European Journal of Medicinal Chemistry 130 (2017) 26–38

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Combination of amino acid/dipeptide with ligustrazine-betulinic acid as antitumor agents

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ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form
12 February 2017
Accepted 13 February 2017
Available online 17 February 2017

Keywords:
Betulinic acid
Amino acids derivatives
Dipeptide derivatives
Selective cytotoxicity
Structure-activity relationships
Apoptosis

ABSTRACT

The lead compound **TBA**, 3β-Hydroxy-lup-20(29)-ene-28-oic acid-3, 5, 6-trimethylpyrazin-2-methyl ester, which exhibited promising antitumor activity and induced tumor cell apoptosis in various cancer cell lines, had previously been reported. Moreover, reports have revealed that the introduction of amino acid to betulinic acid could improve selective cytotoxicity as well as water solubility. Thus, a series of novel **TBA** amino acid and dipeptide derivatives were designed, synthesized and screened for selective cytotoxic activity against five cancer cell lines (HepG2, HT-29, Hela, BCG-823 and A549) and the not malignant cell line MDCK by standard MTT assay. Most of the tested **TBA**-amino acid and dipeptide analogues showed stronger anti-proliferative activity against all tested tumor cell lines than **TBA**. Among them, **BA-25** exhibited the greatest cytotoxic activity on tumor cell lines (mean $IC_{50} = 2.31 \pm 0.78$ μM), that was twofold than the positive drug cisplatin (**DDP**), while it showed lower cytotoxicity on MDCK cell line than DDP. Further cell apoptosis analyses indicated **BA-25**-induced apoptosis was associated with loss of mitochondrial membrane potential and increase of intracellular free Ca^{2+} concentration.

1. Introduction

Cancer is one of the major diseases which threats human life and health seriously [1,2]. The cytotoxic agents are still one of the main clinical treatments for the malignant tumor. However, these drugs usually have some severe side effects with poor patients compliance, therefore the discovery of the targeted anti-tumor drugs is of great significance [3–6]. Because of their strong selective cytotoxicity and potent apoptosis induction activity, pentacyclic triterpenoids and their derivatives had become the focus of scientific interest [7–10].

Ligustrazine (2,3,5,6-tetramethylpyrazine, TMP), a major effective component of traditional Chinese medicine Rhizoma Chuanxiong (*Ligusticum, chuanxiong Hort*), has been used for the treatment of cardiovascular and cerebrovascular diseases in the clinic in China for many years [11–13]. Recently, ligustrazine was found to possess anticancer activity *in vivo* and *in vitro,* it could induce cancer cell apoptosis and reverse multidrug resistance in tumors [14–16]. Meanwhile, recent researches revealed that the introduction of ligustrazine to the anti-tumor components could increase their cytotoxicity and selectivity [17,18]. This has stimulated interest in using ligustrazine as the scaffold to synthesize new anticancer agents by combination it with other anti-tumor ingredients [16,19–21]. In our previous study, we successfully synthesized a series of novel ligustrazine-triterpenes derivatives and observed that these derivatives possessed potent selective cytotoxicity, of which, 3β-Hydroxy-lup-20(29)-ene-28-oic acid-3, 5, 6-trimethylpyrazin-2-methyl ester (**TBA**) displayed promising selective cytotoxicity ($IC_{50} < 5.23$ μM) [17–19,21,22]. In addition, reports have shown that the introduction of amino acid or dipeptide to triterpenes could improve selective cytotoxicity as well as water solubility [8,21,23–25]. Based on the above, we attempted the synthesis of several **TBA** amino acids and dipeptide derivatives **BA-X** by introducing various amino acids or dipeptides to the C3 of **TBA**, in order to improve its antitumor activities and tumor targeting. All newly synthesized compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, HRMS and tested for cytotoxic activity against a panel of tumor cell lines and normal cell line, including HepG2, HT-29, Hela, BGC823, A549 and MDCK. Meanwhile, the preliminary anti-tumor mechanisms of the most potent compound were also investigated by fluorescence staining observation and flow cytometric analysis

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http://dx.doi.org/10.1016/j.ejmech.2017.02.036
0223-5234/

in present study. In addition, the structure-activity relationships of these derivatives were briefly discussed.

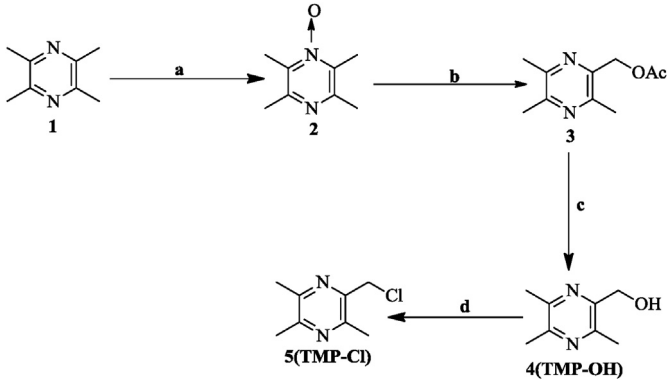
2. Results and discussion

2.1. Chemistry

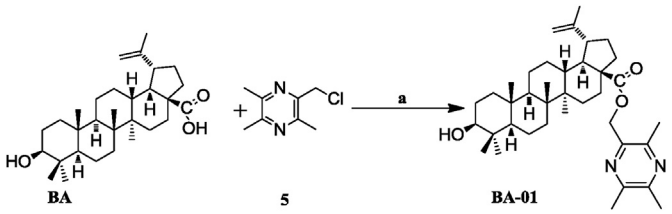
The designed derivatives were prepared following the procedures in Schemes 1–4. The compound **TBA** (**BA-01**) was prepared according to our previous study with some modifications [19]. According to the literature procedure, the intermediate (3,5,6-trimethylpyrazin-2-yl)methanol (**4**) was successively obtained [26]. Then the intermediate **4** was further reacted with tosyl chloride (TsCl) in tetrahydrofuran (THF) in the presence of triethylamine (TEA) and 4-dimethylaminopyridine (DMAP) to yield the important intermediate 2-(chloromethyl)-3,5,6-trimethylpyrazine (**5**) (Scheme 1). Subsequently, it underwent alkylation reaction in N, *N*-dimethylformamide (DMF) with betulinic acid (BA) to afford the compound **TBA** (**BA-01**) (Scheme 2).

The **TBA** amino acid derivatives **BA-02**–**BA-15** (Scheme 3, Table 1) were obtained by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) mediated esterification from the corresponding protected (*N*-Boc, N-Cbz) amino acids and **TBA**. Deprotection was performed with trifluoroacetic acid (TFA) in dry dichloromethane (DCM) or by treating the compounds with Pd/C (10%) in methanol (MeOH). It was worth noting that, to avoid the formation of undesirable by-products, the hydroxyl groups of Cbz-L-serine and Cbz-L-threonine should be protected with tert-Butyldimethylsilyl chloride (TBDMSCl) before esterification with **TBA** in the synthesis of **BA-14** and **BA-15**. Removal of the TBDMS groups was achieved using 1M tetrabutylammonium fluoride (TBAF) solution in THF (Scheme 3).

In similar fashions, the TBA dipeptide derivatives **BA-16**–**BA-27** were prepared according to the procedures in Scheme 4. In brief, the TBA amino acids derivatives **BA-02**, **BA-04**, **BA-06** and **BA-12** underwent peptide coupling reactions with the corresponding *N*-Boc protected amino acids (L-Gly, L-Sar, L-Pro, L-Ala) in the presence of EDCI, 1-hydroxybenzotriazole (HOBt) and N, *N*-Diisopropylethylamine (DIPEA) in dry DCM to afford the corresponding **TBA** dipeptide intermediates, which were further treated with TFA in dry DCM to give the final compounds **BA-16**–**BA-27** (Table 2). The structures of all target derivatives were confirmed by spectral (^{1}H NMR, ^{13}C NMR and HRMS) analysis.



Scheme 1. Synthesis of the intermediate 2-(chloromethyl)-3,5,6-trimethylpyrazine (**5**). *Reagents and Conditions*: (a) aceticacid (AcOH), 30% H2O2, reflux, 90 °C, 6 h; (b) acetic anhydride (Ac2O), reflux, 105 °C, 2 h; (c) THF: MeOH: H2O = 3:1:1, NaOH, 1 h; (d) THF, TsCl, TEA, DMAP, 12 h.



Scheme 2. Synthesis of the derivative **TBA (BA-01)**. *Reagents and Conditions*: (a) dry DMF, dry K2CO3, 25 °C, 12 h.

2.2. Biology

2.2.1. Cytotoxicity assay

The *in vitro* antitumor activity of **TBA** amino acids and dipeptide derivatives was evaluated on five tumor cell lines (HepG2, HT-29, Hela, BCG-823, A549) using the standard MTT assay, and their toxicity was tested using MDCK cells. The IC_{50} values were summarized in Table 3. As shown in Table 3, after combination with amino acid or dipeptide, most of the targeted compounds showed significantly improved cytotoxicity on all tested tumor cell lines compared to the starting material **TBA**. The cytotoxicity detection also revealed that most of **TBA** amino acids derivatives (such as **BA-02**, **BA-09**, **BA-10**, **BA-12** et al.) and nearly all **TBA** dipeptide derivatives exhibited better antiproliferative activities than the positive drug DDP, while they showed lower cytotoxicity than DDP on MDCK cell line. Among the candidates, **BA-25** was the most active one, which exhibited perfect antiproliferative activities (mean $IC_{50} = 2.31 \pm 0.78$ μM) on all tested cancer cell lines. For example, the IC_{50} values of **BA-25** for HT-29, Hela and BGC-823 (1.70 ± 0.34 μM, 1.74 ± 0.99 μM, 1.79 ± 0.28 μM) is much lower than those of the positive drug cisplatin (DDP) (4.10 ± 1.17 μM, 5.60 ± 0.78 μM, 4.25 ± 0.32 μM). Meanwhile **BA-25** exhibited higher cytotoxic selective towards MDCK cells ($IC_{50} = 10.84 \pm 0.27$ μM) than DDP. It is worth noting that, although the compound **BA-10, BA-23** and **BA-24** exhibited slightly lower cytotoxicity than **BA-25** on the tested cancer cells, their pronounced cytotoxic selective (therapeutic index (TI) > 10) towards MDCK cells distinguished them from this series. Their IC_{50} value for MDCK cells was more than 40 μM (see Table 3).

From the obtained results, it was observed that **TBA** aliphatic amino acids derivatives exhibited better anti-proliferative activities than those aromatic amino acids and heterocyclic amino acids derivatives, as exemplified by **BA-02**, **BH-04**, **BA-10**, **BA-12** > **BA-03**, **BA-11**; Structure-activity relationship analysis among the **TBA** aliphatic amino acids derivatives also revealed that the compounds with small molecule aliphatic amino acids seemed to be more active than those with high molecular weight aliphatic amino acids, such as **BA-02**, **BA-04** > **BA-07**, **BA-08**. In addition, it was observed the **TBA** basic amino acids derivatives seemed to be more active than those acidic amino acids derivatives, such as **BA-10** > **BA-05**. The result also revealed that the **TBA** dipeptide derivatives were more active than the amino acids derivatives. Potency is an important criterion for assessing leads, but it is not the only consideration when selecting a lead compound for further optimization into a drug [27,28]. The selectivity, solubility and hydrophobicity were also essential to the perfect drug candidates, because these properties were closely associated with absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the compounds [29–31]. Thus the compound **BA-10**, **BA-23**, **BA-24** and **BA-25** was selected for further pharmacodynamics and pharmacokinetic evaluation, including the *in vivo* antitumor activity and plasma stability, *in vivo* pharmacokinetics, in the hope of producing drug candidates for drug development. And the results