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Trilobolide–porphyrin conjugates: On synthesis and biological effects evaluation

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ABSTRACT

Trilobolide (Tb), a potent natural counterpart of thapsigargin, is a sesquiterpene lactone of guaianolide type isolated from horse caraway (*Laser trilobum*, L. Borkh). Tb exerts remarkable pharmacological properties based on irreversible inhibition of sarco/endoplasmic reticulum calcium ATPase (SERCA), thus being of increasing interest for cancer cure. Additionally, another pharmacological activity of Tb, as well as of thapsigargin, was reported in several studies, Tb as being an effective inductor of nitric oxide and cytokine production. These extraordinary biological properties move these molecules in further pre-clinical evaluation.

Because of ubiquitous character of SERCA expression, development of specifically targeted bioactive molecules is inevitable. Since it is well known that porphyrins are preferentially taken up by cancer cells, we have designed and synthesized novel Tb–porphyrin conjugates. Copper-catalyzed azide–alkyne cycloaddition was used to link Tb with porphyrin at once. Two model conjugates of Tb and porphyrin were synthesized and properly characterized. Employing naturally occurring fluorescence properties of porphyrins, we investigated the intracellular localization of the conjugates employing fluorescence microscopy in living cells. Intriguingly, the prepared conjugates localized both in mitochondria and lysosomes of HeLa and LNCaP cells. Furthermore, the cytotoxicity of Tb–porphyrin conjugates was assessed in a number of human cancer cell lines and rat peritoneal cells. Likewise in cancer cell lines, viability of rat peritoneal cells was not affected by the tested conjugates. Interestingly, we observed dose-dependent nitric oxide (iNOS) production induced by the tested conjugates. The effect was related to the type of a linker used and the overall size of the molecule. Another potent immunobiological effects are under evaluation.

In summary, the results presented here indicate notable immunobiological potential of the prepared Tb conjugates. Moreover, they could help to decipher the molecular mechanism of Tb for its possible biomedical applications.

1. Introduction

Porphyrins are a class of naturally occurring compounds which have become intensively studied in last decades due to their unique photochemical properties. The ability of porphyrins to passively target tumors *in vivo* enables their utilization in cancer therapy and diagnostics [1]. The passive tumor targeting by porphyrins is based on enhanced retention and permeability effect of solid tumors [2]. Photodynamic therapy (PDT) is a non-invasive therapeutic approach based on a use of light-sensitive molecules, photosensitizers. PDT is used worldwide for treatment of a number of diseases, including age-related macular degeneration, psoriasis and cancer [3–8]. Moreover, the photophysical properties of porphyrins allow visualization of their localization as well as of their conjugates both *in vitro* and *in vivo*. Conjugation of the macrocycle with counterpart of choice facilitates its transport via drug- or receptor-mediated endocytosis, affects delivery to a specific location within the cells and generally improves biological effects. Therefore, many drugs based on porphyrins are designed as prodrug systems to enhance their physico-chemical and pharmacokinetic properties [9]. Many groups have focused on conjugation of porphyrins with other biologically active compounds, such as

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saccharides [10,13], peptides [12,14], steroids [15] and polymers [16]. Conjugation is also responsible for specific accumulation in cells [10], which determines biological effects of the conjugate. Mitochondria [11], endoplasmic reticulum [10] and nucleus [12] are believed to be the main organelles for the pharmacotherapeutic intervention. Unfortunately, until now most of the conjugates accumulate in endosomes and lysosomes, as it was described for porphyrins conjugated to saccharides [10,13], peptides [14], dendrimers [15,16], and retinoids [17].

In this study, we present synthesis of two porphyrin conjugates containing a sesquiterpene lactone, trilobolide (Tb). Kmoníčková et al. [18] reported immunostimulatory properties of Tb, which is able to induce interferon gamma (INF-γ) secretion and nitric oxide (NO) release [18]. Another pharmacological feature of Tb is its potency to inhibit sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). The SERCA inhibition leads to accumulation of calcium ions in cytosol, and a sustained increase of Ca^{2+} induces apoptotic pathway and cell death. [19]. We imaged the transfer of the prepared compounds in cells of various tumor origins. Biological activities of the bioconjugates, such as induction of NO release in rat macrophages have also been evaluated. Moreover, we assessed cytotoxic potency of Tb–porphyrin conjugated in cancer cell lines of various histogenic types.

2. Results and discussion

2.1. Chemistry

We chose copper-catalyzed azide-alkyne cycloaddition [20,21] (CuAAC) as the key reaction of the designed synthesis. Thus, both parts of the conjugate, Tb and porphyrin, were modified to provide intermediates suitable for click chemistry. The chemical transformation of Tb is displayed in Scheme 1 and all of the experimental synthetic details are described in Supplementary Section 1. We synthesized the carboxymethyloxime (CMO) derivative **1c** in three steps. Briefly, Tb was transformed to its demethylbutanoyl derivative (**1a**) by mild solvolysis and successively the sole secondary hydroxy group was oxidized using pyridinium chlorochromate (PCC). Obtained 8-oxo-Tb derivative **1b** was transformed to Tb–CMO **1c** by the reaction with *O*-(carboxymethyl)hydroxylamine hemihydrochloride under pyridine catalysis. Finally, we introduced terminal alkyne moiety by the reaction of **1c** with propargyl alcohol in presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC). Obtained propargylester **1d** was used in subsequent click reaction.

The synthesis of 5-(4-carboxyphenyl)-10,15,20-triphenylporfyrin derivatives is displayed in Scheme 2. First we prepared the basic porphyrin according to a previously described method [22], see Supplementary information 1.2.5 and Scheme S1. Thereafter, we introduced azido PEG_3–amine and propargyl moiety into the porphyrin molecule using EDC chemistry (see Scheme 3).

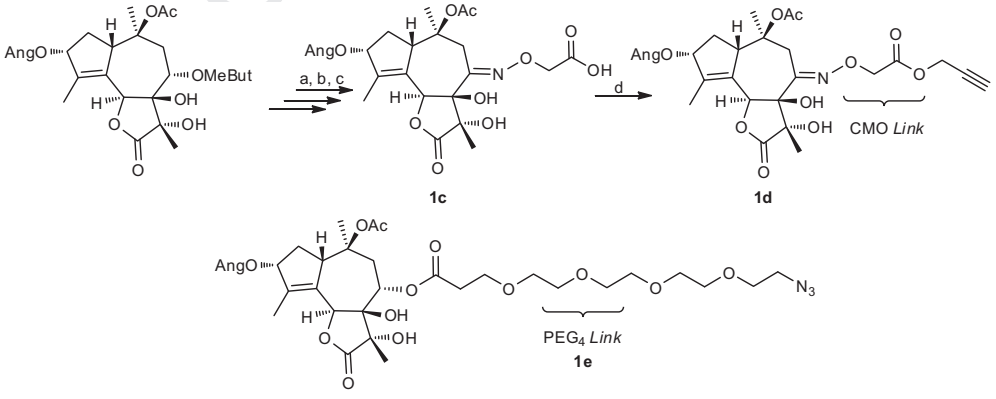
Desired Tb–porphyrin conjugates were prepared using standard click chemistry protocol with CuI and Tris[(1-benzyl-1*H*-1,2,3-triazolyl)-methyl]amine (TBTA) [23] as an accelerator of the product formation. The analytical data including NMR, IR, HRMS, and optical rotation characteristics are described in Supplementary Section 1.2. Both conjugates showed absorbance maxima at 424 nm (Soret band) and emission maxima at 605 and 655 nm (excitation wavelength of 429 nm; see Supplementary 1.3 and Fig. S1), which are typical porphyrin spectral properties. Prior to biological testing, all samples were re-purified by column chromatography and their purity was checked by chromatographic methods.

2.2. Intracellular localization of Tb–porphyrin conjugates

After successful synthesis of Tb–porphyrin conjugates, we studied their intracellular localization in several human cancer cell lines: HeLa, LNCaP, U-2 OS, MCF-7, and MiaPaCa-2. We performed live-cell fluorescence microscopy of these red-emitting conjugates (**3** and **4**) for time intervals ranging from 20 min up to 24 h. Detectable fluorescent emission intensity of the tested derivatives **3** and **4** was observed only after 2 h of incubation with the model cell lines. Thus, the kinetics of Tb–porphyrin conjugates was significantly decreased when compared with the rapid intracellular uptake of Tb-Bodipy occurring already after 20 min of incubation with human cancer cells reported by Jurášek et al. [24]. In HeLa cells, Tb–porphyrin **3** (5 μM) localized in network-like patterned organelles, probably of mitochondrial origin, after 2 h of incubation. The localization of **3** did not change over prolonged incubation periods of 5 h (see Fig. 1), 16 h, and 24 h. Compound **4** (5 μM) was distributed in vesicles of endosomal or lysosomal origin in HeLa cells after 2 h and the intracellular distribution was retained at least up to 24 h. Compound **4** occurred partially also in large fluorescent aggregates probably caused by decreased water solubility. We have observed similar behavior of both compounds in living prostatic cancer cells (LNCaP), data shown in Supplementary Section in Fig. S2, and in human osteosarcoma cells (U-2 OS, see Fig. S3), the only difference was more pronounced aggregation of compound **4**.

2.3. NO production in primary macrophages

Within a group of sesquiterpene lactones, Tb possesses strong activity in stimulating nitric oxide (NO) production by immune



Scheme 1. Synthesis of functionalized Tb via carboxymethyloxime derivative. Reagents and conditions: (a) Et_3N, MeOH, 17 h, rt, yield 69%; (b) PCC, CH_2Cl_2, rt, 2.5 h, yield 74%; (c) *O*-(carboxymethyl)hydroxylamine hemihydrochloride, pyridine, MeOH, rt, 70 min, yield 89%; (d) propargyl alcohol, EDC, HOBt, DMAP, CH_2Cl_2, 0 °C → rt, 12 h, yield 86%. Compound **1e** was available in our laboratory.

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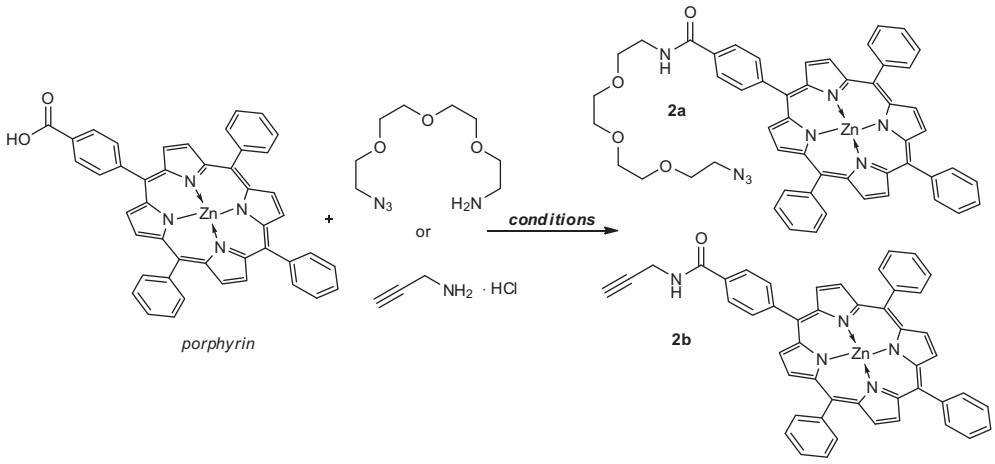
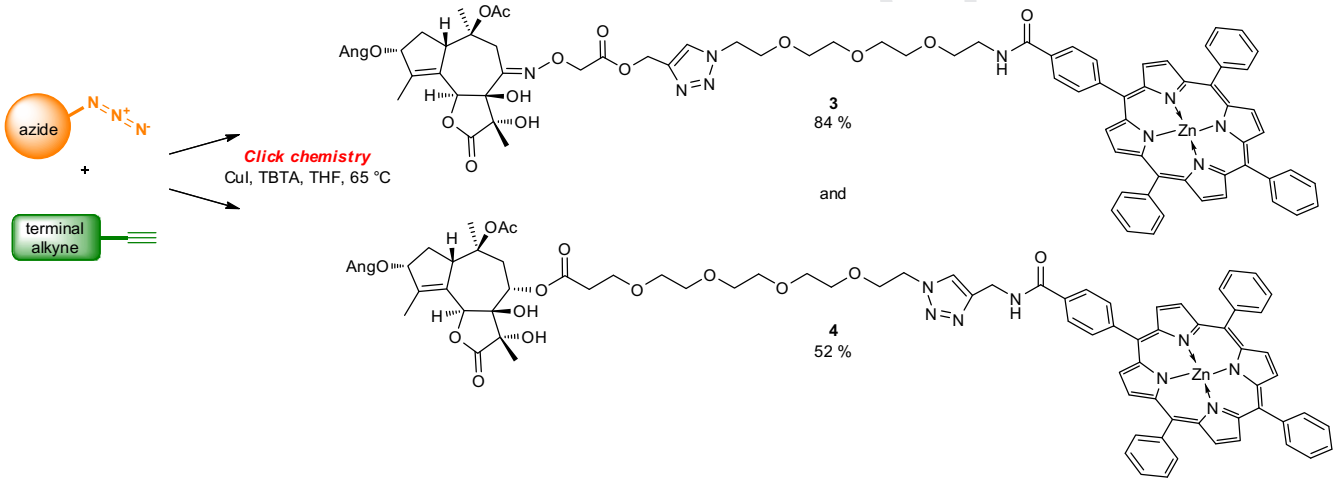


Scheme 2. Synthesis of functionalized porphyrin. Reagents and conditions: EDC, DMAP, HOBt, CH_2Cl_2, 0 °C → rt, 15 h, yield for **2a** 55% and for **2b** 84%.



Scheme 3. Synthesis of Tb–porphyrin conjugates via CuAAC reaction.

cells [25]. Based on this, we investigated nitric oxide release induced by Tb–porphyrin conjugates (Fig. 2). We found different effects of compounds tested in range of 0.01–100.0 μM. The highest NO release was induced by Tb–CMO derivative (**1d**). Also, the potency of compound **1d** with CMO link is closer to Tb itself than to **1e** containing PEG_4 link. The ability of derivatives **1d** and **1e** to induce NO production started at concentration of 1 μM and continued up to 40 μM concentration. It would be easy to simply conclude that also Tb–porphyrin conjugates **3** and **4** possessed an immunological activity even though weaker in comparison to Tb itself or Tb non-fluorescent derivatives. But together with these findings, we mentioned that porphyrin alone in concentration 10–100 μM was able to release NO to supernatant in macrophages. In fact, this result is probably caused by physico-chemical properties of porphyrin. Standard analysis using Griess reagent was applied to measure nitrite concentration. This spectrophotometric method is routinely used to assess NO production; absorbance is recorded at 540 nm. Unfortunately, this wavelength coincided with the absorption spectra of the tested porphyrins. Q-bands of Tb–porphyrin conjugates **3** and **4** in visible spectra overlap with the absorbance wavelength (540 nm) for NO detection. Other than spectrophotometric methods used for NO detection in cell-free samples are just under evaluation.

The effect of naturally occurring zinc protoporphyrin (ZnPP) was studied with respect to inducible HO-1 (heme oxygenase) gene expression and activity [26,27]. ZnPP acts as competitive inhibitor of HO-1. HO-1 is known to work opposite to iNOS and both enzymes are studied in pro-inflammatory and anti-inflammatory conditions. It is interesting that no article has mentioned possible interference of ZnPP with measurement of nitrite concentration using Griess reagent recorded at 540 nm, so far. Chow et al. [26] and Li et al. [27] conclude that ZnPP directly inhibits inflammatory iNOS expression (inducible nitric oxide synthase)/NO production in macrophages. However, we noticed that Tb–porphyrin conjugates **3** and **4** induced slight up-regulation of NO production stimulated by lipopolysaccharides (LPS, 1000 pg mL^{-1}) in rat macrophages. More detailed study is needed to thoroughly explain biological activities of porphyrin conjugates and the role of porphyrins in immune cells, as well as Tb itself in their estimated pharmacological applications [28].

2.4. Cytotoxicity assays

The Tb–porphyrin conjugates were tested for *in vitro* cytotoxicity against rat peritoneal cells and four human tumor cell lines: LNCaP (prostate carcinoma), U-2 OS (osteosarcoma), MCF-7 (breast carcinoma), and MiaPaCa-2 (pancreatic carcinoma). The cytotoxic activity was determined using WST-1 assay based on mitochondrial activity causing conversion of tetrazolium salt into a colored product, formazan. The results are presented in Supplementary

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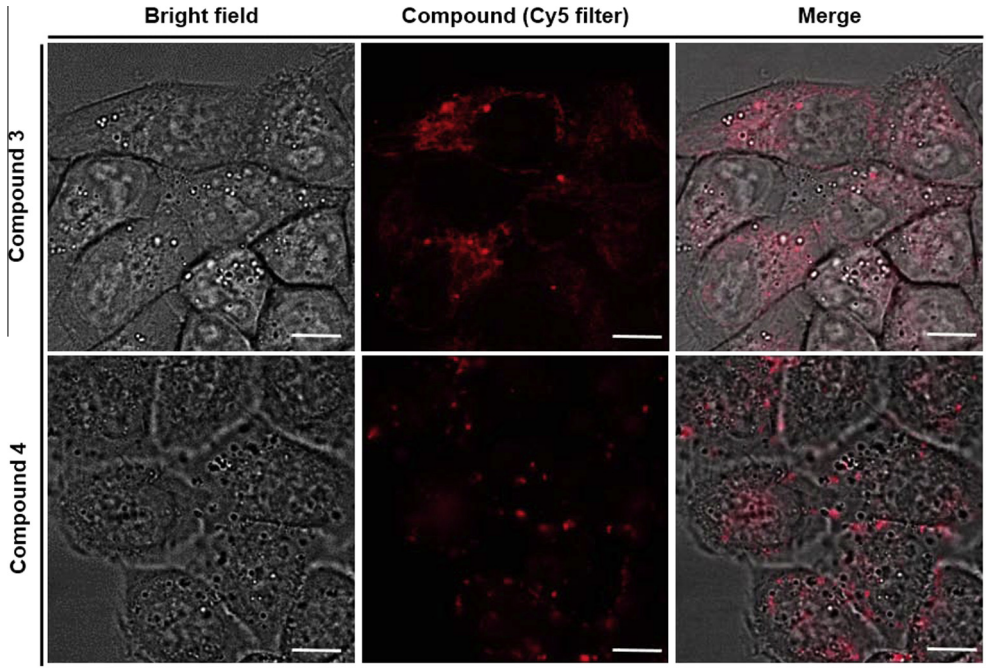

Fig. 1. Intracellular localization of Tb–porphyrin conjugates **3** and **4** in human cervix cancer cells (HeLa). Fluorescence microscopy images of living cells incubated with 5 μM concentration of compounds **3** and **4** for 5 h. Individual columns represent: bright field, fluorescence emission of Tb–porphyrin conjugates (Cy5 filter) and merge, respectively. The scale bar corresponds to 10 μM.

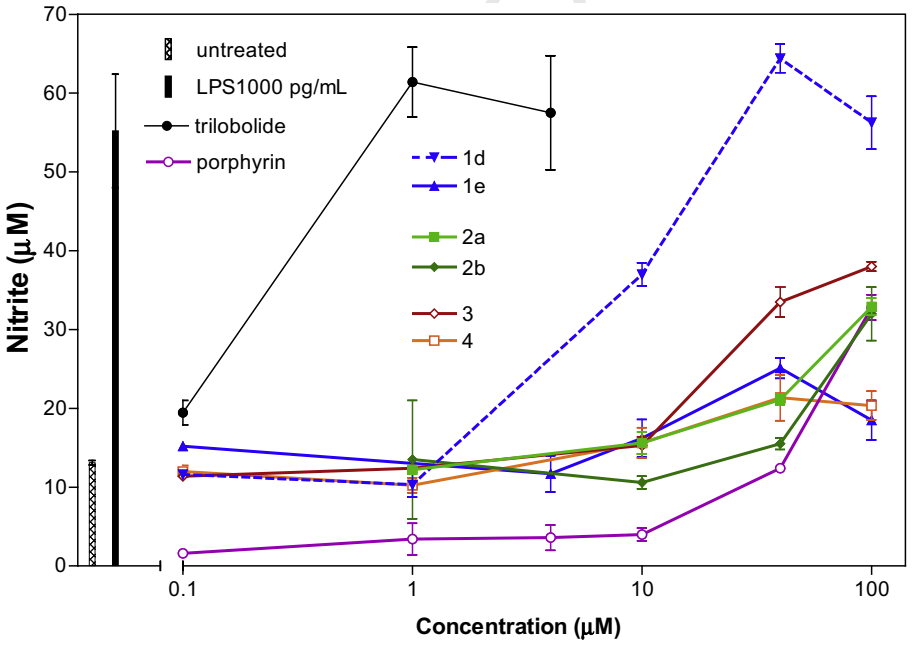

Fig. 2. NO production in rat peritoneal cells induced by Tb and its derivatives. The cells were treated with Tb derivatives (**1d**, **1e**), porphyrins (**2a**, **2b**) and with Tb–porphyrins (**3**, **4**) for 24 h. For evaluation of potential effects of Tb–porphyrin conjugates, Tb and lipopolysaccharide (LPS, 1000 pg mL^{-1}) were used. The results represent the mean ± SEM of 2 independent experiments, $n = 4$.

Section in Fig. S4. Interestingly, we found that compounds **3** and **4** were not cytotoxic after 24 h and 48 h of incubation with the model cell lines. The IC_{50} value was not reached in any of the tested cell lines up to 50 μM concentration of the studied conjugates. Similar data for compounds **3** and **4** were found in primary rat peritoneal cells measured after 24 h (data not shown). Thus, the innate original cytotoxicity of pristine Tb reported by Kmoníčková et al. [18] was not retained upon porphyrin conjugation. It might be caused by steric reasons and inefficient membrane penetration in contrast to the original molecule in immune cells and cancer cell lines as well.

3. Conclusion

In conclusion, this is the first report on conjugation of a sesquiterpene lactone trilobolide with a red-emitting porphyrin moiety.

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Conjugates **3** and **4** were prepared in good yields and were thoroughly characterized. On the basis of previously reported immunostimulatory activities of a pristine Tb, we evaluated immunobiological potency of the prepared Tb–porphyrin conjugates in rat macrophages as a measure of NO production. The more pronounced NO production was induced by Tb-derivative **1d** than by Tb–porphyrin conjugates in comparison with Tb. Moreover, these compounds did not exhibit cytotoxic effect *in vitro* in a number of model cell lines, such as primary macrophages, LNCaP, MCF-7, U-2 OS, and MiaPaCa-2. The IC_{50} value was not reached up to 50 μM concentration of the tested compounds. Utilizing the inherent fluorescence properties of porphyrins, we examined intracellular localization of the Tb–porphyrin conjugates in living cells in real time. Interestingly, the compounds **3** and **4** localized in mitochondria and lysosomes of the model cancer cell lines in 5 μM concentration already after 2 h. Further biological analysis is needed for differentiation between physico-chemical properties and possible immunobiological effect (NO production) of porphyrin and its conjugates in macrophages.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.08.024.

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