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Tailor-Made Fluorescent Trilobolide To Study Its Biological Relevance

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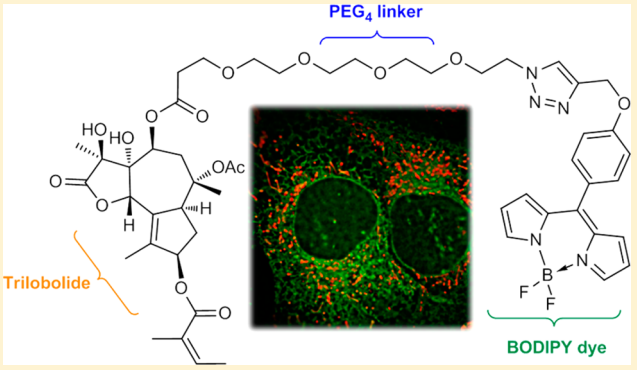
Supporting Information

ABSTRACT: Trilobolide (Tb) is a potent natural counterpart of thapsigargin, which has shown promising results in cancer clinical trials. Here, we report a rational approach to study intracellular localization and biological activity of this sesquiterpene lactone. We conjugated Tb with a green-emitting Bodipy dye attached by alternative linkers of different lengths. The live-cell imaging of the prepared bioconjugates brought clear evidence that Tb–Bodipy localized in the endoplasmic reticulum (ER) of various cancer cell lines. The localization signal was compared with ER-specific dyes. Cytotoxicity of Tb conjugates and impact on the mitochondrial physiology and nitric oxide release were also studied. The nitric oxide production and cytokine secretion in rat peritoneal cells indicate immunobiological potential of these lactone bioconjugates. In summary, our Tb–Bodipy conjugates could help us to reveal the molecular mechanism of trilobolide for its further potential use in biomedical applications.



INTRODUCTION

Trilobolide (Tb), a structurally related compound to intensively studied thapsigargin (Tg), is a sesquiterpene lactone isolated from *Laser trilobum* (L.) Borkh. Previous studies have revealed that Tb is an effective inducer of nitric oxide (NO) production as well as of cytokines.[1] Both lactones cause striking expression of interferon γ (INF-γ) cytokine in human and rat macrophages. Further, it has been reported that stimulatory potential of these lactones depends on activation of MAP kinases p38, ERK 1/2, and transcription factor NF-κB.[2,3] It is generally accepted that these biological effects are most likely caused by the presence of 7,11-dihydroxyguaianolide skeleton and fixed acyl substituents at positions C-3, C-8, and C-10 (ref 4) in both Tb and Tg. Another attractive pharmacological feature of these substances is their inhibition of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). The topography of SERCA binding site of Tg was described by Skytte et al.[5] It is assumed that Tb interacts in a similar way due to the high similarity to Tg (there are only minor structural differences). Empirical structure–activity relationship (SAR) studies of Tg indicate that chemical modifications at O-8 and O-2 positions do not hamper its biological activity. However, Tb lacks the octanoyl moiety present in Tg at C-2 position; therefore, derivatization of Tb molecule remains amenable merely at O-8. Solvolysis of (*S*)-methylbutanoate yields a "triol" which lacks the SERCA inhibitory activity.[5] The subsequent esterification of this product results in a regioselective insertion of an acyl group to preferred O-8 position. This partially restores the original activity. Because SERCA protein is present in all body tissues, targeted delivery based on a Tb-based prodrug form must be developed. This way, the tumor cells should be specifically recognized and killed by the activated inhibitor. In order to characterize the cellular uptake and intracellular localization of Tb for further rational design of effective drug forms, we focused on a preparation and testing of variously modified fluorescently labeled Tb. We altered the length of a linker between Tb and a fluorescent tag that could be replaced with the prodrug counterpart of choice. We synthesized a series of differently linked Tb with a green-emitting Bodipy (boron dipyrromethane) dye, since it is a relatively small, membrane permeable dye often used for labeling of lipoid compounds.[6,7] With the prepared compounds we imaged their distribution in cells of various tumor origins and confirmed the expected intracellular localization in the endoplasmic reticulum (ER). The biological activities of Tb and its novel bioconjugates such as induction of NO release and INF-γ production in rat macrophages and cytotoxic potency across the cancer cell lines of different histogenic origin have been also evaluated.

RESULTS AND DISCCUSSION

Design and Synthesis of Tb–Bodipy Conjugates. A sustainable and straightforward method to obtain fluorescent

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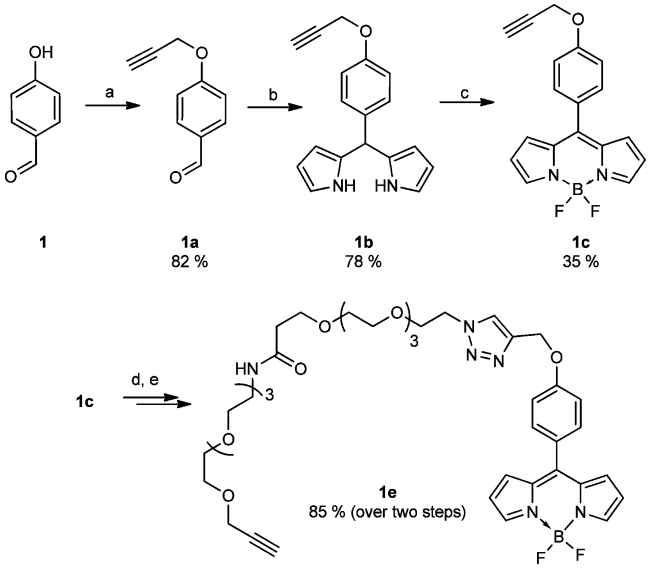
Tb derivatives is the Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction[8,9] taking place between terminal alkynes and azides ("click chemistry", CuAAC). Thus, both Tb and Bodipy dye were tailored to intermediates suitable for click chemistry. Generally, Tb was derivatized with linkers of various lengths containing an azido moiety and a Bodipy dye with a terminal acetylene group.

The synthesis of the Bodipy dye started with *p*-hydroxybenzaldehyde (**1**) which was propargylated[10,11] (**1a**) and transformed to dipyrrole (**1b**) and subsequently to the Bodipy dye (**1c**) according to a previously described method[12,13] (for further details see Supporting Information, section 1.2). Polyethylene glycol (PEG) handles were appended via click chemistry to further extend the variability of linker's length (see Scheme 1).

Scheme 1. Synthesis of Bodipy Dyes[a]



[a]Reagents and conditions: (a) propargyl chloride, K_2CO_3, DMF–toluene, 72 h, 60 °C; (b) (i) pyrrole, TFA, 3.5 h, rt; (ii) K_2CO_3, 30 min; (c) (i) DDQ, $CHCl_3$, 45 min, rt; (ii) BF_3 Et_2O, Et_3N, 3 h, rt; (d) azidoPEG$_4$-acid, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, DMF, microwave, 60 °C, 1 h; (e) acetylenePEG$_4$-amine, EDCI, DMAP, HOBt, CH_2Cl_2, 24 h, 0 °C → rt.

Trilobolide isolated from roots of *Laser trilobum* (L.) Borkh as a white crystalline powder was used as a starting material for the described syntheses.[14] The available data published on a closely related sesquiterpene lactone of Tb, i.e., Tg, show that substitution at O-8 is the most effective option preserving the biological potency of Tb.[5] Solvolysis of trilobolide methylbutanoate at the O-8 position was carried out using *N*,*N*,*N*-triethylamine in methanol according to a previously described method.[5,10,15] Selective acylation of triol **2a** (see Scheme 2) at the same position was accomplished by reaction with organic acids containing azido moiety in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) and *p*-*N*,*N*-dimethylaminopyridine (DMAP) in dimethylformamide (DMF). This way, we synthesized trilobolide-8-*O* azidovalerate (**3a**), azidoPEG$_4$-carboxylate (**5a**), and azidoPEG$_9$-carboxylate (**5b**). Azidoacetate (**4b**) was obtained from bromoacetate (**4a**) by a nucleophilic substitution using sodium azide (see Scheme 2). The yields of the products were inversely related to increasing length of the appended linker. All azido derivatives were fully characterized by NMR, HRMS, IR, and specific rotation (see Supporting Information section 1.3).

Synthesized Tb-azido derivatives were introduced into CuAAC cycloaddition with the prepared Bodipy dyes. All of click reactions were catalyzed by $CuSO_4 \cdot 5H_2O$. Sodium ascorbate served as a reducing agent, and the product formation was accelerated by catalytic amount of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA).[16] The reactions were carried out in DMF under microwave irradiation at 60 °C for 1 h. All the prepared trilobolide–Bodipy conjugates (**4c**, **3b**, **6a**, **6b**, and **7**) were obtained in ≥95% purity. The spectral characteristics and synthetic details are described in Supporting Information, section 1.3, and fluorescence emission spectra are presented in Figure S1. Prior to biological testing the samples were repurified by short column chromatography. The purity was verified by HPLC (see Supporting Information, section 1.4).

Cellular in Vitro Studies. The following in vitro experiments were performed to evaluate the potential biological activity of the compounds prepared in this work: live-cell imaging of cellular uptake and intracellular localization of Tb–Bodipy derivatives, cytotoxicity assay (WST-1), NO release, and cytokine production.

Live-Cell Imaging of Tb–Bodipy Localization in Cancer Cells. To elucidate the intracellular localization of Tb, its fluorescent conjugates (**3b**–**7**) with Bodipy dye were prepared. This type of fluorophore has already been successfully applied to determine in vitro subcellular localization of a thapsigargin derivative.[17,18] Fluorescent Bodipy dyes commonly employed for staining of lipid droplets[19,20] are nontoxic. The intracellular localization of Tb–Bodipy conjugates was examined in a number of human cancer cell lines, such as U-2 OS (osteosarcoma), PC-3 and LNCaP (prostatic carcinomas), MCF-7 (breast ductal carcinoma), HeLa (cervix carcinoma), MiaPaCa-2 (pancreatic carcinoma), A549 (lung carcinoma) cells, one mouse cancer cell line, C2C12 (myoblastoma), and HEK 293T (human embryonic fibroblasts). Cellular uptake of the tested derivatives was followed by means of fluorescence microscopy in living cells at a number of time points: 10, 20, and 40 min and 1, 3, 5, and 24 h. Interestingly, we found that only compounds **3b** and **6a** efficiently localized inside the cells (see Figure S2 (U-2OS cells) in Supporting Information). We observed stable localization of fluorescence signal of these two Tb derivatives (1 μM) already after 20 min of incubation with all the tested cell lines. We have found almost no fluorescence signal in any of the tested cell lines for conjugates **4c**, **6b**, and **7** used at 5 μM concentration even after 24 h of treatment (see Supporting Information Figure S2). This might be due to the extensive size of **6b** and **7**, which probably did not penetrate through the cell plasma membrane. The lack of the fluorescence signal of Tb conjugate **4c** could be caused by too short distance between both counterparts (Tb and Bodipy dye) and/or by prompt clearance from cells. Cells stained only with Bodipy dye were used as a control to exclude the possibility that the localization of the Tb–Bodipy conjugates inside the cells was driven by the Bodipy dye itself. We observed localization of Bodipy dye in living cells in spherical particles, which correspond to cellular lipid droplets. This observation is in coherence with Sook et al.[19] and with the fact that Bodipy is broadly employed for lipid droplets labeling. See example of a Bodipy dye localization in U-2 OS cells in image L of Figure S2 in Supporting Information.

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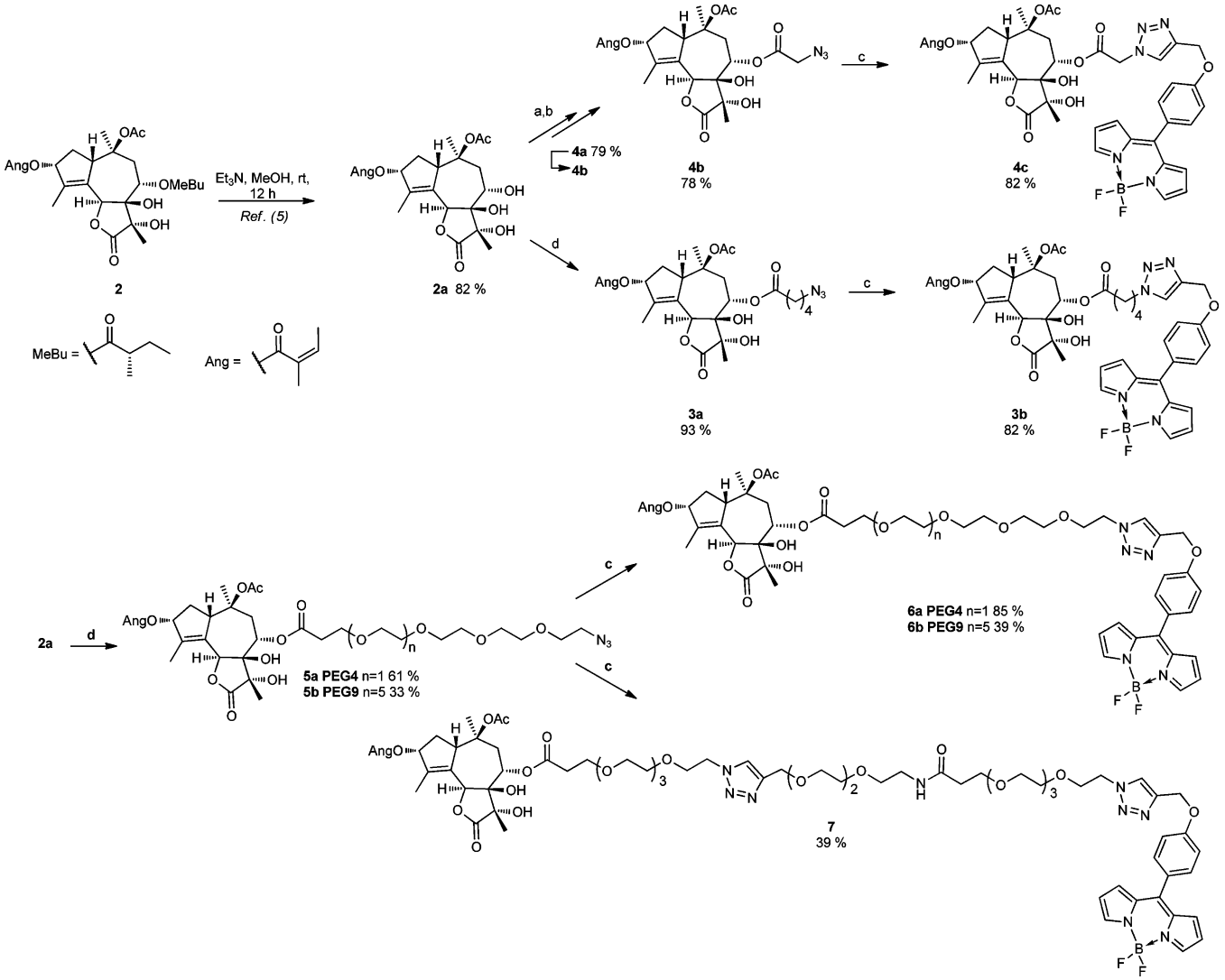
Scheme 2. Synthesis of Trilobolide–Bodipy Conjugates[a]



[a]Reagents and conditions: (a) bromoacetyl bromide, DMAP, CH_2Cl_2, 0 °C, 30 min; (b) NaN_3, DMF, 55 °C; (c) Bodipy **1c** (compounds **4c**, **3b**, and **6a**)/Bodipy dye **1e** (compound **7**), $CuSO_4 \cdot 5H_2O$, sodium ascorbate, TBTA, DMF, microwave, 60 °C, 1 h; (d) 5-azidovaleric acid (3a)/azidoPEG$_4$-acid (**5a**)/azidoPEG$_9$-acid (**5b**), EDCI, DMAP, DMF, rt.

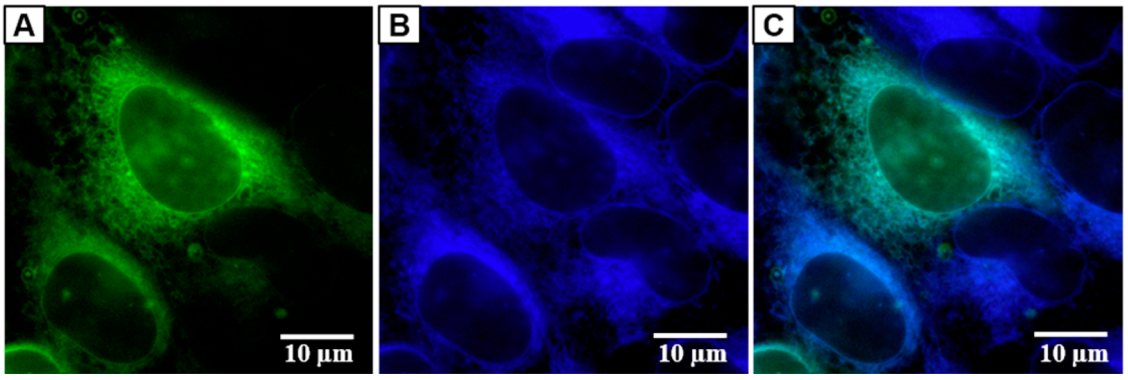


Figure 1. Trilobolide–Bodipy **6a** localization in the endoplasmic reticulum of human osteosarcoma cells (U-2 OS). Shown are fluorescent live-cell images of the endoplasmic reticulum labeled by (A) compound **6a** (1 μM, 1 h), (B) ER-Tracker Blue-White DPX (100 nM, 20 min), (C) merge.

Interestingly, the fluorescence signal of **3b** and **6a** in U-2 OS started to slowly vanish after incubation longer than 40 min; see Figure 1 (strong fluorescence signal of **6a** was apparent only in few cells). We observed this phenomenon only in U-2 OS cells but not in other tested cell lines, as it can be seen from the example in Figure S3 in Supporting Information, where localization of **3b** and **6a** is depicted in two prostatic cancer cell lines, PC-3 and LNCaP, and one pancreatic cancer cell line,

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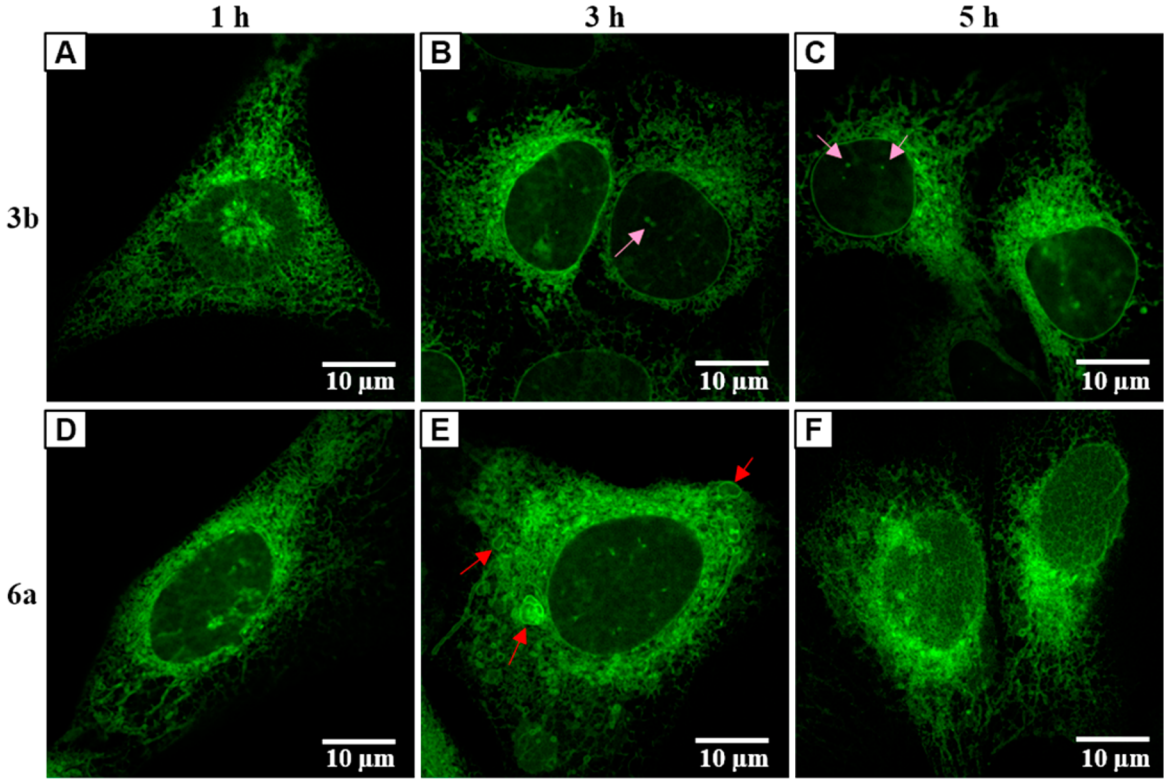

Figure 2. Time-dependent localization of trilobolide–Bodipy (1 μM, 1 h) derivatives in human osteosarcoma cells (U-2 OS). Fluorescent live-cell images of U-2 OS were taken after 1, 3, and 5 h of incubation (in columns). Derivatives **3b** and **6a** are in the upper and bottom rows, respectively. Red arrows depict stressed and damaged the endoplasmic reticulum (notice small pouches), and pink arrows depict numerous of nuclear speckles.

MiaPaCa-2. In Figure S4 in Supporting Information, localization of Tb–Bodipy derivative **3b** is depicted in C2C12 (mouse myoblastoma cells), HEK 293T (human embryonic kidney cells), HeLa (cells from cervical carcinoma), and A549 (lung carcinoma cells). This difference between the cell lines might be caused by the presence of a multidrug resistance protein (e.g., P-glycoprotein), which could result in efflux of Tb and its Tb derivatives. We have previously observed similar behavior for U-2 OS cells labeled by mitochondria-specific dye, MitoTracker Green.[21]

Thus, we performed time- and concentration-based localization study of **3b** and **6a** in U-2OS cells. From Figure S5, showing the localization kinetics of **6a**, it is apparent that at constant concentration (1 μM) this Tb-derivative was localized inside the cells after 10 min, and stable fluorescence signal was observed after 20 min. Nevertheless, already after 30 min, some of the U-2 OS cells lacked any detectable fluorescence signal (see left bottom part of image D in Figure S5; space with cells without fluorescent signal is marked with red arrow). We may hypothesize that this is due to loss of the integrity of the plasma membrane, as the bright field image shows aberrant cell morphology in the lower left corner of the corresponding panel (image C of Figure S5 in Supporting Information). The compounds **3b** and **6a** might be also effluxed from the U-2 OS cells (e.g., hypothetically via multidrug resistance proteins), since after 45 min of incubation at 1 μM concentration, we observed translocation of most of the fluorescence signal of **6a** from intracytoplasmic structures to the plasma membrane (image F of Figure S5). A minor proportion of the cells still exhibited fluorescence in the intracellular space in the endoplasmic reticulum (upper corner of image F in Figure S5 and some other images not shown). The U-2 OS treatment with **6a** (1 μM) for 60 min resulted in almost complete vanishing of the fluorescence signal from the cell interior in most of the cells (at some regions of interest, there were still cells present with Tb–Bodipy intracellular localization). We have also used fluorescence microscopy analysis to determine the saturation plateau of these compounds' efflux in U-2 OS cells. After assessment of wide range of time points (0.5–24 h) and **3b** and **6a** concentrations, we have found that fluorescence signal inside U-2 OS cells reached the plateau at 2 μM concentration (data not shown). This phenomenon was independent of the time period tested. We assume that above 2 μM concentration the capacity of the efflux pumps might be saturated.

To determine precise organelle localization of Tb–Bodipy conjugates, we costained **3b**- and **6a**-treated U-2 OS, PC-3, LNCaP, and HeLa cells with organelle-specific dyes. We found that conjugates **3b** and **6a** colocalized with both ER-Tracker Blue-White DPX and ER-Tracker Red. Thus, we conclude that both **3b**, **6a**, and most probably also trilobolide, localized in ER of the examined cell lines (see Figure 1 and Figure S6 in Supporting Information; data for HeLa cells are not shown). These data accord with similar observations of Bodipy conjugated to Tb counterpart, thapsigargin, reported by Ong et al.[17]

After prolonged incubation (3 and 5 h) of **3b** and **6a** with the tested cells, we observed that both compounds caused ER stress manifested by formation of small vesicles originating from the ER network. This effect was more pronounced after treatment with Tb–Bodipy **6a**; see depiction by red arrows in lower panel of Figure 2. This was probably a consequence of Tb active conjugates accumulation in ER. These data are in agreement

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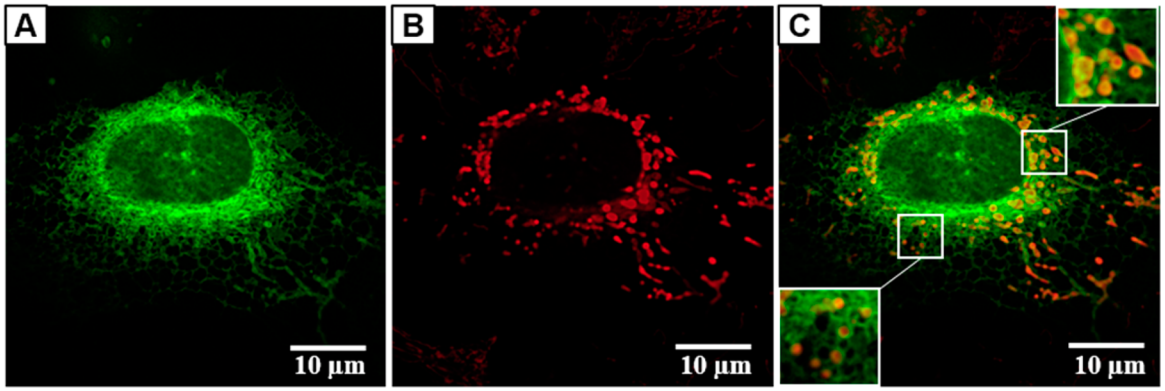

Figure 3. Trilobolide–Bodipy **3b** localization in the endoplasmic reticulum of human osteosarcoma cells (U-2 OS). Shown are fluorescent live-cell images of (A) the endoplasmic reticulum labeled by derivative **3b** (1 μM, 1 h), (B) the mitochondria stained by red-emitting MitoTracker Red FM (100 nM, 10 min), (C) merge, where fragmentation of the mitochondrial network is apparent in the enlarged areas.

Table 1. Evaluation of in Vitro Dark Cytotoxicity of Trilobolide and Its Fluorescent Conjugates[a]

		IC_{50} [μM][b]						
compd	time [h]	LNCaP	PC-3	C2C12	U-2 OS	MCF-7	MiaPaCa-2	HT-29
2	24	3.4	9.6	4.0	12.5	0.2	17.4	41.4
	48	0.5	1.3	1.0	0.2	2.5	6.0	9.8
	72	**0.4**	**0.5**	**0.3**	**0.7**	**2.1**	**1.5**	**9.5**
3b	24	12.7	4.2	>50	25.6	>50	>50	>50
	48	9.7	4.6	7.5	13.2	12.8	43.5	>50
	72	**>50**	**3.8**	**15.1**	**9.1**	**10.8**	**38.3**	**>50**
4c	24	>50	2.2	>50	>50	>50	>50	>50
	48	3.5	2.9	>50	>50	>50	>50	>50
	72	**>50**	**3.2**	**>50**	**>50**	**>50**	**>50**	**>50**
6a	24	7.4	2.5	8.0	>50	7.4	>50	>50
	48	0.4	3.1	6.5	5.4	6.3	9.9	14.2
	72	**20.5**	**4.1**	**14.3**	**9.1**	**6.0**	**6.5**	**>50**
6b	24	>50	>50	>50	>50	>50	>50	>50
	48	>50	>50	>50	>50	48.0	50.0	>50
	72	**>50**	**>50**	**>50**	**>50**	**47.3**	**41.1**	**>50**
7	24	>50	>50	>50	>50	>50	>50	>50
	48	>50	>50	>50	>50	>50	>50	>50
	72	**>50**	**>50**	**>50**	**>50**	**>50**	**>50**	**>50**
Bdp	24	>50	1.5	>50	>50	>50	>50	>50
	48	>50	3.1	>50	>50	>50	>50	>50
	72	**>50**	**5.6**	**>50**	**>50**	**>50**	**>50**	**>50**

[a] IC_{50} values are expressed as concentrations of the tested compounds required for 50% inhibition of the cell viability without light exposure after 24, 48, and 72 h of incubation with the tested compound. See text for explanation of the boldface values. [b] SD is up to 10%.

with action of thapsigargin, known as an ER stress inducer in HepG2 cells by Matsumoto et al.[22]

Interestingly, we have found that **3b** and **6a** caused accumulation of small speckles in nuclei (nuclear speckles) of U-2 OS cells. Unfortunately, because of the various ranges and dynamic character of nuclear compartments (Cajal body, PML bodies, gems, paraspeckles, nucleoli, etc.) we were not able to determine the exact type of these nuclear speckles. Nevertheless, it is known that other natural compounds from actinomycetes cause enlargement and induce formation of nuclear speckles;[23] thus, Tb and Tb–Bodipy derivatives could act similarly.

Impact of Tb and Tb–Bodipy on the Mitochondrial Network. It is known that Tg has a large impact on balance of calcium ions between ER and cytoplasm.[24,25] Because Tb is expected to perform similarly, the calcium ion imbalance caused by Tb treatment should affect the physiology of the mitochondria, which have only limited buffering capacity for elevated level of calcium ions (reviewed by Demaurex et al.[26]). To visualize the mitochondria, MitoTracker Red FM was employed for costaining studies with Tb–Bodipy **3b** and **6a**. It is unlikely that Tb derivatives reside in the mitochondria, as the mitochondria-specific red-emitting dye did not colocalize with green-emitting Tb–Bodipy conjugates (see Figure 3 and Figure S7 in Supporting Information). Importantly, we detected mitochondrial fragmentation in U-2 OS and PC-3 cells treated with **3b** and **6a** (1 μM) for at least 40 min (see Figure 3 and S7 in Supporting Information). Compound **6a** caused more pronounced disruption of the mitochondrial network in these cells (Figure S7).

Cytotoxicity Assay. In the next step, we assessed cytotoxic activity of Tb and its derivatives in vitro. The dark cytotoxicity of the compounds (concentrations up to 50 μM) was measured using WST-1 assay based on conversion of tetrazolium salt into

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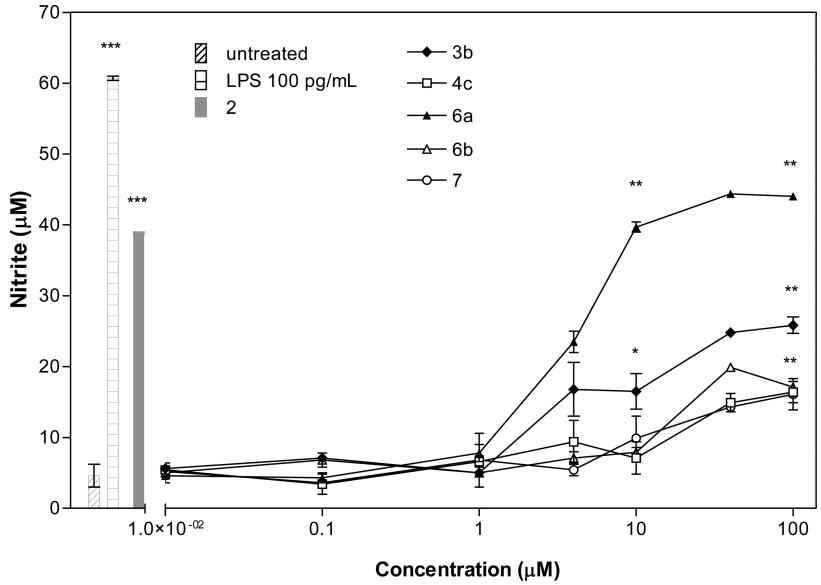

Figure 4. NO production in rat peritoneal cells induced by the tested compounds. The cells were treated with fluorescent conjugates for 24 h. For evaluation of potential effects of compounds **3b**, **4c**, **6a**, **6b**, and **7**, lipopolysaccharide (LPS, 100 pg·mL^{-1}) and nonfluorescent trilobolide (compound **2**, 0.1 μM) were used. The results represent the mean ± SEM of three independent experiments, $n = 6$. Values for 10 μM compounds **3b** and **6a** are significantly different from untreated cells ((∗) $P < 0.05$ and (∗∗) $P < 0.01$, respectively). All values of 40 and 100 μM compounds **3b**, **4c**, **6a**, **6b**, **7** are significantly different from untreated cells ((∗∗) $P < 0.01$).

a colored product, formazan, by the mitochondrial activity. Only Tb and its derivatives **3b** and **6a**, which localized inside various cell lines, exhibited a cytotoxic effect. This experiment was performed using LNCaP, PC-3, C2C12, U-2 OS, MCF-7, MiaPaCa-2, and HT-29 after 24, 48, and 72 h of treatment; results are summarized in Table 1. As expected, compounds **4c**, **6b**, and **7** that did not penetrate into the cells were not cytotoxic. The cytotoxicity of fluorescent conjugates **3b** and **6a** was slightly lower compared to Tb itself. This might be caused by the enlargement of the original Tb molecule by the linkers and the fluorescent dye.

Interestingly, the cytotoxic effect of compounds **3b** and **6a** fluctuated over time in LNCaP cell line; e.g., the IC_{50} values for 24, 48, and 72 h were 7.41, 0.39, 20.53, respectively. A possible explanation of this phenomenon might be related to the long generation time of LNCaP cells which is approximately 60 h, both according to our experience and consistent with data published by Horoszewicz et al.[27] We present the IC_{50} values based on the data collected using the WST-1 assay that reflects the cell metabolic activity. It seems likely that the intracellular concentration of the compound may be affected by the cell number that doubles between the 48 and 72 h time periods. Also the overall metabolic activity could be affected by the cell division step during the transition to mitosis occurring in the same time frame. This is supported by the fact that a similar discrepancy was observed also for the only other slowly dividing cell line used in this study, i.e., HT-29. Nevertheless, explanation of the real cause of this phenomenon would require extensive experimental work exceeding the scope of this paper. As numerous published papers present the IC_{50} values for 72 h of treatment, we refer to these values as the relevant ones (indicated in bold in Table 1).

In primary peritoneal rat cells, cytotoxic activity of Tb significantly declined (by 65% for 10 μM Tb). It is in accordance with our previous data obtained for murine and rat peritoneal cells treated with Tg.[28] On the basis of results with cancer cell line cultures, the fluorescent conjugates were applied at 40 μM concentration to primary immune cells. Mild decrease (by 20%) of viability was found only for compound **3b**. Other compounds including Bodipy itself were not cytotoxic (**4c**, **6a**, **6b**, **7**); see Figure S8 in Supporting Information.

Microscopic Evaluation of Nitric Oxide Evaluation Release in Cancer Cell Lines. Nitric oxide, a very important effector of the immune system but also a source of free radicals, plays a pivotal role in a number of essential cellular processes. Monitoring of NO is very difficult because, in biological systems, it is unstable and present only in very low concentrations. For NO imaging in living cells, we have chosen a nitric oxide specific red-emitting fluorescent probe, DAR-2. An apparent increase of NO was observed in cells (PC-3, LNCaP, MCF-7, and U-2 OS) stimulated by Tb or its fluorescent derivatives **3b** and **6a** (see Figure S9 and S10 in Supporting Information). These results confirm our data on NO release determined by spectrophotometric measurement (next paragraph). The induction of NO production is probably regulated by intracellular calcium ions,[29] whose release was caused by Tb and its derivatives treatment, which is a subject of further investigation.

Nitric Oxide Release in Primary Macrophages. Within a group of sesquiterpene lactones, Tg and Tb possess strong activity in stimulating nitric oxide (NO) production by immune cells.[28,30] Recently, we investigated modified trilobolide molecules prepared as fluorescent conjugates in rat peritoneal cells. We found a dose-dependent (0.01−100.0 μM) NO production induced by fluorescent conjugates (see Figure 4). While the potency of Tb to induce NO release was in the submicromolar range, the ability of fluorescent conjugates to induce NO production started in the micromolar range. All fluorescent conjugates significantly stimulated NO release at 40 μM or higher concentration ((∗∗) $P < 0.01$). Of these compounds, mainly **3b** and **6a** showed evident and significant effects in the range of 1−10 μM concentration to induce production of NO by cells when compared to untreated controls ((∗) $P < 0.05$ for 4 μM **3b** and (∗∗) $P < 0.01$ for 4 μM

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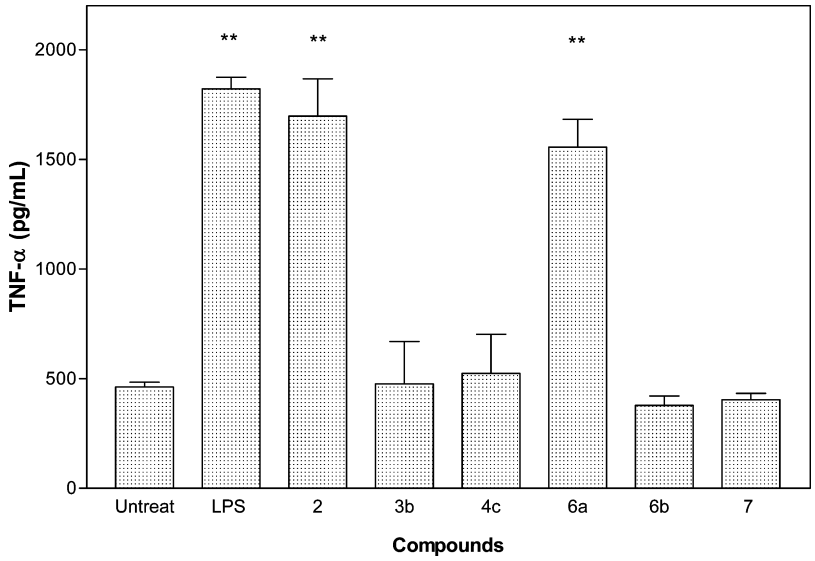

Figure 5. Secretion of cytokine TNF-α in rat peritoneal cells induced by conjugates **3b**, **4c**, **6a**, **6b**, and **7**. The cells were treated with fluorescent compounds (10 μM) for 24 h. For evaluation of potential effects of compounds, lipopolysaccharide (LPS, 1000 pg·mL^{-1}) and nonfluorescent trilobolide (compound **2**, 1 μM) were used as positive controls. Cytokine secretion was analyzed by ELISA. The results represent the mean ± SEM of two independent experiments, n = 4. (**) Values are significantly different from untreated cells (P < 0.01).

of **6a**). Values of NO production in the presence of compound **6a** reached the commonly achieved values induced by Tb or lipopolysaccharide in rat resident peritoneal cells.[30] The effects of fluorescent compounds on nitric oxide release were not observed for Bodipy itself during 24 h (data not shown).

Cytokine Production. On the basis of findings that Tb and its conjugates induced NO release in primary immune cells, we continued with immunobiological characteristics. To compare the immunostimulatory potential of fluorescently modified Tb, rat peritoneal cells were used for the evaluation of secretion of TNF-α, which is a critical cytokine involved in regulation of cancer and immune system. Lipopolysaccharide and Tb served as positive controls. Bodipy itself had no effect on cytokine secretion at 0.01−40 μM concentrations (data not shown). In contrast, secretion of TNF-α (see Figure 5) was significantly induced by conjugate **6a** (10 μM, 24 h culture, (**) P < 0.01 vs untreated cells). This correlated well with substantial effect of the compound **6a** on NO release and justified our original choice of NO for immunomodulatory screening.[31]

CONCLUSIONS

In summary, five fluorescent conjugates of a potent anticancer compound, sesquiterpene lactone trilobolide, with a green-emitting dye Bodipy were designed, synthesized, and properly characterized. Their biological relevance was also examined in vitro using several cancer cell lines.

We found that two of these conjugates, **3b** and **6a**, are functional derivatives of Tb. These two Tb conjugates localized in the endoplasmic reticulum of LNCaP, PC-3, U-2 OS, HeLa, MCF-7, MiaPaCa-2, and C2C12 cells. These findings correspond with the fact that Tb's primary target, SERCA, resides in the endoplasmic reticulum of eukaryotic cells. We also demonstrated that **3b** and **6a** conjugates caused fragmentation of the mitochondrial network in a similar manner as Tb does. These two conjugates induced also nitric oxide release in both cancer cell lines and primary immune cells. Moreover, we confirmed the ability of the modified trilobolide molecules **3b** and **6a** to induce cytokine secretion in immune cells.

Overall, our study provides new fluorescently labeled natural compound that could be used for live-cell imaging and to further expand the scarce knowledge about the molecular mechanism of Tb action.

ASSOCIATED CONTENT

Supporting Information

Synthesis and compound characterization (NMR, HRMS, IR); detailed cell culture and microscopy procedures; supplementary results from trilobolide−Bodipy intracellular localization, colocalization studies, mitochondrial fragmentation, cytotoxicity, microscopic and biochemical evaluation of nitric oxide release, and cytokine secretion. This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ERK, extracellular signal-regulated kinase; TFA, trifluoroacetic acid; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; HRMS, high-resolution mass spectrometry; IR, infrared spectroscopy; MAP, mitogen activated protein; NF-κB, nuclear factor κ light-chain enhancer of activated B cells; TNF-α, tumor necrosis factor α; NMR, nuclear magnetic resonance; NO, nitric oxide; PEG, polyethylene glycol; SAR, structure−activity relationship; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase

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