdom. A variety of biological activities have been ascribed to betulinic acid including anti-inflammatory, antibacterial, antimalarial and antioxidant properties. However, betulinic acid is most highly regarded for its anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines. Previous experimental and epidemiological studies have indicated that betulinic acid as well as its analog- 23-hydroxybetulinic acid (HBA, **1**, Fig. 1) may be developed as potent anti-HIV and antitumor drugs [2–4].

The 23-hydroxybetulinic acid, isolated from the root of *Pulsatilla chinensis*, displayed similar anti-HIV and antitumor activities as its analog betulinic acid [5]. To date numerous derivatization studies have been performed on betulinic acid leading to the production of an array of betulinic acid derivatives [3]. Nevertheless, few studies have been devoted to the structural modifications of 23-hydroxybetulinic acid.

In general, due to various reasons original natural products are not particularly good candidates, and a need therefore exists for improving their drug-like properties by chemical modification [1]. It has been found that heterocyclic ring-fused triterpenoids at C-2 and C-3 position impart the desired characteristics [6]. Nitrogen heterocycles are important pharmacophores in drug design, especially pyrazine derivatives, which are among the most frequently cited heterocyclic compounds [7]. Previously, Urban et al. described the partial synthesis of triterpenic heterocycles, in which some lupane pyrazine derivatives were significantly cytotoxic *in vitro* to warrant the extension of the *in vitro* studies to *in vivo* testing in mice [8]. The cytotoxic activity of some triterpenic compounds was also successfully increased by using heterocyclic modifications [9–11].

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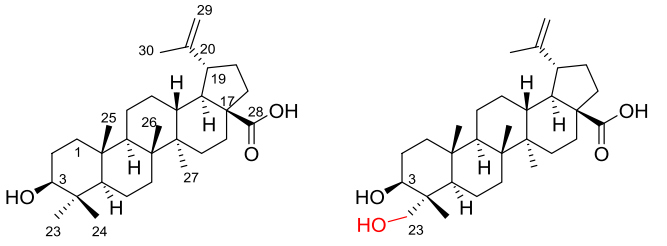

Fig. 1. Chemical structures of betulinic acid and 23-hydroxybetulinic acid.

Previously, a set of C-17-carboxylic acid-modified 23-hydroxybetulinic acid ester and amide derivatives have been reported by our group [12,13]. The promising anti-proliferative of triterpenic pyrazines encouraged us to continue to design and synthesize novel betulinic pyrazine derivatives from 23-hydroxybetulinic acid. Base on the structure–activity relationships (SARs) studies of betulinic acid and 23-hydroxybetulinic acid, further modifications were carried out by substitution of C-28 carboxyl group by ester and amide linkage to enhance the antitumor activity. The obtained derivatives were evaluated for their *in vitro* antitumor activity against five cancer cell lines. Furthermore, the *in vivo* antitumor activity of the representative compound was validated in mice with H22 liver cancer and B16 melanoma. The primary antitumor mechanism was also investigated.

2. Results and discussions

2.1. Chemistry

The 23-hydroxybetulinic acid was isolated from the roots of *P. chinensis* (Bge) Regel [5,14], and characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectra. As described in Scheme 1, it was treated with BnBr and K_2CO_3 in DMF to yield 28-benzyl-23-hydroxybetulinic ester **2**, the hydroxyl group of **2** was protected using tert-butyldimethylsilyl chloride in the presence of DMAP to give siloxane **3**. Oxidation of **3** with pyridinium chlorochromate afforded the ketone **4**, which was then converted to pyrazine derivative **5** in the presence of ethylenediamine and sulfer in morpholine [15]. Deprotection of **5** with hydrochloric acid in acetone gave alcohol **6**. The NMR spectrum displayed readily recognizable signals for the fused pyrazine ring [proton signals at δ 8.35 (H-2′), 8.38 (H-1′) and δ 2.45 (H-1), 3.08 (H-1′) and olefinic carbon signals at δ 152.3 (C-2), 157.8 (C-3), 142.2 (C-1′), 141.5 (C-2′)].

Previous study indicated that substitution of C-23 hydroxyl group may benefit the potency [16]. Therefore, a simple acetyl group was introduced to this position and selected for further investigation. The carboxylic pyrazine **8** was obtained by debenzylation of ester **7** in THF with Pd/C as catalyst under atmospheric pressure of hydrogen. Following deacetylation of **8** produced pyrazine derivative **9**.

Derivatives **10a**-**10e** were synthesized by the reaction of compound **9** with corresponding bromides under basic condition. Hydrolysis of derivative **10d** and **10e** afforded compound **10f** and **10g**. To synthesize derivatives **12a**-**12d**, C-28 carboxylic group was converted to acyl chloride intermediate **11**, which was further reacted with the corresponding alcohols and amides (Scheme 2).

2.2. Biological evaluation

2.2.1. In vitro antiproliferative activity

The *in vitro* antiproliferative activity of these novel betulinic pyrazine derivatives was evaluated on five cancer cell lines (HL-60 human promyelocytic leukemia cell, BEL-7402 human hepatocellular carcinoma, HeLa human cervical adenocarcinoma, SF-763 human brain adenocarcinoma and B16 mice melanoma cells) by MTT assay with doxorubicin as the positive control. The results summarized in Table 1 are presented as the concentration of drug inhibiting 50% cell growth (IC_{50}).

The data indicated that all of the derivatives exhibited stronger antiproliferative activities than parent compound 23-hydroxybetulinic acid in the five cancer cell lines, especially against human brain adenocarcinoma SF-763 and mice melanoma B16 cells. Pyrazine-fused compound **9** was about 2- to 3-fold more potent against five cancer cell lines than HBA, indicating that introduction of the pyrazine ring imparted potent antiproliferative activity. The acetylated derivative **8** showed lower IC_{50} values than compound **9**, which is consistent with the case of benzoylation [16]. Compounds **10f, 12a** and **12d** were the most promising derivatives with IC_{50} values lower than 10 μM on all tested cell lines, in particular compound **12a** was about 7- to 10-fold more potent against five cancer cell lines than HBA and 2- to 4-fold than **9**. These findings revealed that electron-donating and/or polar substituents especially groups bearing the amide linkage at C-28 side chain would benefit the potency. The results also demonstrated that the existence of a structural constraint such as benzyl, cyclohexyl, biphenyl and phenyl at C-28 is favorable to the inhibitory activity, which is exemplified by compounds **6**, **10g**, **10f** and **12d**. The obtained structure–activity relationships of C-28 position have also confirmed the results from previous comparative molecular field analysis (CoMFA), in which the contour maps illustrated that bulky and/or electron-donating groups at C-28 would be favorable to the antitumor activity [17].

Furthermore, selectivity of compound **12a** was assessed on normal human hepatocyte (HL-7702 cell line). The selectivity index was calculated by IC_{50} value in HL-7702 cell line (75.43 ± 2.05 μM) divided by IC_{50} value in cancer cell lines (see supplementary material). It was observed that **12a** was 11–20 times more selective towards cancer cells than to non-tumor cell. The dose–response curves for the test compound towards different tumor cell lines and non-tumor (HL-7702) cell line are given in Fig. 2.

2.2.2. Compound *12a* induces cell cycle arrest

To determine whether the suppression of cell growth by triterpenic pyrazines is caused by a cell-cycle effect, we detected the DNA content of cell nuclei by flow cytometry (Fig. 3). B16 cells were treated with compound **12a** at concentrations of 1.25, 2.5, and 5 μM, which resulted in accumulation of 29.67, 34.67, and 33.46% of cells at the G1 phase, respectively. These results indicated that compound **12a** inhibited the growth of the cancer cells by inhibiting the cell cycle via G1-phase arrest.

2.2.3. Compound *12a* induces apoptosis

To clarify whether the loss of cancer cell viability promoted by triterpenic pyrazines is associated with apoptosis, an annexin V–FITC/propidium iodide (PI) binding assay was performed. As shown in Fig. 4, compound **12a** exhibited potent dose-dependent activity in the induction of apoptosis. Treatment of B16 cells with **12a** at 1.25, 2.5, and 5 μM for 72 h resulted in 16.51, 31.62, and 57.44% apoptotic cells (early and late), as compared with 5.66% in an untreated vehicle control, indicating that compound **12a** was able to induce apoptotic cell death in B16 cells.

2.2.4. In vivo antitumor activity of compound *12a*

Based on the *in vitro* results and intensive mechanistic studies, we further tested the antitumor activity of compound **12a** *in vivo* by performing an assay in mice with H22 liver cancer and B16