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Trilobolide-steroid hybrids: Synthesis, cytotoxic and antimycobacterial activity

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ABSTRACT

Sesquiterpene lactone trilobolide is a sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, thus depleting the Ins(1,4,5)P3-sensitive intracellular calcium stores. Here, we describe a synthesis of a series of 6 trilobolide-steroids conjugates (estradiol, pregnene, dehydroepiandrosterone, and testosterone). We found that the newly synthesized Tb-based compounds possess different remarkable biological activities. Cancer cell cytotoxicity and preferential selectivity is represented in our study by a Tb-pregnene derivative. The most cytotoxic clickates of estradiol and pregnene were studied by FACS where impact on cell cycle and RNA synthesis was observed; live-cell microscopy revealed the impact on cell organelle morphology particularly endoplasmic reticulum, mitochondria and nucleus. Further, we have studied the estrogenic and androgenic properties of the clickate molecules using cell-based luciferase assays. Finally, antimycobacterial tests revealed that testosterone and estradiol derivatives potentiated the antimycobacterial activity up to IC50 of 10.6 μM.

1. Introduction

Sesquiterpene lactones (SL) are the active constituents of a variety of plants, frequently used as herbal medicaments. In human cells, these compounds exhibit a number of biological activities mediated via different mechanisms of action, such as alkylation or calcium pump inhibition [1,2]. Trilobolide (Tb) is SL originally isolated from horse caraway *Laser trilobum*, (L.) Borkh [3,4]. In previous work, we demonstrated that the cytotoxicity of Tb is caused by the inhibition of sarco/endoplasmic Ca^{2+}-ATPase (SERCA) [5] similarly to the well-known SL thapsigargin (Tg) [6]. Binding of Tg as well as Tb to SERCA pump antagonizes both Ca^{2+} pumping and uptake by the endoplasmic reticulum (ER). As a result, the elevated concentration of intracellular calcium ions (iCa^{2+}) mediates also an opening of calcium channels in the cell plasma membrane, which facilitates the influx of extracellular calcium ions (eCa^{2+}) inside the cell. These alterations ultimately lead to mitochondrial damage and apoptosis by a cascade of reactions which have not been fully clarified yet [7,8]. In our recent study [5], we revealed the intracellular localization of a fluorescent Tb-bodipy conjugate in ER. On top of that, we have also detected Tb-bodipy induced ER and mitochondria stress which was caused by the elevated calcium levels in cytosol, similarly as was observed in the case of not modified Tb.

The topography of SERCA binding site for Tg has been already described as well as the modifications which do not humper its inhibitory activity [9]. Since we presume the same or closely similar mechanism of action of Tb, there is only one preferentially modifiable position in the Tb skeleton – at C-8 [4]. In one recent study, the importance of an ester moiety at this position, in which ester carbonyl interacts with water molecule in the binding pocket has been reported [10]. Since, the SERCA protein is expressed in all tissues, a modification of SLs resulting in tumor cell-specific uptake is urgently needed. What has riveted the attention lately, is Tg analogue G-202 targeting prostate-specific membrane antigen (PSMA) [11] in glioblastoma and advanced hepatocellular carcinoma,

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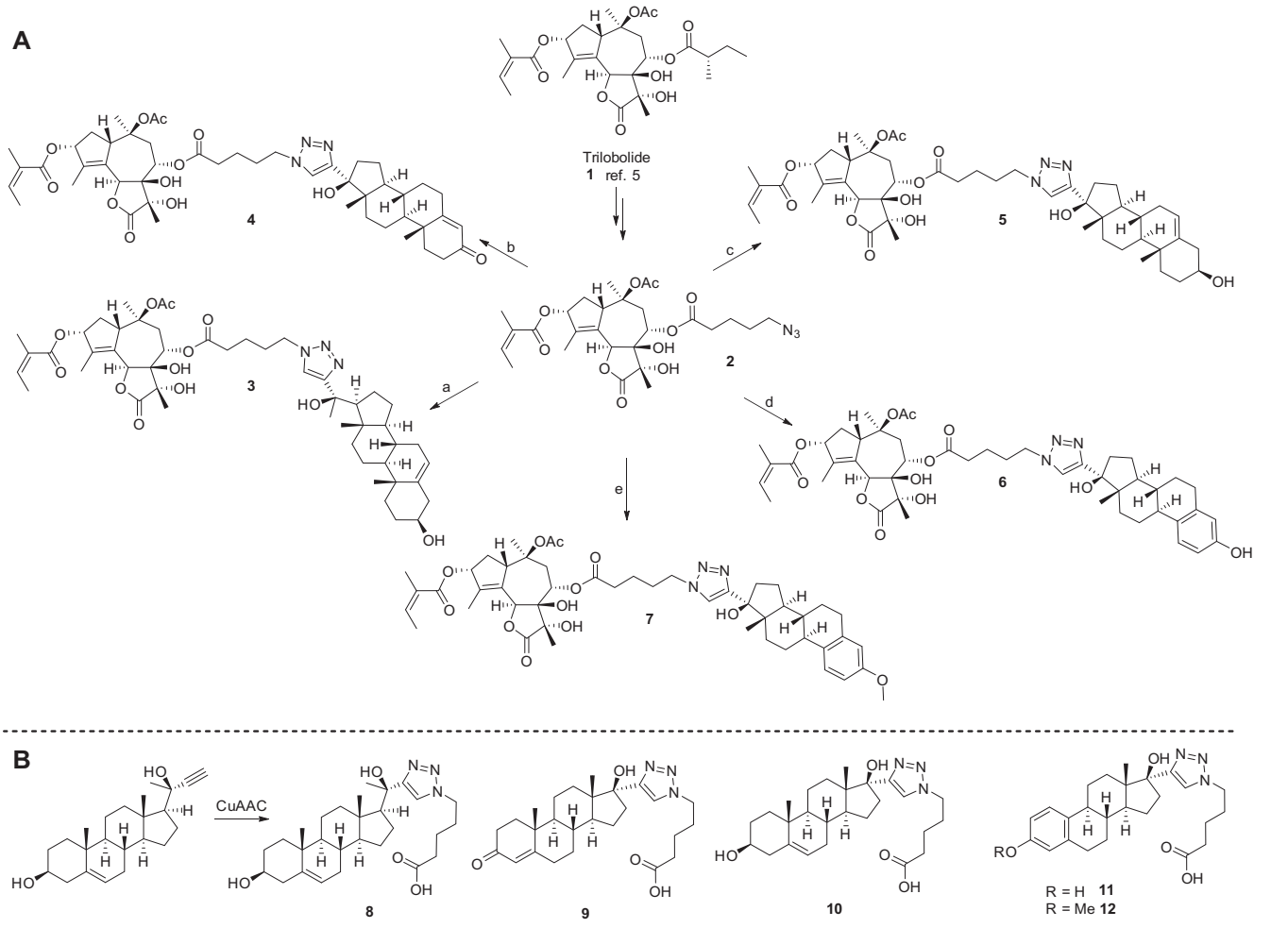
which is now in phase 2 clinical trials. Tb and its analogues have potential to be the next candidates from this class of the compounds for cancer therapy. Therefore, our group is focused on diversity oriented synthesis (DOS) based on Tb. From our previous investigations, we found that Tb-8-*O*-azidovalerate [5] retains the activity of Tb and since the molecule contains an azido group, it is useful for introduction of other moieties using the click chemistry (known as CuAAC reaction) approach. In this article, we present a series of Tb hybrids comprising a bulk steroid moiety. This conjugation is the key factor diversifying the transport and selectivity within different cancer cell lines. We synthesized and properly characterized a miniseries of Tb hybrids composed of selected steroids and Tb-8-*O*-azidovalerate *via* CuAAC. The steroid motifs used, such as estradiol, testosterone, dihydroepiandrosterone (DHEA) and pregnene are originally biologically active, but generally with low toxicity, therefore steroid-sensitive cancer cells might preferably accept these molecular compositions. In order to monitor the differences among the Tb hybrids (compd. **3**–**7**) and the steroid moiety attached, we synthesized also free steroid acids (see Fig. S1 in Supplementary). *In vitro* cytotoxicity of all newly synthesized compounds was examined using WST-1 and MTS assays across 12 cancer cell lines, two fibroblast cell lines and peritoneal rat macrophages. We also tested bacteriostatic and bactericidal activity of the compounds on 8 sensitive and multiresistant bacterial, mycobacterial and yeast strains. Further, the potency of the Tb-conjugates to modulate the transcriptional activity of estrogen (ER) and androgen receptors (AR) was assessed. The most promising compounds were further tracked using FACS for the cell cycle analysis and by live-cell fluorescence microscopy in order to ascertain their impact on cell organelle morphology.

2. Results and discussion

2.1. Chemistry

Cycloaddition of ethinylated steroids (24-norchol-5-en-22-yn-3β-ol, 17α-ethinyltestosterone, 17α-ethinylestradiol, 3-*O*-methyl-17α-ethinylestradiol, 17α-ethinyl-DHEA, see Fig. S1 in Supplementary) and trilobolide-8-*O*-azidovalerate [5] (**2**) was carried out using click chemistry protocol [12,13]. We employed simple procedure with $CuSO_4 \cdot 5H_2O$, sodium ascorbate and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMF under microwave conditions (see Scheme 1, part A). The Tb-steroid hybrids (**3**–**7**) were isolated in good to excellent yields (Section 1.2 in Supplementary). As controls, free steroid acids were synthesized following the procedures described above (compd. **8**–**12**, for chemical structures see Scheme 1, part B). All newly synthesized compounds were properly characterized by NMR (^{1}H, ^{13}C-APT, HMQC, HMBC, COSY, (see Supplementary, Section 4), HRMS, IR and optical rotation (Section 1.2 in Supplementary). Before biological testing, the samples were repurified once and the purity of the test compounds



Scheme 1. Part A. Synthesis of steroid-Tb clickates. (a) 24-norchol-5en-22-yn-3β-ol; (b) 17α-ethinyltestosterone; (c) 17α-ethinyl-DHEA; (d) 17α-ethinylestradiol; (e) 3-*O*-methyl-17α-ethinylestradiol; *Click chemistry – a general procedure:* $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, DMF, MW, 50 °C, 1 h. Part B. Steroid acids synthesized via CuAAC approach.