Steroids xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Synthesis and biological evaluation of nandrolone–bodipy conjugates

Michal Jurášek [a,1], Silvie Rimpelová [b,1], Vladimíra Pavlíčková [b], Tomáš Ruml [b], Oldřich Lapčík [a], Pavel B. Drašar [a,*]

[a] Department of Chemistry of Natural Compounds, Institute of Chemical Technology in Prague, 166 28 Prague, Czech Republic
[b] Department of Biochemistry and Microbiology, Institute of Chemical Technology in Prague, 166 28 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 15 June 2014
Received in revised form 19 September 2014
Accepted 1 October 2014
Available online xxxx

Keywords:
Androgens
Nandrolone
Fluorescent conjugates
Cytotoxicity

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-

* Corresponding author. Tel.: +420 220 443 698.
E-mail address: Pavel.Drasar@vscht.cz (P.B. Drašar).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.steroids.2014.10.002
0039-128X/

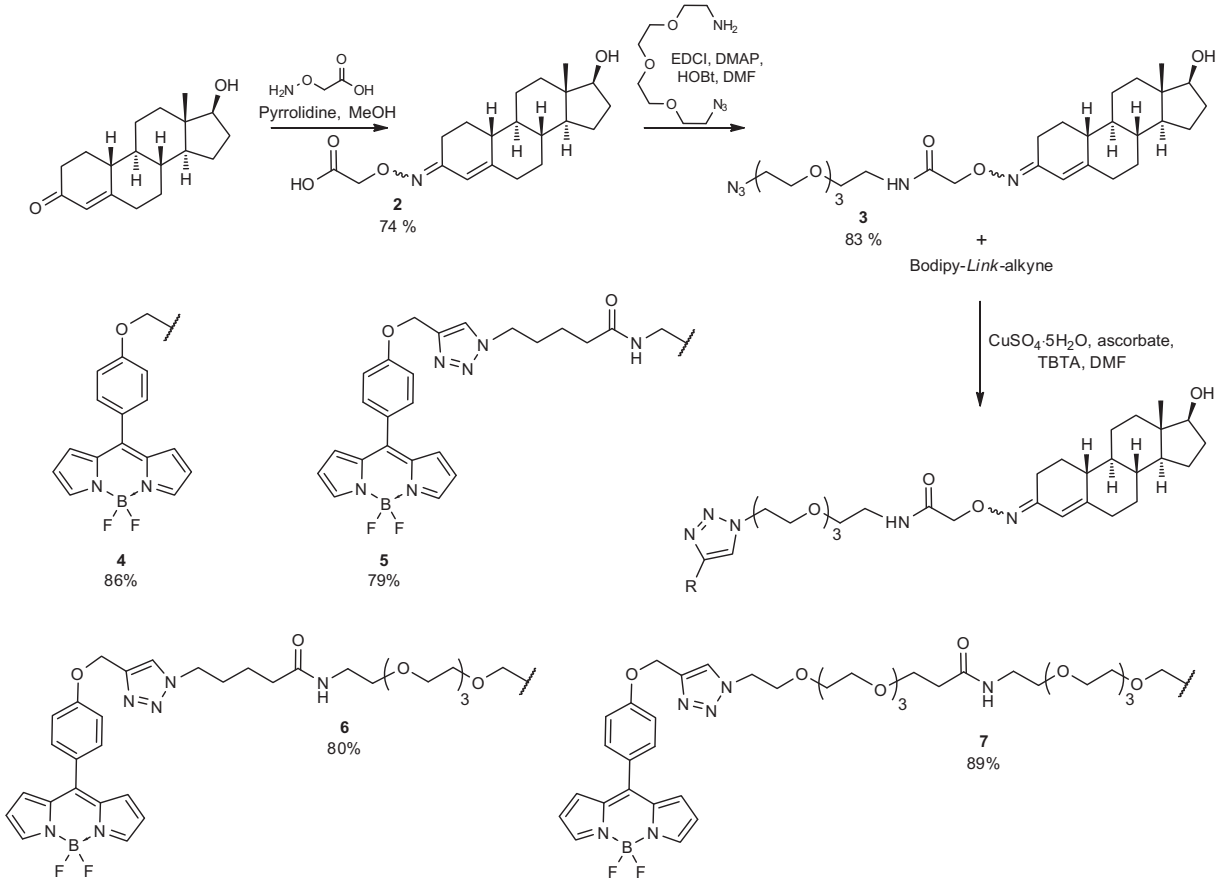
lone analog. This is the first work reporting its successful design, synthesis and application. On top of that, we have prepared a whole series of nandrolone–bodipy derivatives (**4**–**7**) applicable for live-cell fluorescence microscopy.

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.

Please cite this article in press as: Jurášek M et al. Synthesis and biological evaluation of nandrolone–bodipy conjugates. Steroids (2014), http://dx.doi.org/10.1016/j.steroids.2014.10.002

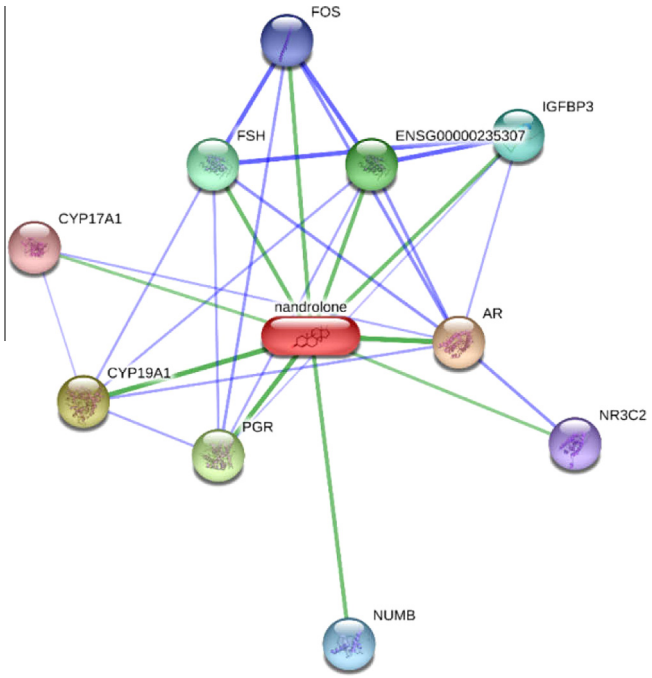


Fig. 1. Functional association network of nandrolone. The nandrolone network was generated using STITCH database (http://stitch.embl.de). The interactions include direct (physical) and indirect (functional) associations derived from genomic context, high-throughput experiments, co-expression, and literature mining (required confidence score 0.4). AR – androgen receptor; CYP19A1 – cytochrome P450, family 19, subfamily A, polypeptide 1; PGR – progesterone receptor; ENSG00000235307 – bromodomain-containing protein 2 (protein RING3) (O27.1.1); FSH – bromodomain-containing protein 2 (protein RING3) (O27.1.1) (801 aa); IGFBP3 – insulin-like growth factor binding protein 3; NUMB – numb homolog (Drosophila); FOS – FBJ murine osteosarcoma viral oncogene homolog; NR3C2 – nuclear receptor subfamily 3, group C, member 2; CYP17A1 – cytochrome P450, family 17, subfamily A, polypeptide 1.

experiments with ER-Tracker™ Red and mitochondria-specific red-emitting dye. From Fig. 2, it is very likely that compound **4** was primarily localized in endoplasmic reticulum (ER) of the tested cell lines. Nevertheless, the overlap of the fluorescent signals of compound **4** and ER-Tracker™ Red was not absolute, there was a remaining fluorescent signal of nandrolone–bodipy outside of ER. We have found also partial colocalization of this signal with mitochondrial sensors (data not shown). Even though that most of the steroidogenic enzymes reside in ER, notably, some of them can be also found in mitochondria of mammalian cells [4], which is in agreement with our findings. It has been recently described [7,8] that mitochondrial DNA contains regions sequentially similar to nuclear hormone response elements (HREs). Mitochondria provide more than 90% of the cell energy supply by oxidative phosphorylation coupled with electron transport chain (ETC) [9], thus yielding oxidative stress response elements, reactive species generation (ROS), and initiating apoptosis [10]. Oxidative phosphorylation (OXPHOS) is a process in which both nuclear and mitochondrially encoded enzymes are required. Complex of a hormone and its receptor binds to HREs of the mitochondrial DNA and induce transcription of mitochondrial OXPHOS genes, thus suggesting the possibility of a direct mechanism for induction of these genes by hormones [11–14].

Another nandrolone–bodipy conjugate, compound **7**, appeared to be localized on the cell plasma membrane of LNCaP, PC-3, MCF-7, and HeLa cells. Thus, colocalization with CellMask™ Deep Red was performed, see Fig. 3. Except for that, we have observed fluorescence emission of all compounds (**4**–**7**) also in small vesicles resembling lipid droplets, where pure bodipy specifically localized (bodipy control localized in lipid droplets-derived vesicles, data not shown). This localization of the nandrolone–bodipy conjugates might be caused by insufficient binding capacity of the specific binding sites inside the cells together with possible excess of applied conjugates. However, this nonspecific localization in lipid droplets does not interfere with the functional studies of nandrolone–bodipy.

The specificity of nandrolone–bodipy derivative **4** (0.25 μM) localization was studied by competitive assay (displacement experiments) using a 50-fold (12.5 μM)) and 100-fold (25 μM)) excess of nonfluorescent nandrolone, and other related steroids: metandrolone, boldenone, trenbolone, and testosterone. Chemical structures of the steroids are depicted in Fig. S28 in Supplementary information. Compound **4** was chosen for this competitive assay as the most effectively and the most rapidly localized one. All the tested parent nonfluorescent steroids displaced large amount of nandrolone–bodipy **4** (decreased fluorescence intensity in ER) from its endoplasmic reticulum localization site within 1 h of incubation with 50-fold and 100-fold excess of the steroids. The displacement was monitored by the decrease of fluorescence intensity of the same (living) cells before and after treatment with the competitors, see Figs. 4 and S29 in Supplementary information. The most pronounced decrease of fluorescence was observed for pure nandrolone, thus confirming functionality of its bodipy derivative **4**. According to our expectations, fluorescent signal from the lipid droplets was not diminished by incubation with nonfluorescent steroids, because in lipid droplets, there is no binding specificity for steroids.

2.3. Cytotoxicity assays

Effect on cell proliferation of the prepared fluorescent nandrolone–bodipy derivatives was examined in two prostatic cancer cell lines, LNCaP and PC-3; one breast cancer cell line (MCF-7) and in

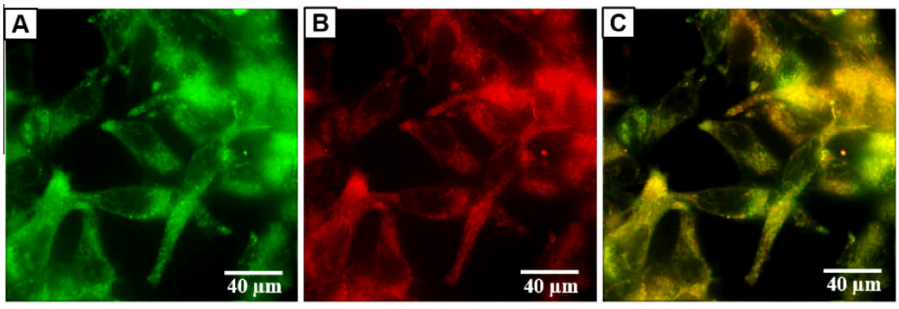


Fig. 2. Fluorescence microscopy images of nandrolone–bodipy conjugate **4** colocalized with ER-Tracker™ Red in human prostatic cancer cells (LNCaP) *in vitro*. (A) compound **4** was applied in 0.25 μM concentration for 3 h; (B) ER-Tracker™ Red in 70 nM concentration for 30 min; (C) merge. From figure C, the colocalization of both compounds is apparent. nandrolone–bodipy **4** localizes in endoplasmic reticulum.

Please cite this article in press as: Jurášek M et al. Synthesis and biological evaluation of nandrolone–bodipy conjugates. Steroids (2014), http://dx.doi.org/10.1016/j.steroids.2014.10.002

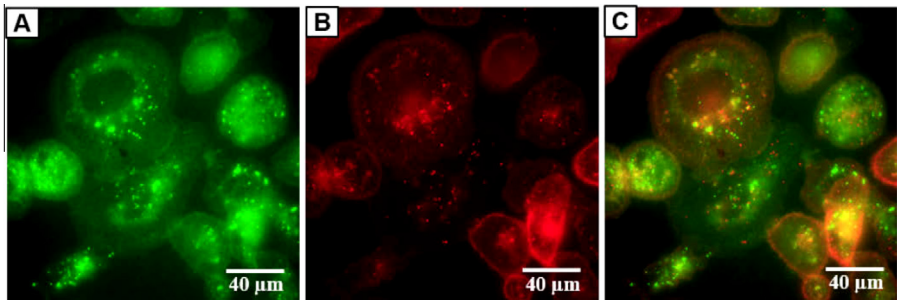


Fig. 3. Fluorescence microscopy images of nandrolone–bodipy conjugate **7** colocalized with CellMask™ Deep Red in human prostatic cancer cells (PC-3) *in vitro*. (A) compound **7** was applied in 2 μM concentration for 3 h; (B) CellMask™ Deep Red in 0.5 μg mL^{-1} concentration for 15 min; (C) merge.

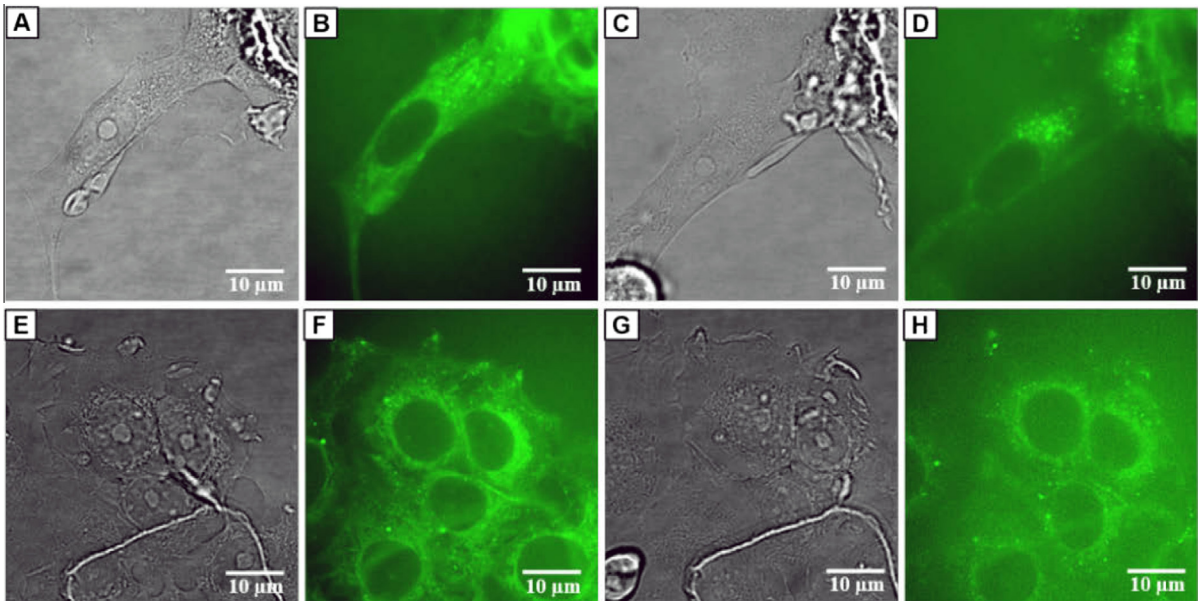


Fig. 4. Competitive assay of nandrolone–bodipy **4** with nonfluorescent steroid derivatives. (A, C, E, G) bright field images of MCF-7 cells; fluorescence microscopy images: (B, F) intracellular localization of nandrolone–bodipy **4** (0.25 μM, 3 h) in MCF-7 cells; displacement of nandrolone–bodipy **4** with 100-fold excess of: (D) nonfluorescent nandrolone (1 h), (H) boldenone (1 h). The same cells were found using Ibidi microscopic dishes with grid (μ-Dish 35 mm Grid-500), because of the cell locomotion over time, the cells do not look the same in the bright field images.

human embryonic kidney cells (HEK 293T) after 24, 48, and 72 h of treatment. The prostatic cells of LNCaP cancer cell line are both androgen and estrogen sensitive [16–18]. These epithelial cells express both prostate specific antigen (PSA) [19] and prostate specific membrane antigen (PSMA) [20]. PC-3 cell line was derived from advanced prostatic cancer which express neither PSA nor PSMA antigen [21]. Third cancer model of our study, MCF-7 cell line, was derived from invasive breast ductal carcinoma. MCF-7 cells are estrogen [22], progesterone, and androgen receptor positive [9,23]. Human embryonic kidney HEK 293T cells do not express any steroid receptors.

Nandrolone stimulated cell proliferation of LNCaP cells up to 5 μM and PC-3, and MCF-7 up to 1 μM concentration after 24 h of treatment. Nandrolone and its CMO derivative did not exhibit any significant toxicity in the tested cells lines after 24–72 h of treatment. The IC_{50} was not reached the maximal tested concentration of 20 μM. Further, we tested the effect on cell proliferation of nandrolone–bodipy conjugates **4–7**. Their actions markedly differed (comparison is given in Supplementary information Figs. S30–S33). To determine the possible impact of the bodipy dye on the conjugate toxicity, pristine bodipy was used as a control (compound **1**; Supplementary Section 1.2). The IC_{50} values of the pure dye **1** did not reach 20 μM concentration in any of the tested cell lines after 24–72 h. Interestingly, from the nandrolone–bodipy conjugates, compounds **4** and **7** were cytotoxic for all tested cell lines, with more pronounced cytotoxicity after 24 h. The IC_{50} values decreased with prolonged treatment.

3. Conclusion

In this work, we designed, synthesized, and tested a series of nandrolone–bodipy derivatives. To our knowledge, this is the first work reporting intracellular localization of a fluorescent nandrolone analog. Compound **4** is suitable for fluorescence microscopic study of intracellular localization and tracking of nandrolone. We have confirmed its suitability for such studies in different cell line models, such as PC-3, LNCaP, MCF-7, and HeLa.

The mechanism of nandrolone interaction with steroid receptors and aromatase will be of interest in our future work, as well as preparation of another nandrolone functional analogs.

Acknowledgements

This work was supported by grants VG20112015045 (Ministry of Interior, Czech Republic) and MSMT No 20/2014 (Ministry of Education, Youth and Sports, Czech Republic).

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.2014.10.002.

References

[1] Bricout V, Wright F. Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances? Eur J Appl Physiol 2004;92:1–12.
[2] Basaria S, Wahlstrom JT, Dobs AS. Clinical review 138: anabolic–androgenic steroid therapy in the treatment of chronic diseases. J Clin Endocrinol Metab 2001;86:5108–17.
[3] Chung BC, Choo HYP, Kim TW, Eom KD, Kwon OS, Suh JW, et al. Analysis of anabolic-steroids using GC/MS with selected ion monitoring. J Anal Toxicol 1990;14:91–5.
[4] Ozer D, Temizer A. The determination of nandrolone and its metabolites in urine by gas chromatography mass spectrometry. Eur J Drug Metab Ph 1997;22:421–5.
[5] Frisch RE, Canick JA, Tulchinsky D. Human fatty marrow aromatizes androgen to estrogen. J Clin Endocrinol Metab 1980;51:394–6.
[6] Bellino FL, Osawa Y. Evidence of direct aromatization of testosterone and different aromatization sites for testosterone and androstenedione in human placental microsomes. Biochemistry-Us 1974;13:1925–31.
[7] Abraham GE, Lobotsky J, Lloyd CW. Metabolism of testosterone and androstenedione in normal and ovariectomized women. J Clin Invest 1969;48:696–703.
[8] Baume N, Avois L, Schweizer C, Cardis C, Dvorak J, Cauderay M, et al. [C-13] Nandrolone excretion in trained athletes: interindividual variability in metabolism. Clin Chem 2004;50:355–64.
[9] Macedo LF, Guo Z, Tilghman SL, Sabnis GJ, Qiu Y, Brodie A. Role of androgens on MCF-7 breast cancer cell growth and on the inhibitory effect of letrozole. Cancer Res 2006;66:7775–82.
[10] Chimento A, Sirianni R, Zolea F, De Luca A, Lanzino M, Catalano S, et al. Nandrolone and stanozolol induce leydig cell tumor proliferation through an estrogen-dependent mechanism involving IGF-I system. J Cell Physiol 2012;227:2079–88.
[11] Pagano RE, Chen CS. Use of Bodipy-labeled sphingolipids to study membrane traffic along the endocytic pathway. Ann Ny Acad Sci 1998;845:152–60.
[12] Lemcke S, Honnscheidt C, Waschatko G, Bopp A, Lutjohann D, Bertram N, et al. DHEA-Bodipy – a functional fluorescent DHEA analog for live cell imaging. Mol Cell Endocrinol 2010;314:31–40.
[13] Jiang J, Zhang H, Li G, Wang Z, Wang J, Zhao H. Preparation of anti-nortestosterone antibodies and development of an indirect heterologous competitive enzyme-linked immunosorbent assay to detect nortestosterone residues in animal urine. Anal Lett 2011;44:2373–93.
[14] Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem Int Ed 2002;41:2596–9.
[15] Hein JE, Tripp JC, Krasnova LB, Sharpless KB, Fokin VV. Copper(I)-catalyzed cycloaddition of organic azides and 1-iodoalkynes. Angew Chem Int Ed 2009;48:8018–21.
[16] Lau KM, LaSpina M, Long J, Ho SM. Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. Cancer Res 2000;60:3175–82.
[17] Takahashi Y, Perkins SN, Hursting SD, Wang TTY. 17β-Estradiol differentially regulates androgen-responsive genes through estrogen receptor-β and extracellular-signal regulated kinase-dependent pathways in LNCaP human prostate cancer cells. Mol Carcinogen 2007;46:117–29.
[18] Castagnetta LA, Miceli MD, Sorci CM, Pfeffer U, Farruggio R, Oliveri G, et al. Growth of LNCaP human prostate cancer cells is stimulated by estradiol via its own receptor. Endocrinology 1995;136:2309–19.
[19] Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, et al. LNCap model of human prostatic-carcinoma. Cancer Res 1983;43:1809–18.
[20] Yao V, Berkman CE, Choi JK, O'Keefe DS, Bacich DJ. Expression of prostate-specific membrane antigen (PSMA), increases cell folate uptake and proliferation and suggests a novel role for PSMA in the uptake of the non-polyglutamated folate, folic acid. Prostate 2010;70:305–16.
[21] Ghosh A, Wang YN, Klein E, Heston WDW. Novel role of prostate-specific membrane antigen in suppressing prostate cancer invasiveness. Cancer Res 2005;65:727–31.
[22] Brooks SC, Locke ER, Soule HD. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J Biol Chem 1973;248:6251–3.
[23] Horwitz KB, Costlow ME, Mcguire WL. MCF-7 – human breast-cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. Steroids 1975;26:785–95.

Please cite this article in press as: Jurášek M et al. Synthesis and biological evaluation of nandrolone–bodipy conjugates. Steroids (2014), http://dx.doi.org/10.1016/j.steroids.2014.10.002