Biochimie 95 (2013) 2097–2106

Contents lists available at ScienceDirect

Biochimie

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

Research paper

New steroidal 17β-carboxy derivatives present anti-5α-reductase activity and anti-proliferative effects in a human androgen-responsive prostate cancer cell line

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ARTICLE INFO

Article history:
Received 7 February 2013
Accepted 31 July 2013
Available online 9 August 2013

Keywords:
Prostate cancer
Benign prostate hyperplasia
Steroidal 5α-reductase inhibitors
Finasteride
LNCaP cells

ABSTRACT

The androgens testosterone (T) and dihydrotestosterone (DHT), besides playing an important role in prostate development and growth, are also responsible for the development and progression of benign prostate hyperplasia (BPH) and prostate cancer. Therefore, the actions of these hormones can be antagonized by preventing the irreversible conversion of T into DHT by inhibiting 5α-reductase (5α-R). This has been a useful therapeutic approach for the referred diseases and can be achieved by using 5α-reductase inhibitors (RIs). Steroidal RIs, finasteride and dutasteride, are used in clinic for BPH treatment and were also proposed for chemoprevention of prostate cancer. Nevertheless, due to the increase in bone and muscle loss, impotency and occurrence of high-grade prostate tumours, it is important to seek for other potent and specific molecules with lower side effects. In the present work, we designed and synthesized steroids with the 3-keto-Δ^4 moiety in the A-ring, as in the 5α-R substrate T, and with carboxamide, carboxyester or carboxylic acid functions at the C-17β position. The inhibitory 5α-R activity, in human prostate microsomes, as well as the anti-proliferative effects of the most potent compounds, in a human androgen-responsive prostate cancer cell line (LNCaP cells), were investigated. Our results showed that steroids **3**, **4** and **5** are good RIs, which suggest that C-17β lipophylic amides favour 5α-R inhibition. Moreover, these steroids induce a decrease in cell viability of stimulated LNCaP cells, in a 5α-R dependent-manner, similarly to finasteride.

Abbreviations: AR, androgen receptor; ATCC, American Type Culture Collection; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; BPH, benign prostate hyperplasia; BSA, bovine serum albumin; CFBS, pre-treated charcoal heat-inactivated fetal bovine serum; DHT, dihydrotestosterone; DHT-$^{13}C_3$, dihydrotestosterone $^{13}C_3$ solution; DMSO, dimethyl sulfoxide; DTE, 1,4-dithioerythritol; DTT, dithiothreitol; FBS, fetal bovine serum; FDA, Food and Drug Administration; GC–MS, gas chromatography–mass spectrometry; LDH, lactate dehydrogenase; F, finasteride; DLLME, dispersive liquid–liquid microextraction; LNCaP cells, human androgen-responsive prostate cancer cell line; MeCN, acetonitrile; MeOH, methanol; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; MTT, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium; NADPH, nicotinamide adenine dinucleotide phosphate; 5α-R, 5α-reductase; RIs, 5α-reductase inhibitors; RPMI, Roswell Park Memorial Institute; RT, room temperature; SEM, standard error of mean; T, testosterone; T-d_3, testosterone-d_3 solution.

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http://dx.doi.org/10.1016/j.biochi.2013.07.023

1. Introduction

The androgens testosterone (T) and dihydrotestosterone (DHT) (Fig. 1) play an important role not only in normal prostate development, cell proliferation and growth, but also in the genesis and progression of benign prostate hyperplasia (BPH) and prostate cancer [1–3]. BPH is a recurrent disease in 50% of males by the age of 50 and 90% of males by the age of 80, being the major responsible for men morbidity [4], while prostate cancer is the second-leading cause of cancer death in men [1]. T is the most abundant circulating androgen and it is converted in prostate into DHT by the enzyme 3-oxo-5α-steroid-4-dehydrogenase, 5α-reductase (5α-R) (Fig. 1). DHT is the main androgen in the prostate and the major responsible for differentiation and prostate growth [4,5].

As androgens are implicated in the development and progression of BPH and prostate cancer, the treatment of these diseases can be achieved by antagonizing the action of these hormones by preventing the irreversible conversion of T into DHT, through the inhibition of 5α-R [3,4,6]. The 5α-R inhibitors (RIs) can be classified into steroidal and non-steroidal [5]. The development of non-steroidal RIs has increased in the last years, but they did not show promising inhibitory activities of 5α-R when compared to steroidal compounds. The first steroidal RIs were designed by modifications of the T, the natural substrate of the enzyme. One of the main modifications was the substitution of one carbon atom of the A-, B-, C- or D-rings of the steroid framework by a heteroatom, specially nitrogen, leading to the discovery of potent inhibitors of human 5α-R such as 4-azasteroids, 6-azasteroids and 10-azasteroids [7,8]. The 4-azasteroid finasteride (Fig. 2) was the first RI to be clinically approved for the treatment of BPH in 1992 [9,10]. Ten years later, another 4-azasteroid, dutasteride (Fig. 2), was also approved by FDA to be used for the symptomatic treatment of BPH [6]. Finasteride and dutasteride are potent irreversible inhibitors of 5α-R forming strong ternary complexes with 5α-R-NADPH complex [5,11]. Both RIs reduce the intraprostatic DHT levels and, therefore, the prostatic size [3,12], being currently used in the clinic for the treatment of BPH and were also proposed for chemoprevention and treatment of prostate cancer [13,14]. Despite the success of finasteride and dutasteride they still have some disadvantages like the increase in bone and muscle loss, and impotency. Furthermore, different clinical trials have demonstrated that these RIs increased the risk of high-grade prostate cancer, which prevent FDA approval of finasteride and dutasteride to be used for prostate cancer treatment [5,15]. For this reason, it is important to seek for other potent and specific molecules with lower side effects.

Previous studies highlight the importance of the 3-keto-Δ^4-androstan-17β-carboxamide steroids as 5α-R inhibitors [16–18]. It has also been found that substitution at C-17β position of steroids by lipophilic side chains, containing amide or ester groups, enhances 5α-R inhibitory activity by binding to a lipophilic pocket on the enzyme [6]. In the present work, it was designed and synthesized steroids with the 3-keto-Δ^4 moiety in the A-ring, as in T, and with carboxamide, carboxyester or carboxylic acid functions at the C-17β position (Scheme 1). The rationale particularly focus on the carboxamide analogues of finasteride and dutasteride (Fig. 2)

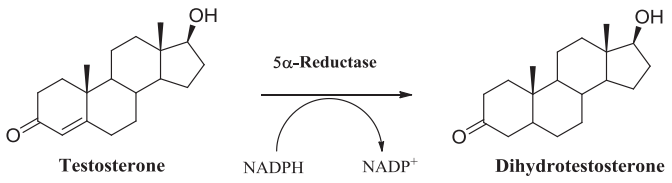

Fig. 1. Biosynthesis of dihydrotestosterone from testosterone.

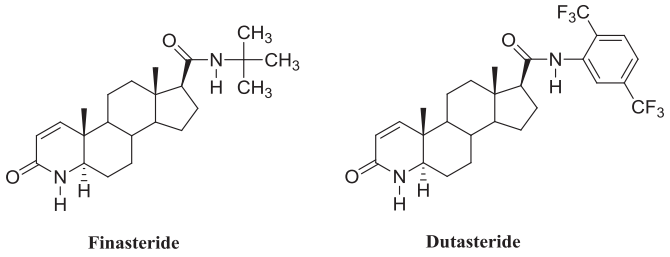

Fig. 2. Steroidal 5α-reductase inhibitors, finasteride and dutasteride.

obtained by combining in the same molecule, the A-ring of the substrate T with the C-17β carboxamide group of the referred RIs. In addition, the biochemical activity of the synthesized compounds was determined in human prostate microsomes, by a new methodology recently developed by our group [19]. The antiproliferative effects of the most potent RIs and of finasteride were also investigated in a human androgen-responsive prostate cancer cell line (LNCaP cells).

2. Material and methods

2.1. Chemistry

Reactions were controlled by TLC using silica gel 60 F254 plates. Melting points (MPs) were determined on a Reichert Thermopan hot block apparatus and were not corrected. IR spectra were recorded on a Jasco 420 FT/IR spectrometer. The ^{1}H NMR spectra were recorded at 600 MHz, on a Varian Unity 600. The ^{13}C NMR spectra were recorded at 150 MHz on a Varian Unity 600. Chemical shifts were referred in δ values in parts per million (ppm) downfield from tetramethylsilane as an internal standard. All J values are given in Hz. Mass spectra ESI were obtained with a mass spectrometer QIT-MS Thermo Finningan, model LCQ Advantage MAX. Purity was determined by HPLC using a Liquid Chromatograph of High Performance Thermo Finningan. 4-Androsten-3-one-17β-carboxylic acid (**1**) was purchased from Fountain Limited (Malta). Reagents and solvents were used as obtained from suppliers without further purification, with exception of dichloromethane, and pyridine, which were dried through reflux and distillation from CaH_2, being stored away from light in a brown bottle with type 4 Å molecular sieves, under an atmosphere of dry N_2.

All compounds possess a purity superior to 98%. The purity was checked by HPLC with a C18-reversed phase column and water/acetonitrile 30:70 as solvent. The purity of individual compounds was determined from the area peaks in the chromatogram of the sample solution.

2.1.1. 4-Androstene-17β-carboxylic acid (*2*)

Sodium borohydride (590.3 mg, 15.6 mmol) was added in small portions to a cooled and stirred mixture of trifluoroacetic acid (3.6 mL), glacial acetic acid (3.6 mL) and acetonitrile (3.6 mL). After this, a solution of compound **1** (1.0 g, 3.16 mmol) in dry dichloromethane (53 mL) was added and the reaction proceeded at room temperature, under a stream of dry nitrogen, until all the starting material had been consumed (3 h 20 min by TLC). The reaction mixture was neutralized with 10% $NaHCO_3$, extracted with chloroform (4 × 100 mL) and the organic layer washed with water (4 × 100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. Crystallization from ethyl acetate/hexane afforded the pure compound **2** (680.5 mg, 71%) as white crystals. $Mp_{(ethyl\ acetate/hexane)}$: 205–208 °C; IR (NaCl plates, $CHCl_3$): υ_{max} = 3399 (O–H carboxylic acid), 3020 (H–C=), 1701 (C=O carboxylic acid), 1676