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Research paper

Synthesis of functionalized new conjugates of batracylin with tuftsin/retro-tuftsin derivatives and their biological evaluation

Wioleta Januchta [a], Marcin Serocki [b], Krystyna Dzierzbicka [a, *], Grzegorz Cholewiński [a], Andrzej Skladanowski [b]

[a] Department of Organic Chemistry, Faculty of Chemistry, Gdansk University of Technology, 11/12 G. Narutowicza Street, 80-233 Gdansk, Poland
[b] Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdansk University of Technology, 11/12 G. Narutowicza Street, 80-233 Gdansk, Poland

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ABSTRACT

New batracylin conjugates with tuftsin/retro-tuftsin derivatives were designed and synthesized using T3P as a coupling agent. The conjugates possess an amide bond formed between the carboxyl group of heterocyclic molecule and the *N*-termini of the tuftsin/retro-tuftsin chain. The *in vitro* cytotoxic activity of the new analogues and their precursors was evaluated using a series of human and murine tumor cells. BAT conjugates containing retro-tuftsin with branched side aminoacid chain, in particular with leucine or isoleucine, were about 10-fold more cytotoxic toward two human tumor cell lines (lung adenocarcinoma (A549) and myeloblastic leukemia (HL-60)). These compounds showed about 10-fold increased cytotoxicity against the two types of tumor cells compared to parent BAT. We have not observed important differences in the mechanism of action between BAT and its cytotoxic tuftsin/retro-tuftsin conjugates. We propose that high biological activity of the most active BAT conjugates is a result of their greatly increased intracellular accumulation.

1. Introduction

Batracylin (8-aminoisoindolo[1,2-b]quinasolin-12(10*H*)-one, BAT) [1] is a heterocyclic amine identified by the drug screening system at the National Institutes of Health (Bethesda, USA) [2]. In preclinical studies, BAT showed high antitumor and cytotoxic activity toward several experimental tumor cell models, including cells which are resistant to standard chemotherapeutics, such as doxorubicin, methotrexate and cisplatin [3–8]. In the recently completed phase I clinical studies, safety profiles of BAT were evaluated in human cancer patients. These studies showed that BAT is well tolerated by human patients up to 400 mg/kg and provided some encouraging data concerning its therapeutic potential [9].

One of the new directions in the synthesis of novel chemical entities with antitumor activity is to combine several different molecules with different functions and/or activities to produce functionalized derivatives [10,11]. The aim of this approach is to obtain compounds with enhanced activity/specificity and improved pharmacologies properties, including increased bioavailability and lowered general toxicity of the conjugate. We recently set out a program aimed at the synthesis of new BAT-tuftsin/retro-tuftsin conjugates which were expected to have improved pharmacological features (such as increased water solubility, lowered general toxicity *in vivo*) and potentially have additional mechanisms of action, including immunostimulatory effect of tuftsin. Tuftsin is a tetrapeptide Thr-Lys-Pro-Arg (TKPR) that been shown to possess immunologic, tumoricidal, and bactericidal activities [12–14]. Accordingly, tuftsin has been successfully used in combination with different antibiotics to treat opportunistic infections caused by bacteria, fungi, and viruses. In addition, it also showed antineoplastics properties [15–32]. Moreover, tuftsin binds to the receptor neuropillin-1 (NRP1) on the surface of cells that participates in several different signalling pathways controlling cell migration and survival [33].

We report here the synthesis of a new series of BAT analogues with tuftsin/retro-tuftsin derivatives containing isopeptide bond between ε-amino group of lysine and carboxyl group of aliphatic amino acids such as Gly, Ala, Val, Leu, Ile. In our method, synthesis of new analogues is based on the modification in the *C*-terminus of the peptide residue by the formation of an amide bond between the

* Corresponding author.
E-mail address: krydzier@pg.gda.pl (K. Dzierzbicka).

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carboxylic group of the respective peptide and the amine group of BAT. We hypothesized that combination of BAT and tuftsin/retro-tuftsin derivatives will allow us to obtain analogues with increased anticancer activity and improved selectivity toward tumor cells. In this paper, we provide data concerning evaluation of the cytotoxic activity as well as other biological effects induced by these compounds in *in vitro* tests and in tumor cells.

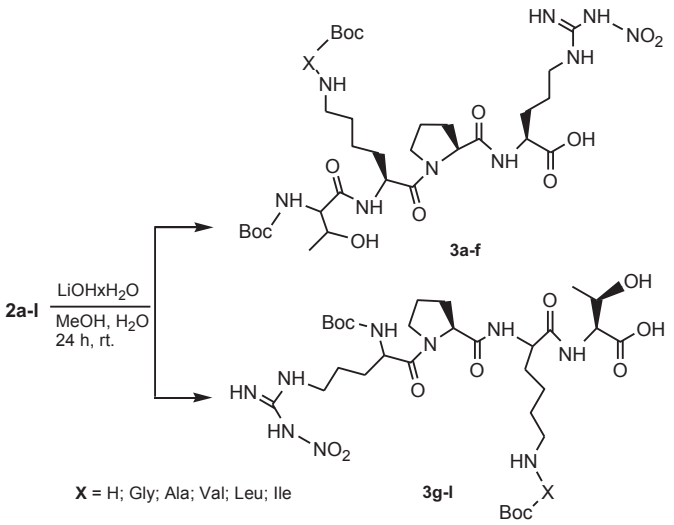
2. Results and discussion

2.1. Chemistry

The synthesis of BAT with tuftsin/retro-tuftsin derivatives was carried out according to reaction presented in Scheme 1, using highly reactive *n*-propanephosphonic acid anhydride (T3P) [34] as a coupling agent in solvent mixture. The condensation between BAT and *C*-termini of tuftsin/retro-tuftsin derivatives **3a**–**l** was achieved during reaction in anhydrous dimethylformamide (DMF) under N_2 for 24 h. T3P (50% solution in DMF) was added to a mixture of **1** and **3a**–**l** in anhydrous pyridine and DMF, and the resulting homogenous solution was held at −15 °C for 4 h. After this time, the reaction was carried out at 45 °C. After 24 h, DMF was evaporated under vacuum. The products **4a**–**l** were purified with preparative TLC, and their identities were confirmed by high resolution ^{1}H NMR (500 MHz) spectroscopy and MALDI-TOF mass spectrometry analysis. The *tert*-butoxycarbonyl (Boc) protecting groups were removed by treatment with HCl in anhydrous Et_2O to give the corresponding hydrochloride as an oil. The presence of final products **5a**–**l** were confirmed by MALDI-TOF mass spectrometry analysis and their purity by HPLC.

BAT **1** was synthesized via modified method based on the Czerniak–Einhorn reaction [20]. In this method, a symmetrically protected 1,4-phenylenediamine derivative has undergone the Czerniak–Einhorn reaction, and after hydrolysis of the protecting groups, BAT **1** was obtained.

The protected tuftsin and retro-tuftsin derivatives **2a**–**l**, tetra- and pentapeptides, were synthesized by the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in anhydrous DMF (Scheme 2) [20–26]. A solution of Boc protected amino acid in anhydrous DMF was cooled to −15 °C and NMM followed by isobutyl chloroformate were added. Five minutes later, amino acid with free carboxyl group or respective peptide in the later stages of a synthesis, neutralized by equivalent amount of NMM or triethylamine (TEA) in anhydrous DMF was added into solution. The reaction mixture was stirred at −15 °C for 4 h then at room temperature for 24 h. After evaporating the solvent *in vacuo*, the crude products were purified by chromatography (SiO_2). Structures of synthesized derivatives **2a**–**l** were established by spectroscopic methods (^{1}H NMR, ^{13}C NMR, MS), optical rotation and melting point.



Scheme 1. Synthesis and chemical structures of BAT analogues **5a-l**.

The Boc protecting groups were removed by treatment with trifluoroacetic acid (TFA). The benzyloxycarbonyl (Z) protecting groups were cleaved by hydrogenolysis (H_2/Pd–C). For recovering free carboxylic group we performed hydrolysis methyl esters of peptides **2a**–**l** (Scheme 3) under mild conditions using LiOH [35] as a reagent.

2.2. Cytotoxic properties

We performed cytotoxic activity testing using different human tumor cells: lung adenocarcinoma (A549), two colon carcinomas (HCT116, HT-29), prostate carcinoma (LNCaP), breast carcinoma (MCF-7), human promyelocytic leukemia (HL-60), as well as two murine leukemias (P388, L1210) and two other murine fibroblast-derived cells (WEHI 1640, NIH-3T3). As presented in Table 1, BAT showed moderate cytotoxic properties, represented by its IC_{50} values between 46.3 μM for A549 lung adenocarcinoma and 90.2 μM for LNCaP prostate cancer. Interestingly, no cytotoxicity was observed for human breast cancer cells (MCF-7) and murine leukemia cells (L1210, P388) even at the highest concentration tested.

For further investigations, we selected two types of tumor cells, which were the most sensitive to BAT from all tumor cells tested i.e. A549 lung adenocarcinoma cells, as a solid tumor and the most sensitive from leukemia cell lines, human promyelocytic leukemia HL-60 cells. Conjugation of BAT with tuftsin alone did not appreciably change the cytotoxicity of the conjugate, compared to the parent compound. In contrast, retro-tuftsin-BAT conjugate was about 2-fold (for HL-60 cells) and about 5-fold (for A549 cells) more cytotoxic that BAT. The majority of BAT conjugates with branched tuftsin or retro-tuftsin were more cytotoxic toward both investigated cell lines than the parent BAT (see Table 2). This was particularly striking for retro-tuftsin conjugates branched with leucine or isoleucine (compounds **5k** and **5l**) which showed about 10 times or more increased cytotoxicity against both tumor cells than the parent compound BAT and its branched BAT-tuftsin analogues. Importantly, branching tuftsin but also retro-tuftsin with other non-polar aliphatic aminoacids (Gly, Ala, Val) had a very variable effect on the cytotoxic activity of BAT conjugates and this was also tumor cell type dependent.

2.3. Effect of studied compounds on the catalytic activity of human type I and II DNA topoisomerases

Previous studies showed that BAT acts as an inhibitor of both type I and II DNA topoisomerases [29,30]. Therefore, we next evaluated whether BAT conjugates with tuftsin and retro-tuftsin, branched with the same chain aminoacids (Leu and Ile) influence the activity of purified DNA topoisomerases. Our results presented on Fig. 1 (upper panel) show that studied compounds did not appreciably inhibit DNA relaxation mediated by type I DNA topoisomerase. Effect of studied compounds on DNA relaxation mediated by topoisomerase IIα enzyme was much more pronounced and drug dose-dependent (Fig. 1, lower panel). In this case, the inhibition of DNA topoiosmerase II correlated with the cytotoxicity toward tumor cells. BAT and its **5f** derivative which showed moderate cytotoxic properties, were able to a certain degree inhibit DNA relaxation *in vitro*, at the concentration 10 μM. In contrast, 100 μM