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Synthesis and biological evaluation of nandrolone–bodipy conjugates

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ABSTRACT

Here, we report synthesis and biological evaluation of fluorescent nandrolone-3-carboxymethyloxime derivatives conjugated with green-emitting bodipy dye via PEG linkers. All the newly-synthesized compounds were evaluated for their effect on cell proliferation *in vitro* in MCF-7, LNCaP, PC-3 and HEK 293T model cell lines using WST-1 assay. By means of live-cell fluorescence microscopy, the intracellular localization of nandrolone–bodipy conjugates was revealed in endoplasmic reticulum. Moreover, we performed competitive localization study with nonfluorescent nandrolone, metandrolone, boldenone, trenbolone, and testosterone.

1. Introduction

Norsteroids are xenobiotics possessing both androgenic and anabolic properties. Originally these substances were designed for the treatment of hematological and post-surgical conditions and substitutive supplementation [1]. Anabolic androgenic steroids (AAS) are widely used in medicine for treatment of various conditions, such as male hypogonadism, chronic wasting conditions, cancer, burns, renal and hepatic failure, anemia, cachexia, and AIDS [2]. Nevertheless, there has been still increasing illicit misusage of these steroids by athletes and bodybuilders. Nandrolone (also known as 19-nortestosterone or 17β-hydroxy-19-nor-4-androsten-3-one) is one of the most abused androgenic anabolic steroids (AAS), especially its commercially available form, 17β-decanoate [3]. This performance-enhancing drug is banned in sports by International Olympic Committee [4]. In addition to the androgen receptor, the effects of nandrolone are associated with progesterone receptor and several other signaling pathways (see Fig. 1). Nandrolone naturally occurs in a tiny amount in the human body since it is one of metabolites of testosterone aromatization [5–8].

Generally, androgens and estrogens have opposing effects on the growth and development of malignant human breast tissues. Androgens, such as testosterone, dihydrotestosterone, and androstenedione, exert an inhibitory effect and estrogens, such as estradiol and its derivatives, have mitogenic effect. Androgen receptors are present in 50–90% of breast cancers and their overexpression has been associated with better response to hormone therapy and longer survival of the patients [9]. Recently, Chimento et al. [10] reported that AAS are involved in progression of testicular cancer. Previous study dealing with the function of androgens in growth of MCF-7 cell line (a model of invasive breast ductal carcinoma) showed that androgens can inhibit cell proliferation *in vitro* [9].

Nandrolone as well as many other important steroids naturally bear oxo-group in C-3 position of the structure which might serve as convenient point for chemical modifications. In this work, we present syntheses of a series of green-emitting bodipy-labeled nandrolone-3-carboxymethyl-oxime (CMO) derivatives containing PEGylated linkers of different length. Bodipy is a small organic, lipophilic and membrane permeable dye often used for fluorescent lipid labeling [11,12]. Different spacing between nandrolone and bodipy was used in order to minimize the interference of the dye and the steroidal part of the molecule. We assessed the impact on cell proliferation of the newly synthesized derivatives in MCF-7, LNCaP, PC-3 and HEK 293T cells. Further, intracellular trafficking of the derivatives was performed using live-cell fluorescence microscopy.

2. Results and discussion

To our knowledge, information about the intracellular trafficking and localization of nandrolone is very scarce. Therefore, the aim of our study was to develop a functional fluorescent nandro-

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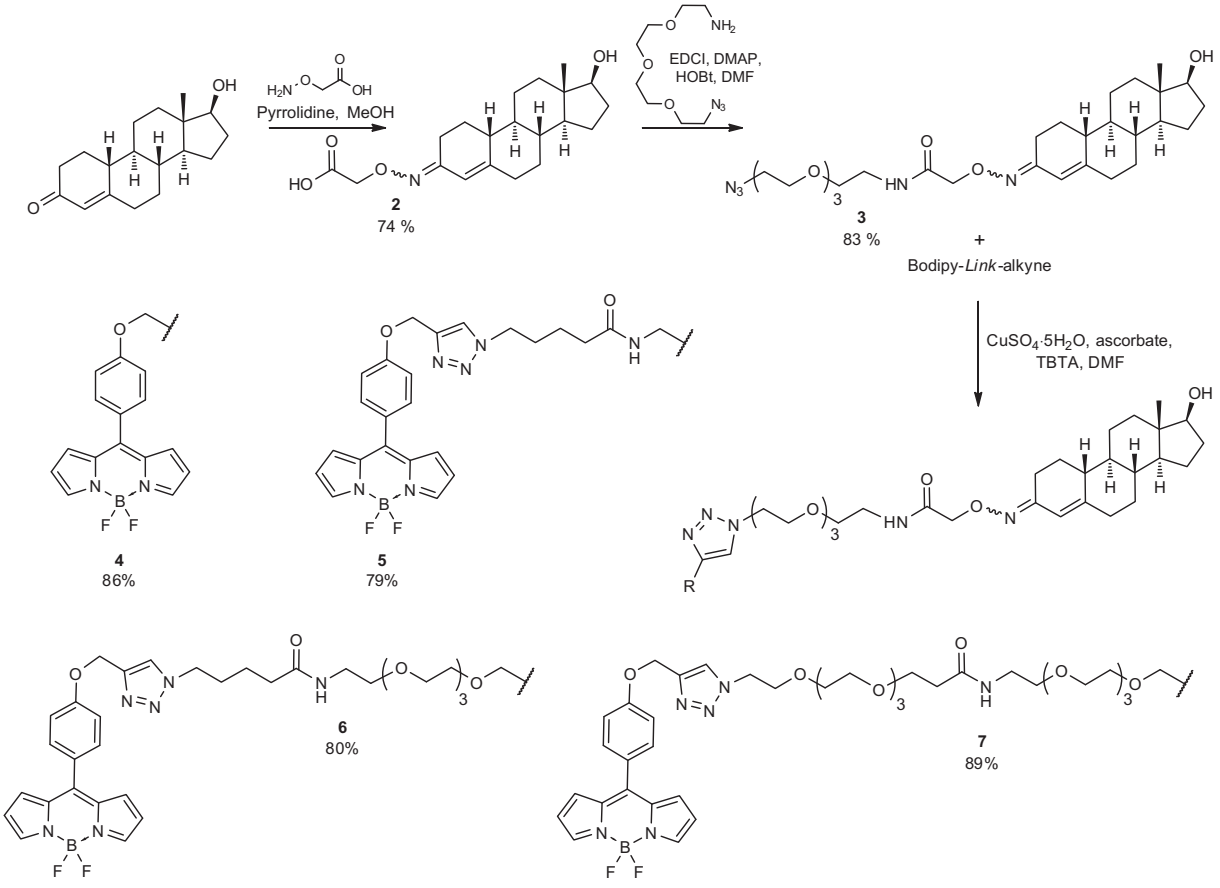
lone analog. This is the first work reporting its successful design, synthesis and application. On top of that, we have prepared a whole series of nandrolone–bodipy derivatives (**4**–**7**) applicable for live-cell fluorescence microscopy.

2.1. Chemistry

The synthetic route of nandrolone–bodipy derivatives is displayed in Scheme 1. For the synthetic approach of bodipy-*Link*-alkynes see Section 1.2 in Supplementary information, Scheme S1. Synthesis of nandrolone-3-carboxymethyloxime **2** has been already described [13]. We elongated this derivative by amide condensation of azidoPEG$_3$-amine using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride chemistry (EDCI) to obtain derivative **3** containing azido moiety (strong vibration in IR spectra at 2105 cm^{-1}). Further, to attach the fluorescent label, we used standard click chemistry (CuAAC) protocol [14]. Here, we chose a green emitting dye difluoro(2-{[4-(prop-2-yn-1-yloxy)-phenyl](2*H*-pyrrole-2-ylidene-κ*N*)methyl}-1*H*-pyrrolato-κ*N*)boron (bodipy) with sharp emission maxima at 516 nM (Section 1.2.5 in Supplementary information and Fig. S6). Four examples of differently handled bodipy dyes containing terminal acetylene moiety were prepared by group previously (for structures and chemical synthesis see Section 1.2.1 in Supplementary information). Click reactions were performed using $CuSO_4{\cdot}5H_2O$, sodium ascorbate and tris((1-benzyl-1*H*-1,2,3-triazolyl)-methyl)amine (TBTA) [15] to generate *in situ* copper(I) acetylide which undergoes regioselective cyclization with azides to stable 1,4-disubstituted 1,2,3-triazoles. This way, we synthesized four fluorescent 19-nortestosterone-like derivatives **4**–**7** in good to excellent yields (see Scheme 1; and Section 1.2 in Supplementary information). Prior to biological testing, the samples were repurified by short column chromatography and the purity checked by HPLC (Section 1.2.4 in Supplementary information).

2.2. Intracellular localization of nandrolone–bodipy

The intracellular localization of nandrolone–bodipy **4**–**7** derivatives was examined in five cell lines: LNCaP, PC-3, MCF-7, HeLa, and HEK 293T cells. Fluorescence microscopy of these green-emitting conjugates was performed in living cells for number of time intervals: 0.5, 1, 2, 3, 6, 16, and 24 h (concentrations ranging from 0.2–1 μM). Detectable fluorescence emission of the tested derivatives **4**–**7** was observed already after 0.5 h of incubation with the model cell lines, but a stable signal was achieved only after 2 h. The fluorescence intensity of the nandrolone–bodipy conjugates increased up to 3 h of incubation with the cells, when it reached a plateau, which was retained at least up to 24 h (the longest incubation time tested for live-cell microscopy). Interestingly, at low concentrations (0.2 μM), only compound **4** was intracellularly localized in all of the tested cell lines, compound **7** was retained at the cell plasma membrane, which might be caused by its size (the longest linker used) resulting in prolonged binding to cytoplasmic membrane surface receptors. Compounds **5** and **6** did not localize inside the cells at 0.2 μM concentration after 2 h (data not shown). In Fig. S27 in Supplementary information, there are representative images of intracellular localization of compounds **4** and **7** (0.5 μM) after 3 h of incubation with LNCaP, PC-3, MCF-7, and HeLa cells. Due to the network-like pattern of the intracellular fluorescence of compound **4**, we assessed colocalization



Scheme 1. Synthetic route to fluorescent nandrolone-like derivatives.