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Chemistry and Biological Activity of Steroids

Edited by Jorge António Ribeiro Salvador and Maria Manuel Cruz Silva

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Meet the editors

Jorge António Ribeiro Salvador has a degree in Pharmaceutical Sciences, a Master's degree in Organic and Technological Chemistry, and a PhD in Pharmaceutical Chemistry. He has a position as Full Professor at the Faculty of Pharmacy, University of Coimbra, Portugal, and is a non-executive member of the board of CHEM4PHARMA, a start-up pharmaceutical company located in Portugal. His research activity is focused on the development of new drugs, mainly for cancer treatment. He has published about 100 SCI journal papers, 13 book chapters, and 10 patents (two US patents). In addition, he is the author/coauthor of 250 presentations in national and international conferences and workshops. Over the last few years he has conducted several research projects in the field of pharmaceutical/medicinal chemistry, which have contributed to his significant expertise in drug discovery.

Maria Manuel Cruz Silva has a degree in Pharmaceutical Sciences, a Master's degree in Drugs Technologies, and a PhD in Pharmaceutical Chemistry from the University of Coimbra in collaboration with the CNR, Milan, Italy. She has a position as Assistant Professor at the Faculty of Pharmacy of the University of Coimbra, Portugal. Her research is currently devoted to the synthesis of novel bioactive steroids. She is the author and coauthor of 25 publications in peer-reviewed journals and three book chapters.

Contents

Preface

Chemistry and Biological Activity of Steroids puts together recent contributions of scientific research to understand the physiological roles of steroids and discover new bioactive steroidal molecules.

The chapters that compose this book encompass the biotransformations of steroids, taking advantage of the striking regio- and stereoselectivity of oxidizing enzymes affording functionalized steroids that would be very difficult to obtain by conventional methods.

Estrone sulfatase is a clinically validated drug target in estrogen-dependent cancers and its inhibition by synthetic steroids is reviewed. The role of steroids in the somatotropic–liver axis, intermediate metabolism, or gender dimorphism is also discussed. Finally, exploitation of the steroid structure to develop supramolecular systems and nanomaterials for biomedical application is examined.

The editors sincerely thank the authors, who have shared their knowledge throughout the chapters of this book.

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Chapter 1

Introductory Chapter: Chemistry and Biological Activity of Steroids - Scope and Overview

Maria Manuel Cruz Silva and
Jorge António Ribeiro Salvador

1. Introduction

Steroid compounds are widely present in living organisms playing an important role in their vital activities.

The steroidal basic structure is constituted by a common chemical skeleton of four fused rings, consisting of three six-membered rings and a five-membered ring. This hydrocarbon scaffold contains 17 carbons and has the cyclopentanoperhydrophenanthrene basic structure [1, 2]. The four steroid rings are labelled as A, B, C and D, and their carbon atoms are numbered according to the universal convention (International Union of Pure and Applied Chemistry/International Union of Biochemistry Joint Commission on Biochemical Nomenclature). Angular methyl groups at C13 and C10 are designated as 18-CH_3 and 19-CH_3, respectively, and alkyl substituents at C17 are the steroid side chain. The 18- and 19-methyl groups stand above the plane of the steroid skeleton and, by convention, have β-configuration. Therefore, other atoms or substituents located above this plane also have β-configuration, while those below it have α-configuration [3].

Steroids interact with enzymes and receptors in a strikingly specific manner. Small changes in the steroid structure afford major biological differences.

Several natural and synthetic steroids are important therapeutic tools for a wide range of diseases [4, 5]. The steroid classes present in drug therapy include, among others, corticosteroids, neurosteroids, sexual hormones, bile acids, vitamin D and cardiotonic steroids [4].

Hundreds of steroid compounds have been isolated from natural sources, and many thousands of them have been obtained synthetically over the last decades, and their chemical and biological investigation continues to be very active. In fact, the steroid scaffold continues to be the structural basis of new drugs for a variety of targets and diseases.

The book *Chemistry and Biological Activity of Steroids* aims to provide an updated overview of the recent advances in the medicinal chemistry of steroids.

Novel synthetic methods of steroids through the use of microorganisms as carriers of strikingly selective enzyme catalysts, able to promote reactions that would be very difficult by conventional chemical methods, continue to be an area of intensive research and enormous industrial interest. Several biotransformations at industrial scale have been applied in the production of steroids, through chemo-, regio- and stereoselective reactions, namely, hydroxylations. The chapter concerning steroid biotransformations gives an overview of the recent achievements in this field.

The steroid hormones were discovered almost a century ago and have been found to be involved in important physiopathological conditions, being therefore important starting points for the development of drugs.

Oestrogens and androgens are two classes of steroid sex hormones responsible for female and male differentiation, respectively, and continue to be a source of questions and opportunities in deciphering the mechanisms of homeostasis and disease.

A chapter concerning the discovery of novel inhibitors of oestrone sulphatase, a clinically validated drug target in oestrogen-dependent cancers, presents the medicinal chemistry rational behind the design, synthesis and safety assessment of anticancer drug candidates for this pharmacological target. Furthermore examples of dual aromatase-sulfatase inhibitors are given, disclosing the potential of a synergistic dual inhibition.

On the other hand, sex steroids have important physiological actions, not limited to the reproductive organs. They exert important physiological roles, including the regulation of somatotropic-liver axis, intermediate metabolism or gender dimorphism. This is in part because the liver is a sex steroid-responsive organ where sex steroid- and growth hormone-dependent signalling pathways connect to regulate complex gene expression networks. Deficiency of sex steroid- and GH-dependent signalling pathways has an impact on the mammalian liver physiology. This interesting and vast topic is discussed in Chapter 4.

Finally, the usefulness of steroids in the cutting-edge technology of supramolecular systems and nanomaterials for biomedical application is discussed in the fifth chapter. The renewable and economic natural steroid compounds can be employed as building blocks in the design and construction of steroid-based supramolecular systems. Interesting characteristics of steroids, concerning physicochemical and biological properties, biocompatibility and bioactivities, make them attractive as building blocks of supramolecular systems to be employed in biomolecular recognition/sensing and biomolecular transportation.

The chemical and biological properties of steroids include a large variety of topics. This book contributes with a selection of different chapters that give updated information and critical discussions, illustrating the novelty of this old class of molecules.

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Chapter 2

Biotransformation of Steroids Using Different Microorganisms

Arturo Cano-Flores, Javier Gómez and Rigoberto Ramos

Abstract

The introduction of a hydroxyl group "biohydroxylation" in the steroid skeleton is an important step in the synthesis of new steroids used physiologically as hormones and active drugs. There are currently about 300 known steroid drugs whose production constitutes the second category within the pharmaceutical market after antibiotics. Several biotransformations at industrial scale have been applied in the production of steroid hormones and drugs, which have functionalized different types of raw materials by means of *chemo-*, *regio-*, and *stereo*selective reactions (hydroxylation, Baeyer-Villiger oxidation, oxidation reactions, reduction of group carbonyl, isomerization, and Michael additions, condensation reactions, among others). In Green Chemistry, biotransformations are an important chemical methodology toward more sustainable industrial processes.

Keywords: biotransformation, steroid compounds, biological transformation, bioconversions, microorganisms

1. Introduction

Steroids (stereos = solids) are organic compounds derived from alcohols, which are widely distributed in the animal and plant kingdoms. Their base skeleton has 17 carbon atoms in a tetracyclic ring system known as cyclopentanoperhydrophenanthrenes (gonane and estrane). In this group of substances, life-vital compounds are categorized, such as cholesterol, bile acids, sex hormones, vitamin D, corticosteroids, cardiac aglycones, and antibiotics, among others.

Some of the most potent toxins are steroid alkaloids. Steroids are responsible for important biological functions in the cell; for example, the steroids derived from androstane, pregnane, and estrane have hormonal activity [1–5]; bile acids are important for the digestion and absorption of fats; and cardiotonic aglycones are used for the treatment of heart disease. Sterols are constituents of the cell membrane, essential for cell stability and development; also, they are precursors of bile acids and steroid hormones.

A large number of steroids are used as anti-inflammatory agents [6], immunosuppressants, progestational agents, diuretics, anabolics, and contraceptives [7–9]. Some are used for the treatment of prostate and breast cancer [10, 11], for adrenal insufficiency [12], for prevention of heart disease [13], as antifungal agents [14], and as active ingredients used for the treatment of obesity [15] and AIDS [16]. Recently, the antiviral activity against the herpes simplex virus type I of some steroid glycosides was determined [17].

The therapeutic action of some steroid hormones has been associated with their interaction with intracellular receptors, which act as transcription factors in the regulation of gene expression [18]. It has been reported that some steroids, such as dehydroepiandrosterone (DHEA), progesterone, pregnenolone and its sulfated derivatives [19, 20], as well as, 17β-estradiol, allopregnanolone and its synthetic derivatives (afoxolaner and ganaxolone) are considered neurosteroids, due to their action at the level of the CNS [19].

The physiological activity of steroids depends on their structure, the type, number, spatial orientation, and reactivity of the different functional groups present in the tetracyclic core as well as the oxidation state of the rings. For example, the presence of an oxygenated function in C-11β is crucial for the anti-inflammatory activity; the hydroxyl function in C-17β determines androgenic properties; the aromatization of ring A confers estrogenic effect; and corticosteroids have the 3-keto-4-ene group and the pregnane side chain at C-17 [21, 22].

Currently, about 300 steroid drugs are known, and this number tends to grow. Their production represents the second category in the pharmaceutical market after antibiotics [24, 25]. Nowadays, steroids represent one of the largest sectors in pharmaceutical industry with world markets in the region of US$ 10 billion and the production exceeding 1,000,000 tons per year [23].

The production of steroid drugs and hormones is one of the best examples of the applications that biotransformations have on an industrial scale [3, 21]. Microbiological transformations are an effective tool for the preparation of various compounds [26], which can be difficult to obtain by conventional chemical methods and have been widely used in the bioconversion of steroids [25]. In 1950, the pharmacological effects of cortisol and progesterone were reported, in addition to the hydroxylation of the latter in C-11α using *Rhizopus* species. This began a very important stage in the development of the synthesis of steroids with biological activity [4, 5].

Currently, a great versatility of microbial systems in the pharmaceutical industry for the commercial production of steroids and other drugs is recognized [27, 28]. Several hundreds of microbiological transformations of steroids have been reported in the literature; also, many bioconversions have been incorporated into numerous partial syntheses of new compounds for their evaluation such as hormones or drugs [21, 29–32]. Chemical derivatives of some steroids are reported to have better therapeutic advantages than the starting materials.

However, the main objectives in the research and development of the steroid drug industry currently consist of the detection and isolation of microbial strains with novel activity or more efficient transformation capacity, where genetic engineering and metabolic engineering can play a prominent role in the metabolism of bacteria, fungi, and plants [33–36].

The aim of the present review is to emphasize the importance of biotransformation using microorganisms to obtain steroid compounds with pharmaceutical interest, as a chemical-biological strategy that alternates with the chemical synthesis, and to highlight the chemical reaction made by different types of microorganisms in the functionalization of the steroid skeleton.

2. Microbiological transformations of steroids

In Green Chemistry, biotransformations constitute an important methodology in organic chemistry [37]. The microbiological transformations of steroids have been an essential chemical tool used for the preparation of many intermediaries and in the generation of new drugs, where chemical functionalization-hydroxylation, Baeyer-Villiger oxidation, reduction, isomerization, Michael additions, and condensation

reactions can be carried out in different positions of the steroid skeleton in *chemo-*, *regio-*, and *stereo*selective ways, being very complicated or even impossible by the classic chemical methods. Currently, any stereogenic center of the steroid skeleton can be specifically hydroxylated stereoselectively. Nowadays, *biohydroxylations* in C-11α, 11β, 15α, and 16α are industrially carried out via a microbial hydroxylation with good yields and enantiomeric excess (ee). Below are some of the microbiological transformations performed on different natural and synthetic steroids [25].

In the literature, it is the well-documented *regio-* and *stereo*selective hydroxylation in C-14 with α orientation in progesterone (**1**) and other steroids by well-functioning fungi, such as *Thamnostylum piriforme* (ATCC 8992), *Mucor griseocyanus* (ATCC 1207a), *Actinomucor elegans* (MMP 3132), and *Zygodesmus* sp. (ATCC 14716).

From the incubation of **1** with *T. piriforme,* 14α-hydroxyprogesterone (**2**, 32%) and 9α-hydroxyprogesterone (**3**, 1.4%) were obtained; whereas in the incubation of **1** with *M. griseocyanus,* **2** (13.4%), 7α,14α-dihydroxyprogesterone (**4**, 6.5%) and 6β,14α-dihydroxyprogesterone (**5**, 2.8%) were obtained. In the biotransformation of **1** using *A. fumigatus* after 24 h of incubation, different mono-and dihydroxylated products were obtained: 11α-hydroxyprogesterone (**6**, 33%), 11α,15β-dihydroxyprogesterone (**7**, 17%), 7β,15β-dihydroxyprogesterone (**8**, 14%), 15β-hydroxyprogesterone (**9**), 7β-hydroxyprogesterone (**10**), where **9** and **10** were detected in minimal quantity. Finally, at 72 h, the main products were 7 (48%) and 8 (25%), with the positions 11α and 15β being hydroxylated more easily than the position 7β in **1** [38, 39].

In the incubation of **1** with *Saprolegnia hypogyna*, 4-androstene-3,17-dione (**11**), testosterone (**12**), and testolactone (**13**) were obtained [40]. The compounds **13** (98%) were also obtained from the bioconversion of **1** using *A. sojae* (PTCC 5196). The biotransformation pathway indicating the presence of Baeyer-Villiger monooxygenase (BVMO) can carry out both oxygenative esterification of 20-ketosteroids and oxygenative lactonization of 17-ketosteroids [41]. The compounds 15α-hydroxyprogesterone (**14**, 47%) and 12β,15α-dihydroxyprogesterone (**15**, 25%) were isolated in the biotransformation of **1** using *Fusarium culmorum* [42]. In the biotransformation of **1** using the bacterium, thermophilic *Bacillus stearothermophilus*, four products of monohydroxylation, 20α-hydroxyprogesterone (**16**, 61%), 6β-hydroxyprogesterone (**17**, 21%) and 6α-hydroxyprogesterone (**18**, 14%), and 9,10-seco-pregnen-3,9,20-trione (**19**, 4%), were isolated [43].

An efficient *regio-* and stereoselectivity was observed in the biotransformation of **1** on a large scale by the system *Mucor* 881 (M881) to give the hydroxylated derivatives **6**, 6β,11α-dihydroxyprogesterone (**20**), and 6β-hydroxypregn-4-ene-3,11,20-trione (**21**). In the literature, it is described that species of the genus *Mucor* and *Rhizopus* can hydroxylate said positions but with lower yields. The fungal system M881 showed the ability to carry out hydroxylation at 6β and 11α positions of 4-ene-3-one steroids (**1**, **11**, **12** and **211**) [44].

Recently, it was reported that in the biotransformation of **1** using *Penicillium aurantiogriseum* for 10 days, **11** and androsta-1.4-dien-3,17-dione (**22**) were obtained. These products were observed in the biotransformation of **1** using *Bacillus sphaericus*; the hydroxylation in C-17 was mainly observed [45, 46]. Biotransformation of **1** using *Geobacillus gargensis* (DSM 15378) has resulted in the production of secoderivatives: **19** and **23** (9,10-seco-4-pregnene-20α-hydroxy-3,9-dione), which are produced by the rupture of the ring B of **1** (**Figure 1**) [47]. Secosteroids are an important group, which exhibits a variety of different biological activities [48, 49].

In the biotransformation of 5β-dihydroprogesterone (**24**) using *T. piriformis,* 14α-hydroxy-5β-pregnan-3,20-dione (**25**, 11.8%), 3β,14α-dihydroxy-5β-pregnan-20-one (**26**, 0.5%), and 14α,15β-dihydroxy-5β-pregnan-3,20-dione (**27**, 0.4%) were characterized, while in the biotransformation of 3β-hydroxy-5β-pregnan-20-one (**28**), **26**

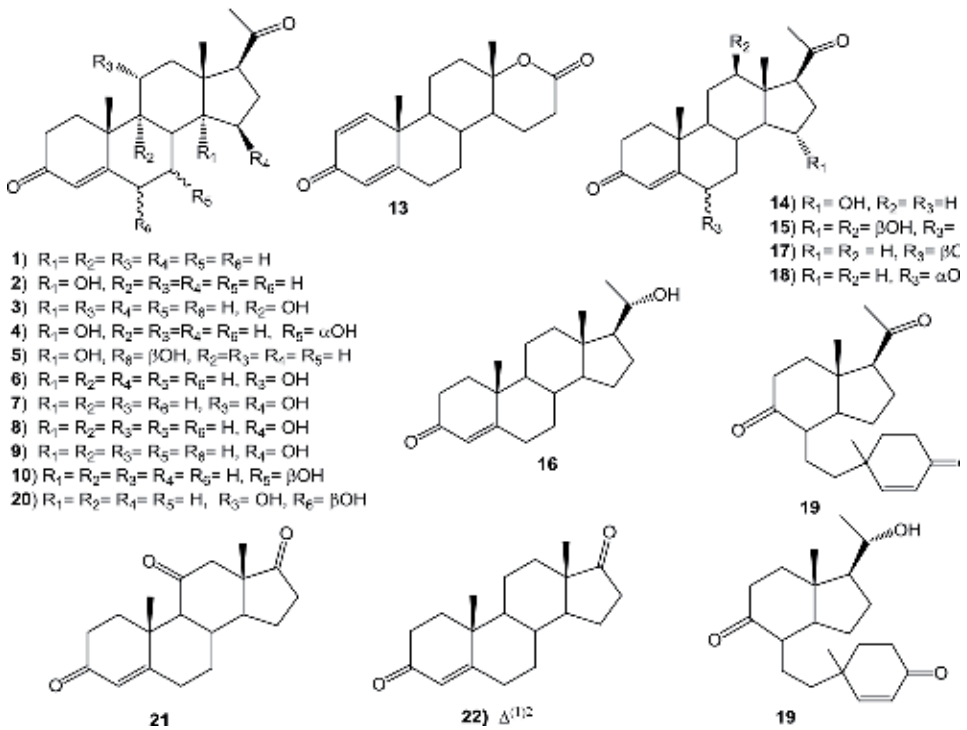


Figure 1.
*Biotransformation products of progesterone (**1**).*

(0.6%) and 3β,9α,14α-trihydroxy-5β-pregnan-20-one (**29**, 16%) were isolated, after being incubated for 96 h. The microbiological transformation of **28** using *Actinomucor elegans* produced the compounds **25** and **28** in lower yield than *T. piriforme* and a minor product identified as 3β,9α-dihydroxy-5α-pregnan-20-one (**30**) (**Figure 2**) [38].

The biotransformation of 16-dehydroprogesterone (4,16-pregnadien-3,20-dione, **31**) using *Mucor piriformis* has been reported to give different hydroxylation products: 14α-hydroxypregna-4,16-dien-3,20-dione (**32**, 1%), 7α,14α-dihydroxypregna-4,16-dien-3,20-dione (**33**, 78%), 3β,7α,14α-trihydroxy-5α-pregna-16-en-20-one (**34**, 3%), and 3α,7α,14α-trihydroxy-5α-pregna-16-en-20-one (**35**, 2%); while the microsomes

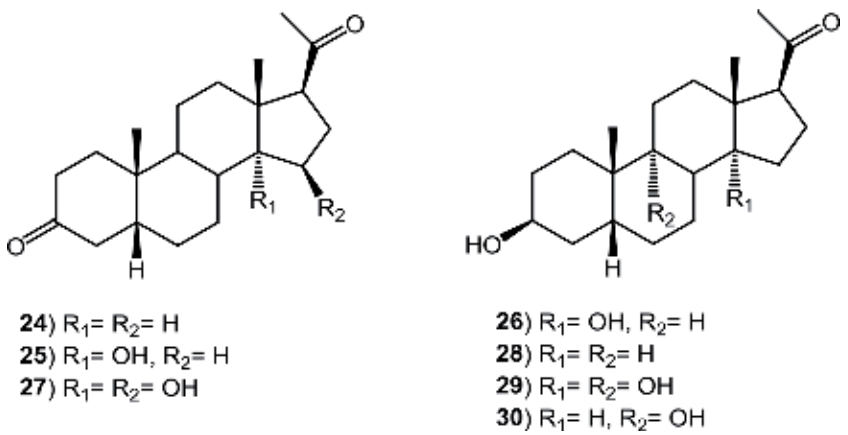


Figure 2.
*Biotransformation products of 5β-dihydroprogesterone (**24**).*

prepared from **31** transformed the hydroxylate to 14α-hydroxy derivative (**32**). Incubation of **32** with *M. piriformis* resulted in the formation of **33–35** (**Figure 3**) [50].

In contrast, in the biotransformation of 17α-hydroxyprogesterone (**36**) using *M. piriformis*, after 48 h of incubation, four compounds were obtained: 17α,20α-dihydroxypregn-4-en-3-one (**37**, 19%), 7α,17α-dihydroxypregn-4-en-3,20-dione (**38**, 25%), 6β,17α,20α-trihydroxy-pregn-4-en-3-one (**39**, 18%), and 11α,17α,20α-trihydroxypregn-4-en-3-one (**40**, 25%); it was observed that *M. piriformis* was able to hydroxylate the C-6, C-7, C-11, and C-14 positions stereospecifically, in addition to reducing the 4-en-3-one system in ring A and the keto group of C-20 (**Figure 4**) [50]. The biotransformation of **36** using *Fusarium culmorum* led to the formation of **14** (47%) and **15** (25%) [42].

Pregnenolone (3β-hydroxypregn-5-en-20-one, **41**), the precursor of many steroid hormones, was biotransformed by *Mucor piriformis* to obtain two metabolites, 3β,7α-dihydroxypregn-5-en-20-one (**42**) and 3β,7α,11α-trihydroxypregn-5-en-20-one (**43**) [51], where **43** (46.4%) was also a bioconversion product of **41** using *Mucor circinelloides* var. *lusitanicus* [52]. Two metabolites of pregnenolone (**41**) obtained from biotransformation of *B. cinereae* were characterized as 3β,11α,16β-trihydroxypregn-5-en-20-one (**44**, 39%) and 11α,16β-dihydroxypregn-4-en-3,20-dione (**45**, 6%). The formation of the hydroxylation products in C-11 and C-16 by *B. cinereae* can be determined by the presence of the acetyl group in C-20 [53]. The biotransformation of **41** using different microorganisms (*Cunninghamella elegans*, *R. stolonifer*, and *G. fujikuroi*) was reported by Choudhary et al. [54]. Incubation of **41** with *C. elegans* produced 3β,7β,11α-trihydroxypregn-5-en-20-one (**46**, 28%), 3β,6α,11α,12β,15β-pentahydroxypregn-4-en-20-one (**47**, 4%), and 3β,6β,11α-trihydroxypregn-4-en-20-one (**48**, 2%), while incubation with *G. fujikuroi,* two products 3β,7β-dihydroxypregn-5-en-20-one (**49**, 3%) and 6β,15β-dihydroxypregn-4-en-3,20-dione (**50**, 2%) were obtained. In the microbiological transformation of **41** using different *Bacillus* strains, **42**, **49**, and 7-oxo-pregnenolone (**51**) were the major products obtained [55], while by using *Fusarium oxysporum* var. *cubense,* **42** was the only product obtained [56]. The biotransformation of pregnenolone acetate (**52**) using *C. elegans* generated **41**, **22**, 6β,15β-dihydroxyandrosta-4-en-3,17-dione (**53**), and 11α,15β-dihydroxypregn-4-en-3,20-dione (**54**), while by using *R. stolonifer*, 11α-hydroxypregn-4-en-3,20-dione (**55**) and **53** were obtained (**Figure5**) [54].

The microbiological transformation of the racemic mixture of 13-ethyl-17β-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (**56**) was tested with different fungi *Rhizopus nigricans*, *R. arrhizus*, *Aspergillus niger*, *A. ochraceus*, and *Curvularia lunata.* The bioconversion of the racemic mixture of **53** by *R. arrhizus* produced only one major product, (±)-13-ethyl-10β,17β-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one

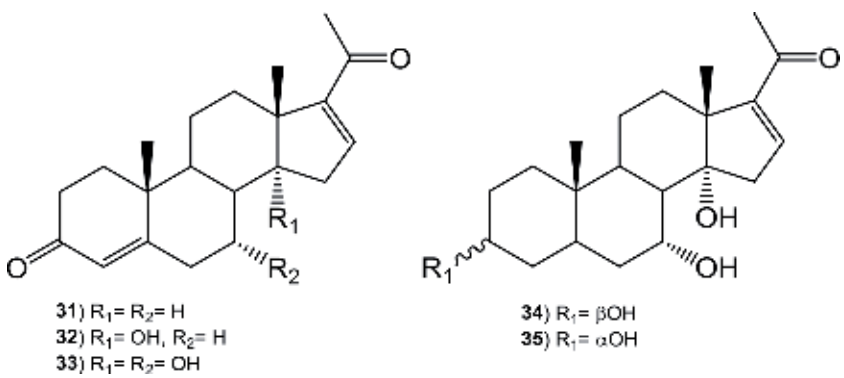


Figure 3.
*Biotransformation products of 16-dehydroprogesterone (***31***).*

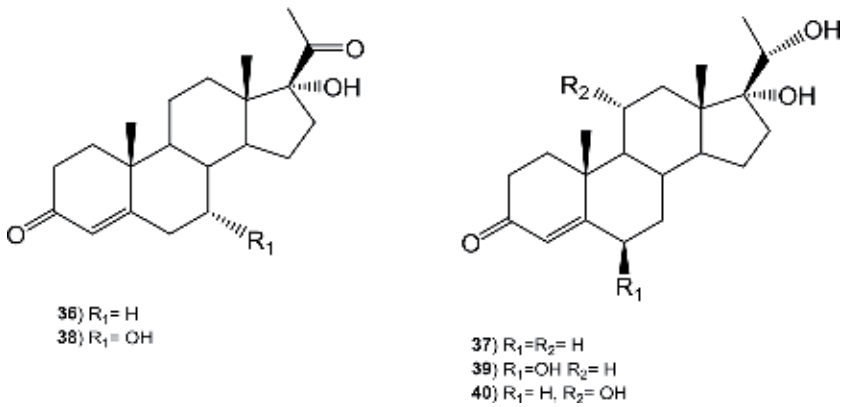


Figure 4.
*Biotransformation products of 17α-hydroxyprogesterone (**36**).*

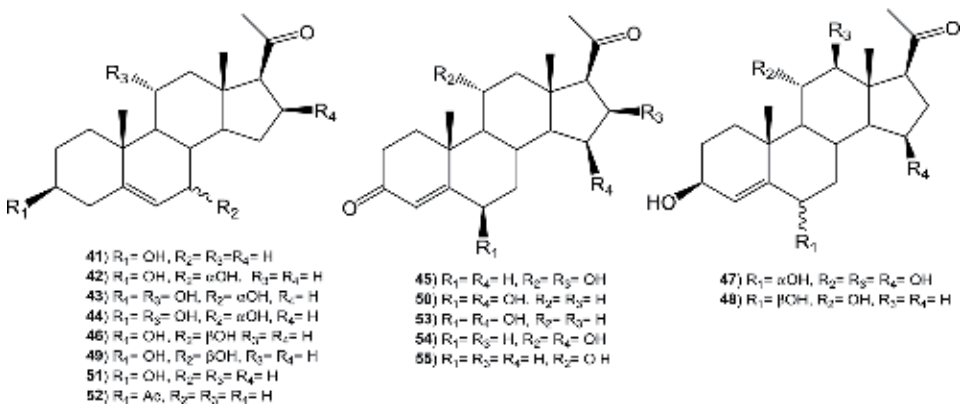
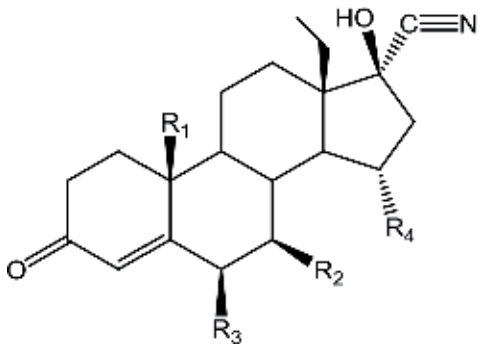


Figure 5.
*Biotransformation products of pregnenolone (**41**) and acetyl derivate (**52**).*

(**57**, 28.4%), whereas *R. nigricans*, *A. niger*, and *C. lunata* biotransformed **56** to **57** more slowly and inefficiently [57].

The racemic mixture (±)-13-ethyl-7β,17β-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (**58**, 4.3%) was obtained as product of incubating mixture **56** with *A. ochraceus*; none of the fungi tested were able to differentiate the two enantiomers of **56** in the course of the hydroxylation reaction; in addition, the absence of the hydroxylated derivative in C-11 is due to the presence of the ethyl group in C-13 or the ethynyl group in C-17 [57]. The microbiological transformation of the racemic mixture and the dextro enantiomer of compound **56** has been described using different species of *Cunninghamella* [58]. For example, the transformation of the racemic mixture of **56** by *C. blakesleeana* (AS 3.910) produced **57** (5.3%), 13-ethyl-6β,17β-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (**59**, 3.6%), 13-ethyl-15α,17β-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (**60**, 3.0%), and 13-ethyl-6β,10β,17β-trihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (**61**, 3.6%), while by using *C. echinulata* (AS 3.1990), **61** (3.2%), **57** (1.2%), and enantiomer dextro of **58** (2.9%) were obtained. The transformation of the enantiomer dextro of **56** using *C. blakesleeana* produced **57** (1.2%), **58** (2.9%), and **61** (3.2%), by using *C. echinulata*, the same compounds were obtained but in lower yield. Therefore, the microbial transformation of the racemic mixture and the *d*-enantiomer of **56** using different *Cunninghamella* species gave poor yields and poor resolutions, which were obtained for the hydroxylation reaction (**Figure 6**) [58].



56) R_1= R_2= R_3= R_4= H
57) R_1= OH, R_2= R_3= R_4= H
58) R_1= R_3= R_4= H, R_2= OH
59) R_1= R_2= R_4= H, R_3= OH
60) R_1= R_2= R_3= H, R_4= OH
61) R_1= R_3= OH, R_2= R_4= H

Figure 6.
*Biotransformation products of (+)-13-ethyl-17β-hydroxy-18, 19-dinor-17α-pregn-4-en-20-yn-3-ona (***56***).*

The biotransformation of danazol (17β-hydroxy-17α-pregna-2,4-dien-20-yno-[2,3-d]-isoxazole, **62**), a heterocyclic steroid drug in which an isoxazole ring is fused with ring-A of a steroid nucleus, using *Fusarium lini*, *A. niger* and *Cephalosporium aphidicola* yielded 17β-hydroxy-2-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**63**) and 17β-hydroxy-2-(hydroxymethyl)-17α-pregn-1,4-dien-20-yn-3-one (**64**); while *Bacillus cereus* afforded **64**, as the only product [59]. Microbial transformation of danazol (**62**) using *C. blakesleeana* yielded four compounds: 14β,17β-dihydroxy-2-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**65**, 1.2%), 1α,17β-dihydroxy-17α-pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**66**, 1.2%), and 6β,7β-dihydroxy-17α-pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**67**, 0.8%) and **64** (1.2%). This involves hydroxilations al C-1, C-6 and C-15, whereas oxidation at C-3, and N-O bond cleavage has also occurred (**Figure 7**) [60].

Norethisterone (17α-ethynyl-19-nortesterone, **68**) is a potent progestin used as a contraceptive agent; its biotransformation with *Cephalosporium aphidicola* (IMI 68689) produced the aromatization of ring A that yielded 17α-ethynylestradiol (**69**), whereas **69** was biotransformed by *Cunninghamella elegans* (NRRL 1392) producing the compounds 19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3,4,17β-triol (**70**), 19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3,7α,17β-triol (**71**), 19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3,11α,17β-triol (**72**), 19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3,6β,17β-triol (**73**), and 19-nor-17α-pregna-1,3,5(10)-trien-20-yn-3,17β-diol-6β-methoxy (**74**) (**Figure 8**) [61].

Mestranol (**75**) and 17β-methoxymestranol (**76**) are the mono- and dialkylated derivatives of **69**, respectively. In incubating **75** with *C. elegans*, two hydroxylated compounds were obtained: 6β-hydroxymestranol (**77**, 2.8%) and 6β,12β-dihydroxymestranol (**78**, 3.6%), inferring that the presence of the methoxyl group in C-3 reduces the number of biotransformation products and introduces hydroxyl groups in C-6 and C-12 with β orientation, while **76** was not biotransformed due to the presence of the methoxyl group in C-17 (**Figure 9**) [62].

Microbial transformation of 6-dehydroprogesterone (**79**) using *A. niger* yielded five metabolites: 6β-chloro-7α,11α-dihydroxypregna-4-en-3,20-dione (**80**, 1.0%), 7α-chloro-6β,11α-dihydroxypregna-4-en-3,20-dione (**81**, 1.33%), 6α,7α,-epoxy-11α-hydroxypregna-4-en-3,20-dione (**82**, 1.33%), 6α,7α,-epoxy-pregna-4-en-3,20-dione (**83**, 2.0%), and 11α-hydroxypregna-4,6-dien-3,20-dione (**84**, 2.33%). Compound 11α-hydroxyandrosta-4,6-dien-3-one (**85**, 15.4%) was obtained through whole cell biotransformation of **79** by *G. fujikuroi* (ATCC 10704). The formation of **80** and **81** is an interesting finding. This route provides an efficient method for the obtention of chlorohydrins from alkene functionality [63]. The compound **84** was obtained through the microbial transformation of **79** using *R. nigricans* [64], *Nigrospora sphaerica*, *Mucor racemosus*, and *Botryosphaeria obtusa*. 6-dehydroprogesterone (**79**)

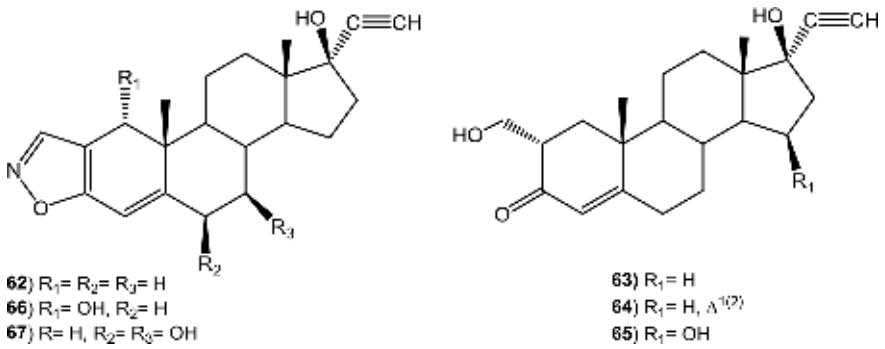


Figure 7.
*Biotransformation products of danzol (***62***)*

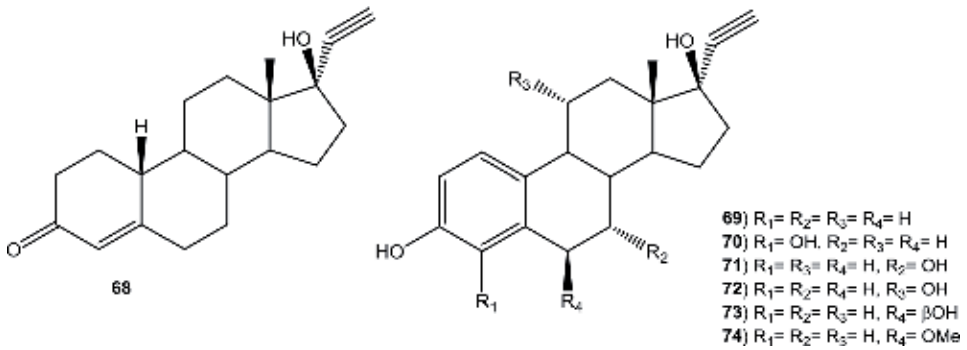


Figure 8.
*Biotransformation products of norethisterone (***68***) and 17α-ethinylestradiol (***69***).*

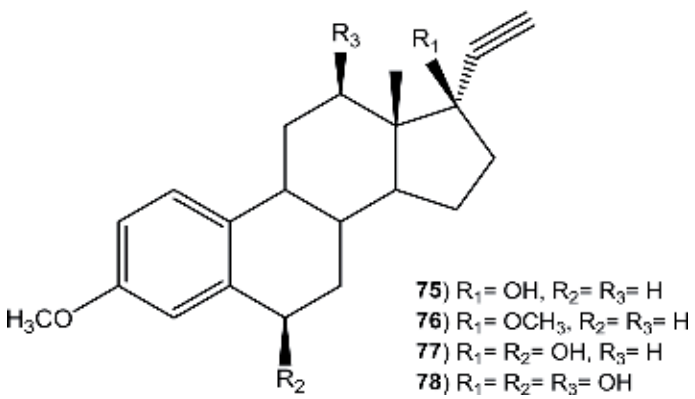


Figure 9.
*Biotransformation of products of mestranol (***75***).*

is a synthetic derivate of progesterone. *Botryodiplodia theobromae* was used for the synthesis of 6-DPH from progesterone (**Figure 10**) [65].

Incubation of melengestrol acetate (**86**) with *C. blakesleeana*, which provides an route for the monohydroxylation of the (**86**) at C-11, yielded a 17α-acetoxy-11β-hydroxy-6-methylenepregna-4,6-diene-3,20-dione (**87**) (**Figure 11**) [66].

Biotransformation of 3β-hydroxy-17β-carboxyethyl-5β-androstenol (**88**) using *T. pyriformis* resulted in the mixture of

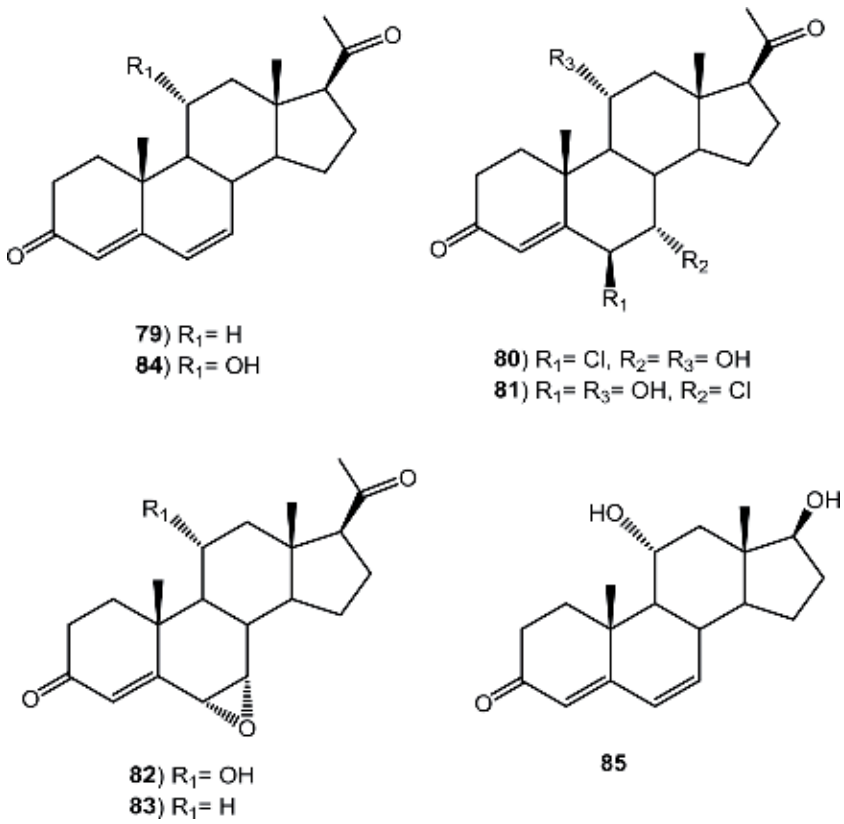


Figure 10.
*Biotransformation products of 6-dehydroprogesterone (***79***).*

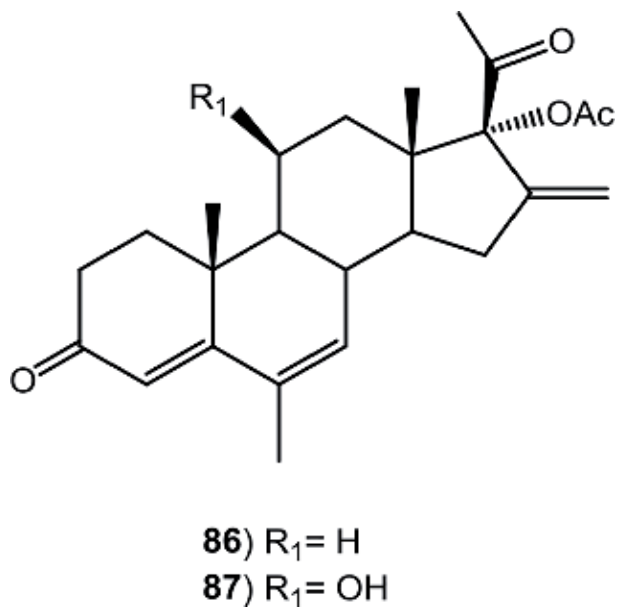


Figure 11.
*Biotransformation products of melengestrol acetate (***86***).*

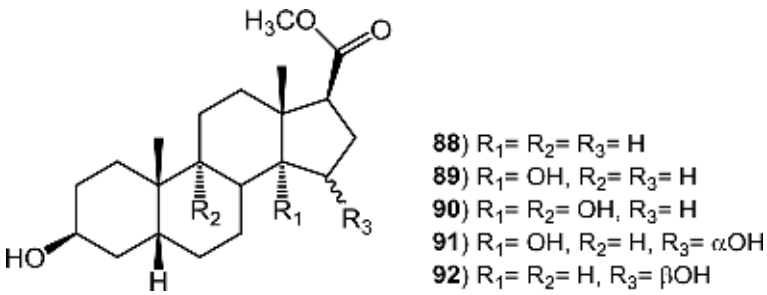


Figure 12.
*Biotransformation products of 3β-17β-carboxyethyl-5β-androsteno (***88***).*

3β,14α-dihydroxy-17β-carboxyethyl-5β-androstenol (**89**, 9%) with 9α,14α-dihydroxy derivative (**90**, 12%) and two minor products 14α,15α-dihydroxy (**91**) and 15β-hydroxy (**92**). Compound **92** was identified as a product of biotransformation using *A. elegans*, *M. griseocyamus*, and *Zygodesmus sp.* (**Figure 12**) [38].

Androst-4-en-3,17-dione (**11**), which plays an important role in the metabolism of drugs, among many other functions, was biotransformed using *M. piriformis* to give one main product, 6β-hydroxyandrost-4-en-3,17-dione (**93**, 13%), and four minor products, 14α-hydroxyandrost-4-en-3,17-dione (**94**, 2%), 7α-hydroxyandrost-4-en-3,17-dione (**95**, 2%), testosterone (**12**, 3%), and 6β-hydroxytestosterone (**96**, 1%). In the biotransformation of **11** using *M. griseocyamus* **94** (9%), **95** (4%) and 14α-hydroxytestosterone (**97**, 9%) were the major products obtained; likewise, **11** and **93** were identified in the mixture of biotransformation products [67]. From the incubation of **11** with *M. piriformis*, **94–97** and 7α,14α-dihydroxytestosterone (**98**) were obtained [38]. Hydroxylated steroids in C-9 are important intermediaries in the synthesis of highly effective anti-inflammatory drugs. The microbiological transformation of **11** to 9α-hydroxyandrost-4-en-3,17-dione (**99**) was studied using *Rhodococcus* sp. in a low-nutrient culture medium at a fixed pH (**Figure 13**) [68]. When **11** was incubated with *Bacillus* strain HA-V6–3, the metabolites **12**, **93–97**,

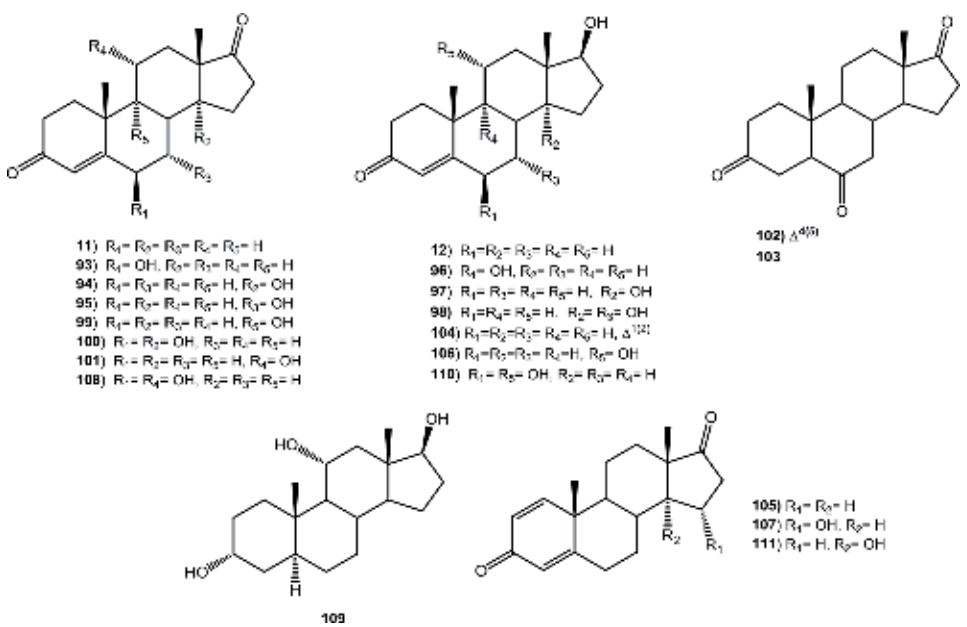


Figure 13.
*Biotransformation products of androst-4-en-3, 17-diona (***11***).*

6β,14α-dihydroxyandrost-4-en-3,17-dione (**100**), 11α-hydroxyandrost-4-en-3,17-dione (**101**), androst-4-en-3,6,17-trione (**102**), and 5α-androst-3,6,17-trione (**103**) were produced as described by Schaaaf and Dettner [69].

In the bioconversion of **11** using *C. aphidicola*, **93** and **94** were obtained [70], while in the fermentation of **11** using *Curvularia lunata,* the products **101** (4%), 17β-hydroxyandrost-1,4-dien-3-one (**104**, 4.4%), androsta-1,4-dien-3,17-dione (**105**, 3%), 11α,17β-dihydroxyandrost-4-en-3-one (**106**, 4%), and **107** (15α-hydroxyandrost-1,4-dien-3,17-dione, 2.8%) were obtained (**Figure 13**) [71]. Biotransformation of **11** using *Beauveria bassiana* was studied in times and with culture media at different pH (pH 6 and 7) [72]. At pH 6, two products were obtained: **106** and 6β,11α-dihydroxyandrost-4-en-3,17-dione (**108**), where the stereoselective hydroxylation was observed at C-11α and C-6β; while at pH 7, the compounds **12**, **106**, 3α,11α,17β-trihydroxy-5α-androstane (**109**), and 6β,11α,17β-trihydroxy-androst-4-en-3-one (**110**) were obtained. Products **93** (14%) and **94** (75%) were isolated from the biotransformation of **11** using *Chaetomium* sp. (**Figure 13**) [73].

Obtaining hydroxylated derivatives in a specific position is one of the objectives of the steroid industry; for example, 14α-hydroxysteroids are shown to have anti-inflammatory, contraceptive, and antitumor activities. With the biotransformation of **11** and **105** using different strains of the fungus, *C. lunata* allowed in the case of **11**, the production of a major product, **94**; while with **105,** 14α-hydroxyandrost-1,4-dien-3,17-dione (**111**, 70%) was obtained (**Figure 13**) [74].

Androsta-1,4-dien-3,17-dione (**105**) is a useful precursor in the chemical or microbiological preparation of other steroid hormones and pharmaceutical. Transformation of **105** by *Colletotrichum lini* (As3.486) produced the hydroxylated compounds at C-11α and C-15α: 15α-hydroxyandrost-1,4-dien-3,17-dione (**107**), 11α,15α-dihydroxyandrost-1,4-dien-3,17-dione (**112**), and 15α,17β-dihydroxyandrost-1,4-dien-3-one (**113**) (**Figure 14**) [75].

Testosterone (**12**) was metabolized by *M. griseocyamus* and *T. piriforme*. In the biotransformation of **12** using *M. griseocyamus*, **97** (35%) and other products were obtained, where **94** was identified as the major product. Conversely, the microbiological transformation of **12** using *T. piriforme* produced **97** (10%), as the main product at 24 h; after 72 h of biotransformation, four products were obtained: **93** (13%), **96** (7%), **97** (13%), and **111** (5%). It was discovered that *T. piriforme* produced smaller quantity of 14α-hydroxy derivatives (**Figure 15**) [38].

In the biotransformation of **12** using *Nectria haematococca*, four substances were isolated, whose performance was dependent on the incubation time; majority of the products were produced at 72 h. The hydroxylated derivatives in C-11 with α

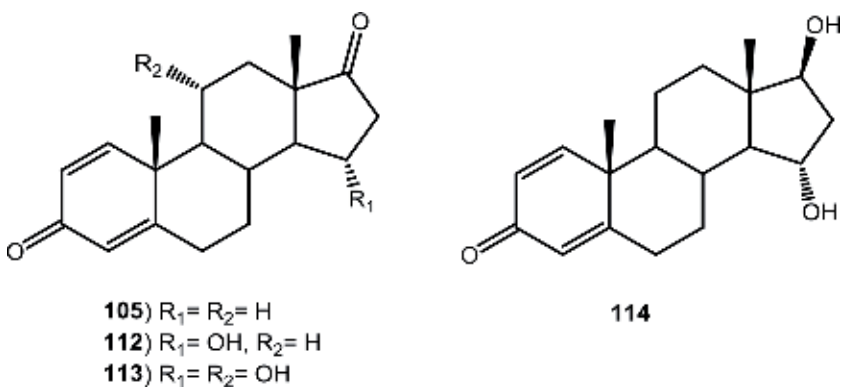


Figure 14.
*Biotransformation products of androsta-1,4-dien-3, 17-dione (***105***).*

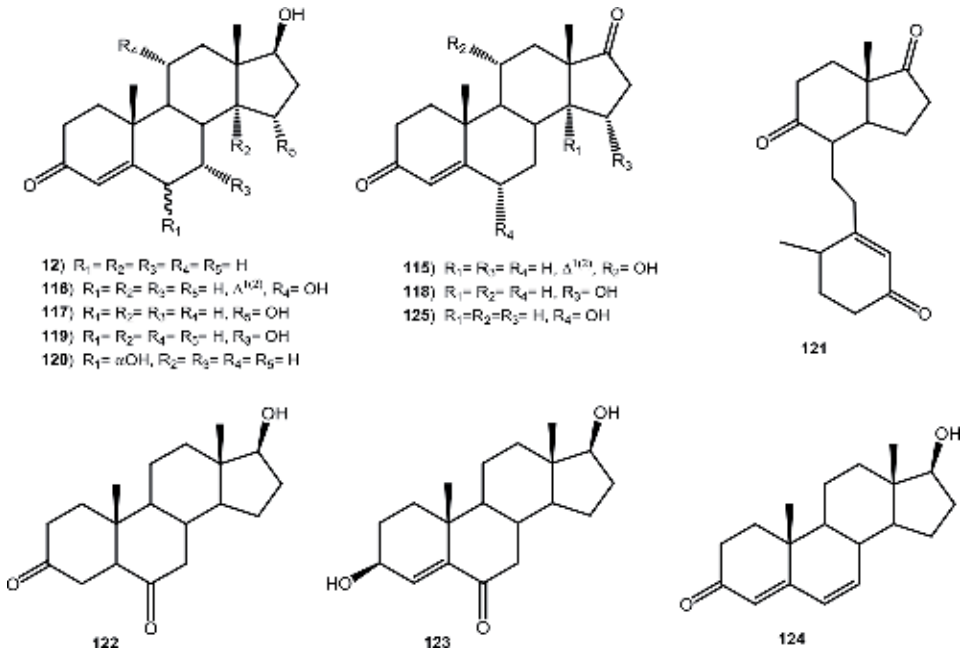


Figure 15.
*Biotransformation products of testosterone (**12**).*

orientation and dehydrogenation in C_1-C_2 resulted in the following compounds: 11α-hydroxyandrost-1,4-dien-3,17-dione (**114**, 8.0%), 11α,17β-dihydroxyandrost-1,4-dien-3-one (**115**, 4.3%), **101** (1.9%), and **104** (2.3%) [76]. Incubation of **12** with *Fusarium culmorum* produced **93** (10%) and **96** (32%) with hydroxylated derivatives at C-6β, including the products, 15α,17β-dihydroxyandrost-4-en-3-one (**116**, 22%) and 15α-hydroxyandrost-4-en-3,17-dione (**117**). Selective hydroxylation of **103** at C-6 with a β orientation and allylic position at the unsaturated 3-keto-system is favored by the system π and the presence of the hydroxyl group at C-17, while hydroxylation at C-15 is a very frequent process carried out by fungi of the genus *Fusarium* [42]. Metabolites **11**, **85**, **105,** and **115** were obtained as oxidation and hydroxylation products of **12** using the fungus *F. oxysporum* var. cubense [56]. The fungus, *Cephalosporium aphidicola,* was hydroxylated with **12** to give the products **96** (47%) and **97** (3%), with hydroxylated derivatives in C-6β and C-14α, respectively [70]. Incubation of **12** with *C. lunata* and *Pleurotus ostreatus* yielded compounds **11** (17%) and **115** (13%), respectively [77]. The phytopathogenic fungus, *Botrytis cinerea,* produced 7β,17β-dihydroxyandrost-3-one (**118**, 73%), as the only biotransformation product of **12**. It seems that the presence of the hydroxyl group in C-17 in the androstane skeleton directed the hydroxylation at C-7 with a β orientation (**Figure 15**) [53].

In the biotransformation of **12** using *Bacillus stearothermophilus*, thermophilic bacterium, the major product obtained was **11** (90.2%); it was generated by the oxidation of C-17, and the hydroxylated derivatives of **11** in C-6 (**93**, C-6β, 1.1%) and (**119**, C-6α, 0.9%) include two monohydroxy derivatives of **12**, **96** (C-6β, 3.9%) and **120** (C-6α, 3.9%). This indicates that hydroxylation with α orientation in C-6 may be a common action of some thermophilic bacteria [78]. Biotransformation of **11** using *B. stearothermophilus* in the presence of hydrolase inducers—salicylic acid, chloramphenicol, cyclodextrin, dexamethasone, riboflavin, and rifampicin—resulted in obtaining a higher concentration of the compounds: 9,10-seco-4-androst-3,9,17-trione (**121**), 5α-androst-3,6,17-trione (**103**), 17β-hydroxy-5α-androst-3,6-dione (**122**), 3β,17β-dihydroxyandrost-4-en-6-one (**123**), and 17β-hydroxyandrost-4,6-dien-3-one

(**124**). For example, the presence of glucose and cycloheximide favored the obtaining of **123**, while the production of **124** was achieved in the presence of rifampicin [79]. The products isolated from the biotransformation of **12** using *Chaetomium sp.* were **93** (21%), **94** (39%), and **99** (19%); after 24 h of incubation, the presence of **11** was detected. Janeczko et al. [73] concluded that the steric factors associated with the substrate determine the location and orientation of the hydroxyl group. For example, the carbonyl group in C-17 at **11** directs the entry of the hydroxyl group at C-14 with α orientation, while the hydroxylation in C-6β is favored by the presence of the hydroxyl group in C-17, as in **12**. In the case of progesterone (**1**), which has an acyl group, dihydroxylated derivatives were observed in C-6 and C-14 (**Figure 15**) [73].

Incubation of **11** and **12** with *C. lini* ST-1 displayed different catalytic characteristics. Biotransformation of **11** afforded two products: 15α-hydroxyandrost-4-en-3,17-dione (**117**, 5%) and 11α,15α-dihydroxyandrost-4-en-3,17-dione (**125**, 64%), while **12** yielded 15α-hydroxyandrost-4-en-3,17-dione (**117**, 60%). Incubation of **1** resulted in the isolation of **14**. Wu et al. [80] concluded that the different hydroxylation sites between **11** and **12** suggested that the hydroxyl group or carbonyl group on the substrate at C-17 had influence on the location of introduced hydroxyl groups (**Figure 15**).

Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, **126**) endogenous prohormone secreted by the adrenal glands is a precursor of androgens and estrogens. Incubation with *M. piriformis* allowed the isolation of five compounds: 3β,17β-dihydroxyandrost-5-ene (**127**), 3β,7α-dihydroxyandrost-5-en-17-one (**128**), 3β-hydroxyandrost-5-en-7,17-dione (**129**), 3β,17β-dihydroxyandrost-5-en-7-one (**130**), and 3β,7α,17β-trihydroxyandrost-5-ene (**131**). The action of the fungus was the stereospecific hydroxylated products at C-7α (**128** and **131**) and the reduction of the carbonyl group at C-17 [51]. From the microbiological transformation of **126** using *Rhizopus stolonifer*, six poducts were isolated: **127** (20%), **128** (12%), **129** (20%), 3β,17β-dihydroxyandrost-4-ene (**132**, 12%), 17β-hydroxyandrost-4-en-3-one (**133**, 34%), and 3β,11β-dihydroxyandrost-4-en-17-one (**134**, 15%) [81]. *Fusarium oxysporum* biotransformed to **126** in a mixture of four hydroxylated derivatives (**127**–**129** and **130**), which were characterized as their acetylated derivatives; the hydroxylation was favorably in C-7 stereospecifically (α orientation) in the 3β-hydroxy-Δ^5-steroids, while *Colletotrichum musae* biotransformed to **126**–**127** by reducing the carbonyl group in C-17 (**Figure 16**) [56].

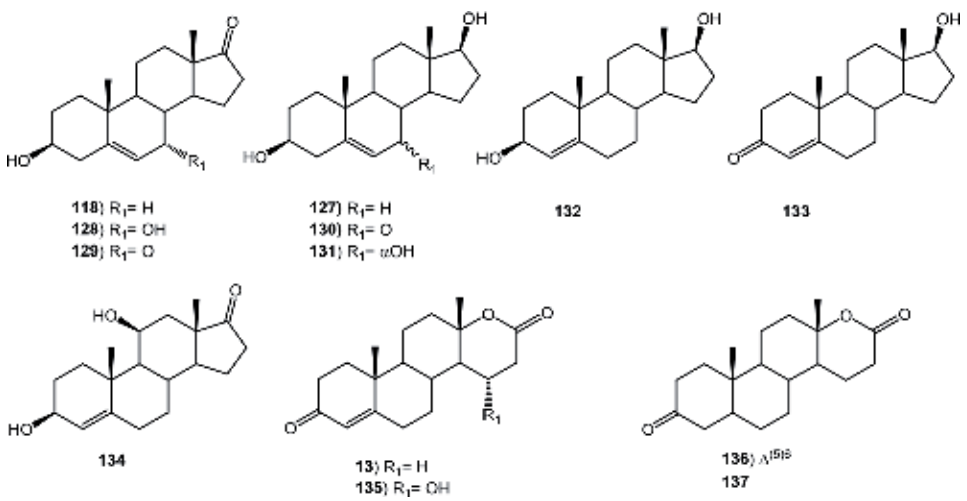


Figure 16.
*Biotransformation products of dehydroepiandrosterone (**126**).*

In the biotransformation of **126** using *Penicillium griseopurpureum* and *P. glabrum*, the following was produced; hydroxylated derivatives in C-7α (**95**), C-14α (**94**) and C-15α (**117**), with **11** being the main product. In addition, *P. griseopurpureum* generated products for the Baeyer Villiger oxidation to give the lactone D ring (testolactone, **13**) and its hydroxylated derivative at C-15α (15α-hydroxy-17α-oxa-D-homo-androst-4-en-3,17-dione, **135**); while *P. glabrum* generated the compounds, 3β-hydroxy-17α-oxa-D-homo-androst-5-en-17-one (**136**) and 3β-hydroxy-17α-oxa-D-homo-5α-androstan-17-one (**137**) (**Figure 16**) [82].

The biotransformation of 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (ethisterone, **138**) and 17α-ethyl-17β-hydroxyandrost-4-en-3-one (**139**) was described using the fungi *Cephalosporium aphidicola* and *Cunninghamella elegans*. The bioconversion of **138** using *C. aphidicola* yielded 17α-ethynyl-17β-hydroxyandrost-1,4-dien-3-one (**140**, 5.5%), while by using *C. elegans*, 17α-ethynyl-11α,17β-dihydroxyandrost-4-en-3-one (**141**, 3.4%) was obtained. The biotransformation of **138** using *C. aphidicola* generated 17α-ethyl-17β-hydroxyandrost-1,4-dien-3-one (**142**, 2.2%). In contrast, when incubating **139** with *C. elegans*, two new products were obtained: 17α-ethyl-11α,17β-dihydroxyandrost-4-en-3-one (**143**, 2.8%) and 17α-ethyl-6α,17β-dihydroxy-5α-androstan-3-one (**144**, 1.6%) (**Figure 17**) [83].

Adrenosterone (**145**) is an inhibitor of the enzyme estrogen synthetase responsible for the formation of estrogen, and it has a great clinical application. Biotransformation of **145** using *C. aphidicola* produced androst-1,4-dien-3,11,17-trione (**146**, 3%), 17β-hydroxyandrost-4-en-3,11-dione (**147**, 2%), and 17β-hydroxyandrost-1,4-dien-3,11-dione (**148**, 17%). **145** (11.2%) and **12** (8.1%) were obtained from the biotransformation of **145** using *Fusarium lini*, while **147** (36.8%) was obtained from the biotransformation of **145** using *Trichothecium roseum* (**Figure 18**) [84].

The biotransformation of mesterolone (1α-methyl-17β-hydroxy-5α-androst-3-one, **149**), a synthetic androgenic steroid, was performed using different fungi as described by Choudhary et al. [85]. From the biotransformation of **149** using *C. aphidicola*, the compounds 1α-methyl-5α-androst-3,17-dione (**150**), 1α-methyl-5α-androst-3,17-diol (**151**), and 1α-methyl-15α-hydroxy-5α-androst-3,17-dione (**152**) were obtained. Incubation of **149** with *Fusarium lini* produced the compounds **152**, 1-methyl-5α-androst-1-en-3,17-dione (**153**), 1α-methyl-6α,17β-dihydroxy-5α-androst-3-one (**154**), 1α-methyl-15α,17β-dihydroxy-5α-androst-3-one (**155**), and 1-methyl-15α,17β-dihydroxy-5α-androst-1-en-3-one (**156**). The products obtained from the biotransformation of **149** using *R. stolonifer* were **150**, **154**, **156**, 1α-methyl-7α,17β-dihydroxy-5α-androst-3-one (**157**), and 1α-methyl-11α,17β-dihydroxy-5α-androst-3-one (**158**) [85]. Bioconversion of **149** using *C. blakesleeana* produced

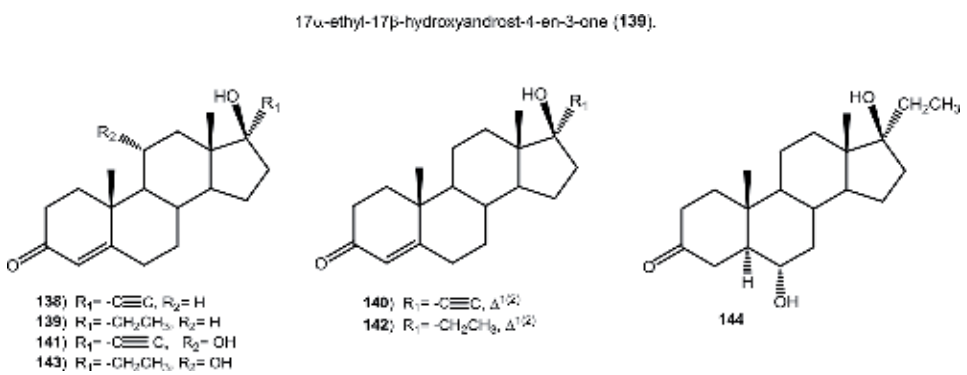


Figure 17.
*Biotransformation products of 17α-ethynyl-17β-hydroxyandrost-4-en-3-one (***138***) and 17α-ethyl-17β-hydroxyandrost-4-en-3-one (***139***).*

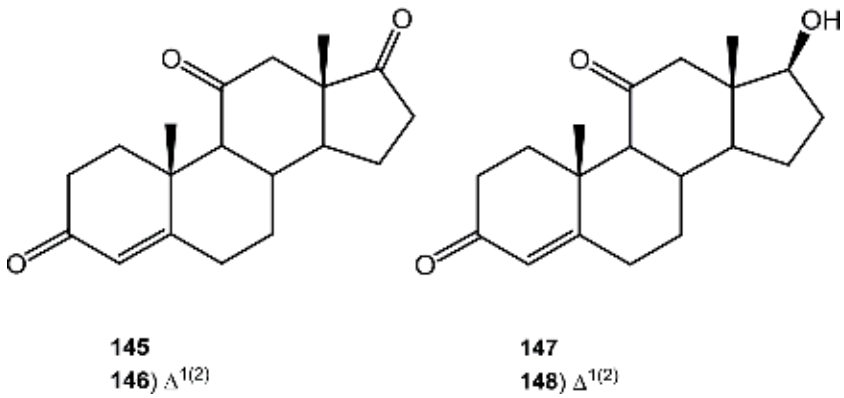


Figure 18.
*Biotransformation products of andresterone (***145***).*

seven biotransformation products, such as **154**, **157, 158**, in addition to 1α-methyl-1β,11β,17β-trihydroxy-5α-androst-3-one (**159**), 1α-methyl-7α,11β,17β-trihydroxy-5α-androst-3-one (**160**), 1α-methyl-1β,6α,17β-trihydroxy-5α-androst-3-one (**161**), and 1α-methyl-1β,11α,17β-trihydroxy-5α-androst-3-ona (**162**). *Macrophomina phaseolina* biotransformed **149** to obtain 1α-methyl-17β-hydroxy-5α-androst-3,6-dione (**155**) [86]. Additionally, the biotransformation of **141** using *C. blakesleeana* (ATCC 8688A) yielded three metabolites: 1α-methyl-11β,14α,17β-trihydroxy-5α-androstan-3-one (**163**, 0.4%), 1α-methyl-7β,17β-dihydroxy-5α-androstan-3-one (**164**, 0.47%), and 1α-methyl-17β-hydroxy-5α-androstan-3,7-dione (**165**, 0.67%). *C. blakesleeana* catalyzed the β-hydroxylation in C-11, and dihydroxylation and oxidations at various positions of steroid skeleton (**Figure 19**) [87].

In the microbiological transformation of 3-hydroxyestra-1,3,5-(10)-trien-17-one (**166**) using *Fusarium oxysporum* var. *cubense*, the compounds, reduced in C-17 (3,17-dihydroxyestra-1,3,5-(10)-triene, **167**) and hydroxylated in C-15 (3,15α-dihydroxiestra-1,3,5-(10)-triene, **168**), were isolated (**Figure 20**) [56].

Prednisone (**169**) is a synthetic corticosteroid (prodrug) used for the treatment of autoimmune, inflammatory and kidney diseases, among others.

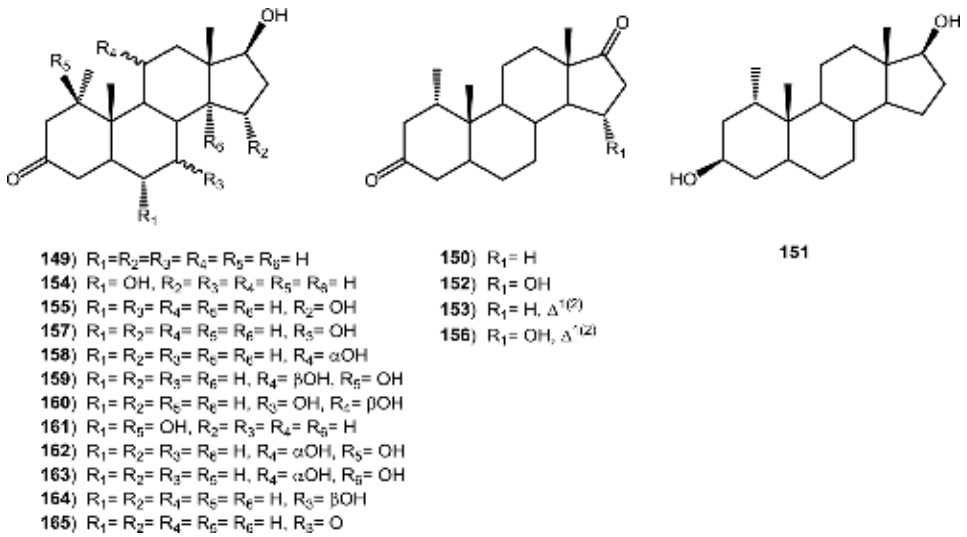
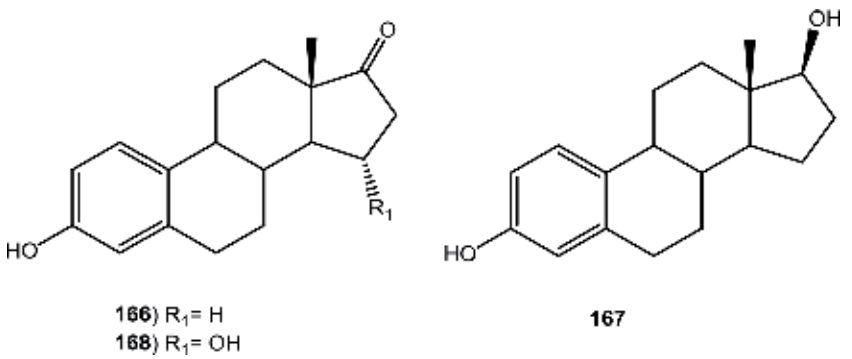


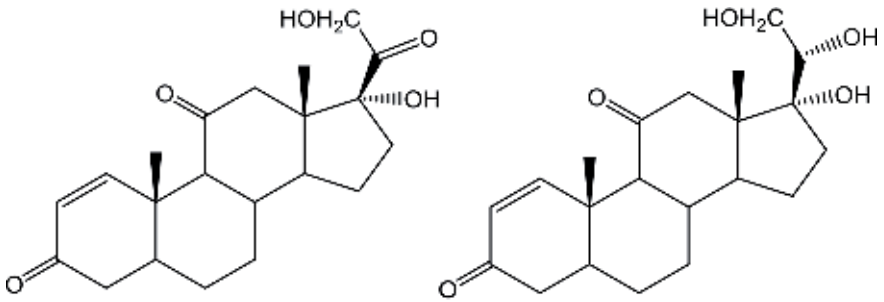
Figure 19.
*Biotransformation products of mesterelone (***149***).*



166) R_1= H
168) R_1= OH

167

Figure 20.
*Biotransformation products of 3-hydroxy-1,3,5-(10)-trien-17-one (***166***).*



169) $\Delta^{4(5)}$
170

171) R_1= H
172) $\Delta^{4(5)}$

Figure 21.
*Biotransformation products of prednisone (***169***).*

Biotransformation of **169** using *C. elegans* occurred by hydrogenation of the $\Delta^{4(5)}$ and reduction of C-20, to produce the compounds 17α,21-dihydroxy-5α-pregn-1-en-3,11,20-trione (**170**, 15.6%) and 17α,(20S),21-trihydroxy-5α-pregn-1-en-3,11-dione (**171**, 6.5%); whereas as the only biotransformation product, **169** using *F. lini* (5.2%), *R. stolonifer* (5.5%) and *C. lunata* (6.2%), was 1,4-pregnadien-17α,(20S),21-trihydroxy-3,11-dione (**172**) (**Figure 21**) [88].

The main chemical transformation carried out by different *Acremonium* species in various steroid compounds have been oxidations, reductions, hydroxylations in different positions, isomerizations, and hydrolysis of the chain in C-17. Hydrocortisone (**173**) is an important anabolic, used clinically as anti-inflammatory and antiallergic drug, besides being a raw material for the synthesis of many steroid hormones. Biotransformation of **173** using *Acremonium strictum* generated the products 11β,17β-dihydroxyandrost-4-en-3-one (**174**, 8%), 11β,17α,20β,21-tetrahydropregn-4-en-3-one (**175**, 11.2%), and 21-acetoxy-17β,17α,20-trihydroxypregn-4-en-3-one (**176**, 7.6%); it was observed that the actions of the said species were as the reduction, acetylation and degradation of the chain in C-17, without modification of the unsaturated ketone-α,β [89]. Biotransformation of **173** using *Gibberella fujikuroi* yielded 11β-hydroxyandrost-4-en-3,17-dione (**177**, 41%), while *B. subtilis* and *R. stolonifer* yielded **175** (15%). The products **173** (45%) and 3β,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (**178**, 31%) were obtained from the bioconversion of **173** using *Bacillus cereus* (**Figure 22**) [90].

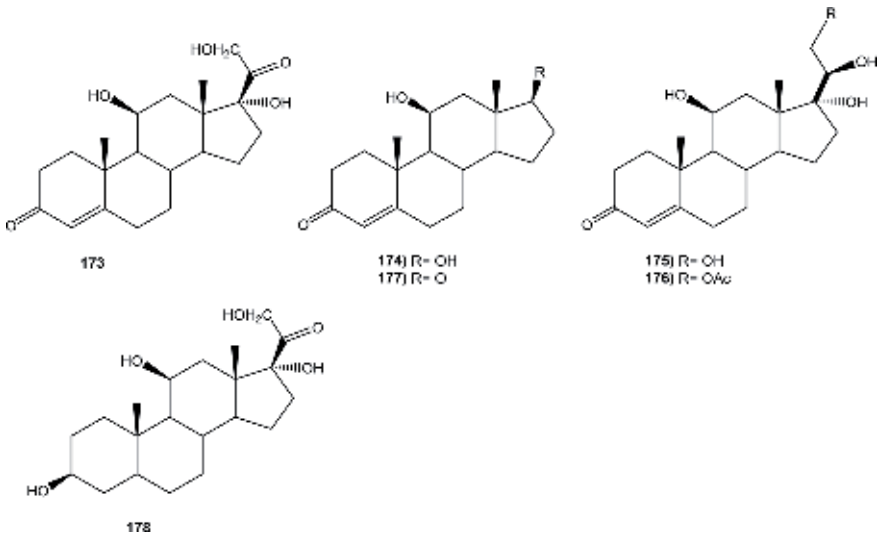


Figure 22.
*Biotransformation products of hydrocortisone (***173***).*

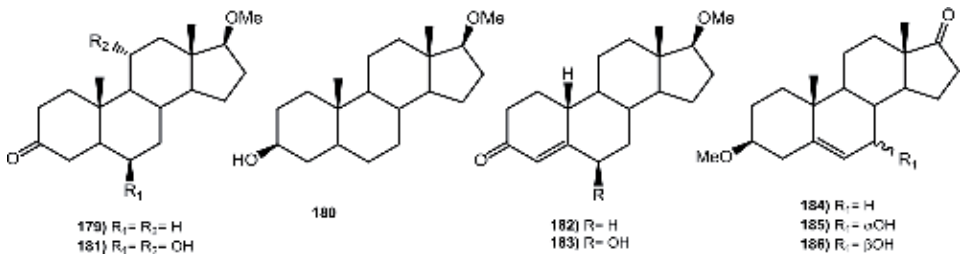
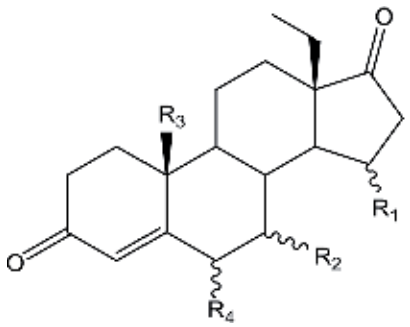


Figure 23.
*Biotransformation products of 17β-methoxy-5α-androst-3-one (***179***).*

Incubation of 17β-methoxy-5α-androst-3-one (**179**) with *Cephalosporium aphidicola* produced 17β-methoxy-5α-androst-3β-ol (**180**) and 6β,11α-dihydroxy-17β-methoxy-5α-androst-3-one (**181**); while the biotransformation of 17β-methoxyestra-4-en-3-one (**182**) using *C. aphidicola* produced a major metabolite 6β-hydroxy-17β-methoxyestra-4-en-3-one (**183**). Similarly, the microbiological transformation of 3β-methoxyandrost-5-en-17-one (**184**) gave a mixture of products: 7α-hydroxy-3β-methoxyandrost-5-en-17-one (**185**) and 7β-hydroxy-3β-methoxyandrost-5-en-17-one (**186**) (**Figure 23**) [91].

In the literature, several species of fungi belonging to the genera *Aspergillus*, *Fusarium*, *Mortierella,* and *Penicillium* and capable of hydroxylating various steroids in C-15 have been described. For example, Jekkel et al. [92] described that more than 3000 fungi hydroxylate 13β-ethyl-4-gonene-3,17-dione (**187**) in C-15 position, the genus being *Fusarium*, particularly *F. nivale*; the fungus preferentially hydroxylated **187** with an α orientation in C-15 (15α-hydroxy-13β-ethyl-4-gonene-3,17-dione, **188**, 77%) and C-7β (7β,15α-dihydroxy-13β-ethyl-4-gonene-3,17-diona, **189**). On the other hand, the biotransformation of **187** using *Mortierella pusilla* produced **188**, **190** (10β-hydroxy-13β-ethyl-4-gonene-3,17-dione) and **191** (6β-hydroxy-13β-ethyl-4-gonene-3,17-dione) (**Figure 24**).

The ethynodiol diacetate (**192**) is a synthetic derivative **1**, used as an oral contraceptive because it inhibits the ovulation process. The microbiological transformation of **192** using *Cunninghamella elegans* produced four hydroxylated compounds



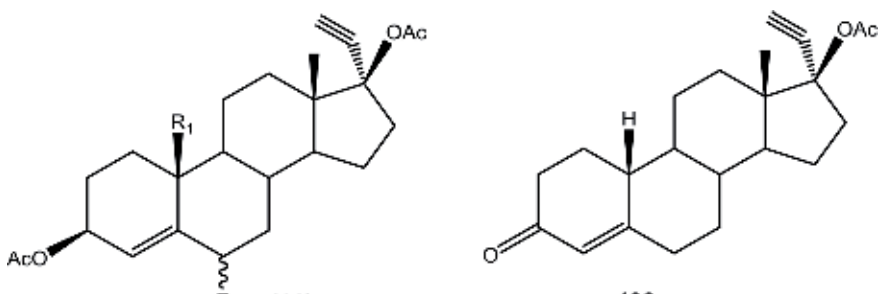
187) R_1= R_2= R_3= R_4= H
188) R_1= αOH, R_2= R_3= R_4= H
189) R_1= αOH, R_2= βOH, R_3= R_4= H
190) R_1= R_2= R_4= H, R_3= OH
191) R_1= R_2= R_3= H, R_4= βOH

Figure 24.
*Biotransformation products of 13β-ethyl-4-gonene-3, 17-dione (***187***).*

characterized as: 17α-ethynylestr-4-en-3β,17β-diacetoxy-6α-ol (**193**, 0.5%), 17α-etynylestr-4-en-3β,17β-diacetoxy-6β-ol (**194**, 1.0%), 17α-etynylestr-4-en-3β,17β-diacetoxy-10β-ol (**195**, 0.5%), and 17α-ethynyl-17β-acetoxiestr-4-en-3-one (**196**, 1.4%) (**Figure 25**) [93].

Desogestrel (13-ethyl-17-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol, **197**) is an orally active third-generation contraceptive steroid drug. Conversion of **197** by *C. blackesleeana* (ATCC 8688 A) yielded four metabolites: 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-6β,15β,17β-triol (**198**), 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3β,6β,17β-triol (**199**), 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3α,5α,6β,17β-tetraol (**200**), and 13-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-6β,17β-dihydroxy-3-one (**201**). Compounds **197** and **198** showed a potent growth inhibition against drug-resistant strains of *S. aureus* (**Figure 26**) [94].

The drugs mexrenone (**202**) and canrenone (**203**) are steroids with a spironolactone in C-17 and are potent antagonists of mineralocorticoids [95]. The biotransformation of **202** and **203** using a wide variety of microorganisms resulted in the production of monohydroxylated products in different positions, where *Beauveria bassiana* generated 11α-hydroxymexrenone (**204**, 67%) as the major product, while 12β-hydroxymexrenone (**205**, 50%) and 6β-hydroxymexrenone (**206**, 33%) were obtained using *Mortierella isabellina*. The dehydrogenation product ($\Delta^{1(2)}$-mexrenone, **207**, 15%) was favored with *Bacterium* cyclooxidants as well as *Rhodococcus equi*, *Nocardia aurentia*, and *Comamonas testosteroni*. From the biotransformation of **203** using *Corynespora cassiicola*, 9α-hydroxycanrenone



192) R_1= R_2= H
193) R_1= H, R_2= αOH
194) R_1= H, R_2= βOH
195) R_1= OH, R_2= H

196

Figure 25.
*Biotransformation products of ethynodiol diacetate (***192***).*

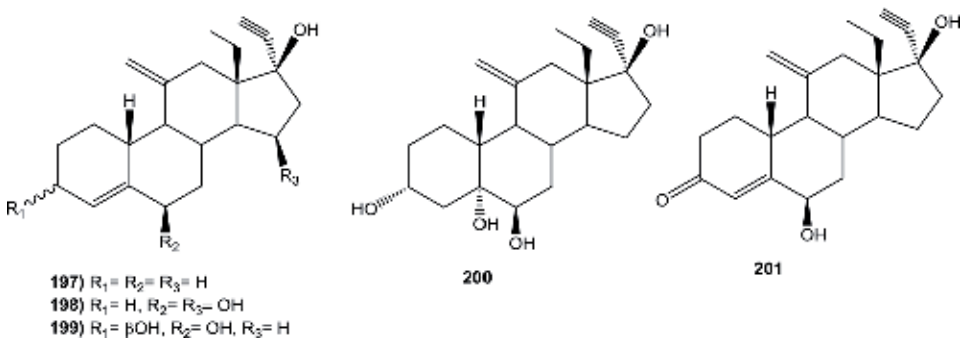


Figure 26.
*Biotransformation products of desogestrel (***197***).*

(**208**, 30%) was obtained, [96]. Conversion of canrenone (**203**) by *Colletotrichum lini* ST-1 gave two hydroxyl compounds, 15α-hydroxy-canrenone (**209**, 22%) and 11α,15α-dihydroxy-canrenone (**210**, 47%) (**Figure 27**) [80].

One of the steroids used in the treatment of breast cancer is exemestane (**211**), an inhibitor of steroidal aromatase. From the transformation of **211** using *Macrophomina phaseolina*, 16β,17β-dihydroxy-6-methylene-androsta-1,4-diene-3-one (**212**), 17β-hydroxy-6-methylene-androsta-1,4-diene-3,16-dione (**213**), and 17β-hydroxy-6-methylene-androsta-1,4-diene-3-one (**214**) were obtained, while by using *Fusarium lini*, the only product obtained was 11α-hydroxy-6-methylene-androsta-1,4-diene-3,17-dione (**215**) (**Figure 28**) [97].

4-Hydroxyandrost-4-ene-3,17-dione (formestane, **216)** is an irreversible aromatase inhibitor and therapeutically used in breast cancer treatment in post-menopausal women. Bioconversion of **216** using *Rhizopus oryzae* (ATCC 1145) resulted in the production of 4β,5α-dihydroxyandrost-3,17-dione (**217**, 8.6%) and 3,5α-dihydroxyandrost-2-ene-4,17-dione (**218**) [98], while the biotransformation of **217** using *Beauveria bassiana* produced 4,17β-dihydroxyandrost-4-en-3-one (**219**, 5.3%), 3α,17β-dihydroxy-5β-androstan-4-one (**220**, 0.9%), and 4,11α,17β-trihydroxyandrost-4-en-3-one (**221**, 2.4%) (**Figure 29**) [99].

Methyltestosterone (**222**), an anabolic steroid, was transformed by *Mucor racemosus* in 5 days to produce two monohydroxylated

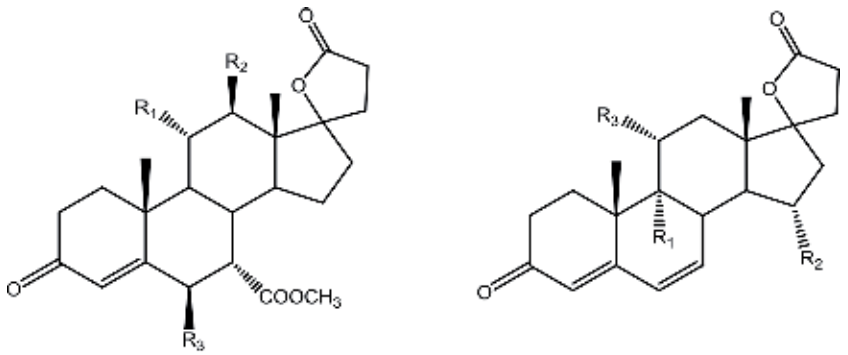


Figure 27.
*Biotransformation products of mexrenone (***202***) and canrenone (***203***).*

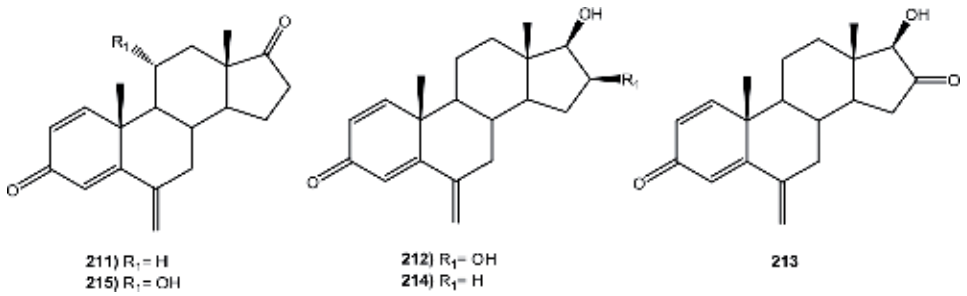


Figure 28.
*Biotransformation products of exemestane (**211**).*

products in the C-7 (7α-hydroxymethyltestosterone, **223**, 35%) and C-15 (15α-hydroxymethyltestosterone, **224**, 21%) positions, plus a dihydroxylated product (12,15α-dihydroxymethyltestosterone, **225**, 22%) [100]. Recently, three additional products were identified: 11α-hydroxy-17α-methyltestosterone (**226**), 6β-hydroxy-17α-methyltestosterone (**227**), and 6β,11α-dihydroxy-17α-methyltestosterone (**228**). Isolation of hydroxylation products have been reported in different carbons from **222** with different orientations, C-6β, C-7β, C-9α, C-11α, C-12β, and C-15α (**Figure 30**).

Dianabol (methandrostenolone, 17α-methyl-17β-hydroxyl-androst-1,4-dien-3-on, **229**) is an oral anabolic steroid that promotes the synthesis of proteins (increasing the muscle tissue). From the biotransformation of **229** using *Cunninghamella elegans*, five bioconversion products were obtained: 6β-hydroxydianabol (**230**), 15α-hydroxydianabol (**231**), 11α-hydroxydianabol (**232**), 6β,12β-dihydroxydianabol (**233**), and 6β,15α-dihydroxydianabol (**234**). The products 17β-hydroxy-17α-methyl-5α-androst-1,4-dien-3,6-dione (**235**), 7β-hydroxydianabol (**236**), 15β-hydroxydianabol (**237**), 17β-hydroxy-17α-methyl-5α-androst-1,4-dien-3,11-dione

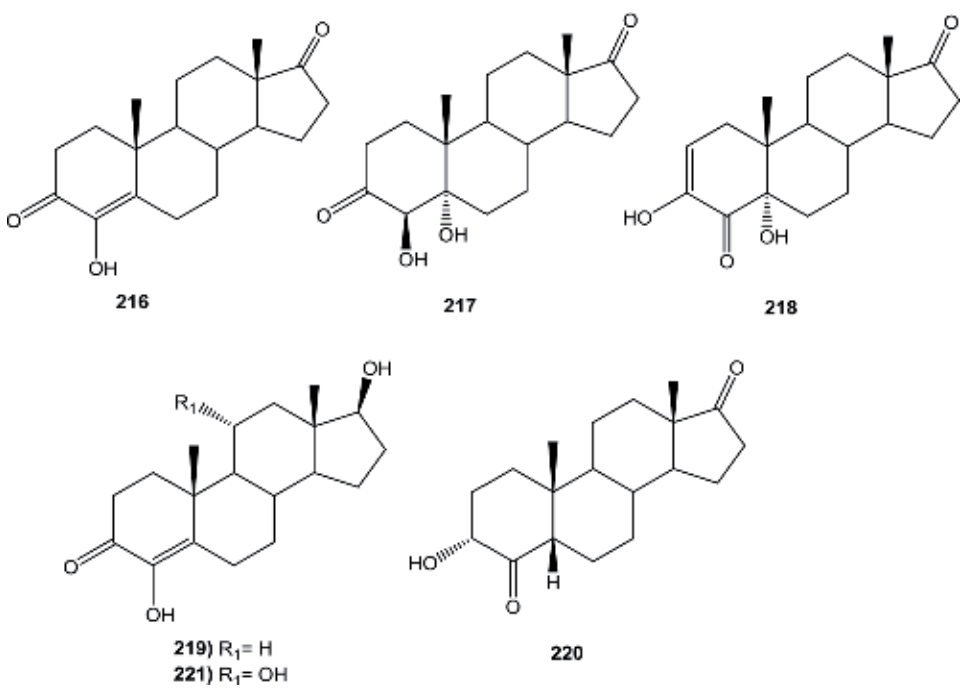
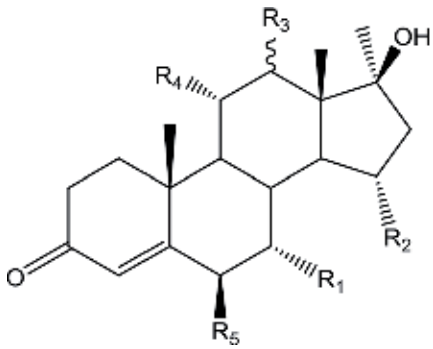
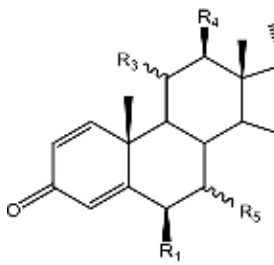


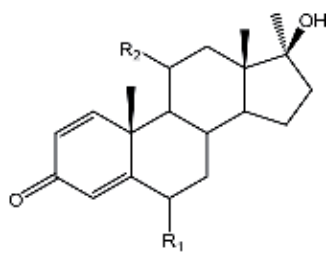
Figure 29.
*Biotransformation products of formestane (**216**).*



222) R_1= R_2= R_3= R_4= R_5= H
223) R_1= OH, R_2= R_3= R_4= R_5= H
224) R_1= R_3= R_4= R_5= H, R_2= OH
225) R_1= R_2= R_5= H, R_2= R_3= OH
226) R_1= R_2= R_3= R_5= H, R_4= OH
227) R_1= R_2= R_3= R_4= H, R_5= OH
228) R_1= R_2= R_3= H, R_4= R_5= OH

Figure 30.
*Biotransformation products of methyltestosterone (***222***).*

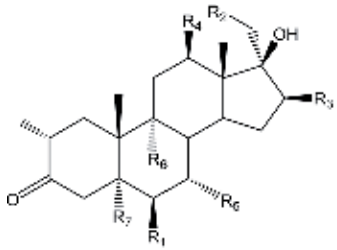


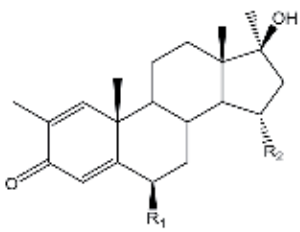


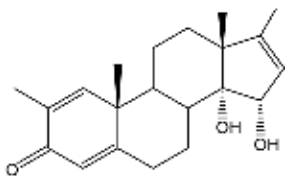
229) R_1= R_2= R_3= R_4= R_5= H
230) R_1= OH, R_2= R_3= R_4= R_5= H
231) R_1= R_3= R_4= R_5= H, R_2= αOH
232) R_1= R_2= R_4= R_5= H, R_3= αOH
233) R_1= R_4= OH, R_2= R_3= R_5= H
234) R_1= OH, R_2= αOH, R_3= R_4= R_5= H
236) R_1= R_2= R_3= R_4= H, R_5= βOH
237) R_1= R_3= R_4= R_5= H, R_2=βOH
239) R_1= R_2= R_4= R_5= H, R_3= βOH
240) R_1= R_2= R_3= R_4= H, R_5= αOH

235) R_1= O, R_2= H
238) R_1= H, R_2= O

Figure 31.
*Biotransformation products of dianabol (***229***).*







241) R_1= R_2= R_3= R_4= R_5= R_6= R_7= H
242) R_1= O, R_2= R_3= R_4= R_5= R_6= R_7= H
243) R_1= R_2= R_3= R_4= R_6= H, R_5= OH
244) R_1= R_2= R_4= R_5= R_7= H, R_3= R_6= OH
245) R_1= R_2= R_3= R_5= R_6= H, R_4= R_7= OH
246) R_1= R_2= R_3= R_5= R_7= H, R_4= R_6= OH
247) R_1= R_2= R_3= R_4= R_5= H, R_6= R_7= OH

248) R_1= R_2= OH, $\Delta^{1(10)}$
249) R_1= H, R_2= OH
252) R_1= O, R_2= H
253) R_1= R_2= H

251

Figure 32.
*Biotransformation products of masthasterone (***241***).*

(**238**), and 11β-hydroxydianabol (**239**) were obtained from the biotransformation of **229** using *Macrophomina phaseolina* [101]. Biotransformation of **229** using several microorganisms has been reported, for example, *Penicillium notatum* [102] transformed **229** into **230** and **231**, while *Trichoderma hamatum* produced **232** [103].

Similarly, *B. bassiana, A. ochraceus*, *Colletotrichum lagenarium,* and *Sporotrichum sulfurreducens* gave a biotransformed product **232** [104]. *Absidia glauca* metabolized **229** in compounds **230**, and **236**–**237** [105]. In contrast, the biotransformation of **229** using *A. coerula* yielded **239** along with 7α-hydroxydianabol (**240**) [106], while by using *B cinerea*, **237** was obtained as the only product (**Figure 31**) [107].

Methasterone (**241**) is a synthetic anabolic steroid, known to gain muscle mass. Microbial transformation of **241** using *M phaseolina* yielded 17β-hydroxy-17α(hydroxymethyl)-2α-methyl-5α-androstane-3,6-dione (**242**), while by using *C. blakesleeana*, 7α-hydroxymethasterone (**243**, 2.0%), 7α,16β-dihydroxymethasterone (**244**, 0.7%), 5α,12β-dihydroxymethasterone (**245**, 1.0%), 7α,12β-dihydroxymethasterone (**246**, 1.5%), and 7α,9α-dihydroxy-methasterone (**247**, 0.5%) were obtained. Incubation of **241** with *Fusarium lini* yielded different metabolites with dehydrogenation in ring A and D: 6β,17β-dihydroxy-2,17α-dimethyl-5α-androst-1,4-diene-3-one (**248**, 1.0%), 15α,17β-dihydroxy-2α,17α-dimethyl-5α-androst-1,4-diene-3-one (**249**, 0.6%), 6β,17β-dihydroxy-2,17α-dimethylandrost-1,4-diene-3-one (**250**, 0.4%), 14α,15α-dihydroxy-2,17-dimethyl-5α-androst-1,4,16-trien-3-one (**251**, 0.3%), 17β-hydroxy-2,17α-dimethyl-5α-androst-5α-1,4-dien-3,6-dione (**252**, 0.3%), and 17β-hydroxy-2,17α-dimethyl-5α-androst-1,4-dien-3-one (**253**, 1.0%) (**Figure 32**) [108].

3. Conclusions

The biotransformation processes of different steroid compounds described in this review, although not exhaustive, aim to highlight the importance of biotransformation through different microorganisms, as a useful chemical-biological tool for obtaining novel derivatives for research purpose and as industrial applications. An example includes obtaining steroid compounds for the pharmaceutical industry.

Biotransformation of steroids has been implemented in an important way in the partial synthesis of new steroids, for their evaluation as hormones and drugs. Currently, there is a wide variety of steroids used as diuretics, anabolic, anti-inflammatory, antiandrogenic, anticontraceptive, antitumor, among other applications. Chemical functionalization in different carbon atoms of the sternum skeleton is related to the biological activity of the molecule. This is why microbiological transformations play an important role in obtaining these compounds through chemical transformations, such as the oxidation of hydroxyl group at C-3 and C-17, isomerization of the double bond $\Delta^{5(6)}$ to $\Delta^{4(5)}$, hydrogenation of double bonds $\Delta^{1(2)}$ and $\Delta^{4(5)}$, and reduction of the carbonyl group at C-17 and C-20 with β orientation. Biohydroxylations performed in different positions of the steroid skeleton—C-11α, C-11β, C-15β, and C-16α—using different species of fungi of the genera *Rhizopus*, *Aspergillus*, *Curvularia*, *Cunninghamella,* and *Streptomyces* with high yields are an important chemical transformation in many synthesis schemes of new steroids with a determined biological activity.

Hydroxylation of steroids—progesterone, testosterone, 17α-methyltestosterone, and 4-androsten-3,17-dione—presenting the 4-en-3-one system, proceeds with a high stereo- and regioselectivity in the C-6 and C-11 positions, with a β orientation in C-6 and α orientation in C-11. The presence of the methyl group in C-10 is necessary for the hydroxylation in C-11, as can be seen in the derivatives of 19-nortesterone.

The interest in the biotransformation of steroid compounds has been increasing in recent years, due to the obtaining of new and useful pharmacologically active compounds. In addition to the development of new genetically modified strains, there is an increase in the availability of immobilized enzymes and the manipulation of culture media.

Biotransformation of steroids proceeds with low to moderate yields in general. One of the main causes is their low solubility in water. Currently, methodologies are developed that allow the incorporation of chemicals—surfactants, ionic liquids, cyclodextrins, liposomes, among others—that contribute to improve the yields of each biotransformation process and the processes friendly to the environment.

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Chapter 3

Estrone Sulfatase Inhibitors as New Anticancer Agents

Svetlana N. Morozkina and Alexander G. Shavva

Abstract

Enzyme steroid sulfatase (STS) is considered as a promising therapeutic target for the treatment of hormone-dependent oncological diseases such as breast, endometrial, prostate cancers, and endometriosis. The discovery of potent and irreversible STS inhibitors stimulated huge efforts of preclinical and clinical work. Various STS inhibitors such as steroid sulfamate, steroid nonsulfamate, nonsteroidal sulfamate, and nonsteroidal nonsulfamate-based inhibitors have been developed. In the review known STS inhibitors from the point of view of their safety, side-effects and perspectives for clinical application are considered. Among STS inhibitors several dual (multitargeted) compounds have huge potential being nonestrogenic and acting in nanomolar levels on the targets. The dual aromatase-sulfatase inhibitors (DASI) approach has a great potential when a synergy between STS and aromatase inhibition is expected and, thus it could address acquired resistance mechanisms. Among STS inhibitors based on steroid skeleton 17α-benzyl-, 17β-arylsulfonamides, 17-diisopropylcarbamoyl-3-O-sulfamates exhibit the best properties, especially as dual anticancer potential drugs. The same modifications result in the increased activity against STS in 2-OMe-3-O-sulfamates as well as 2-OMe-3, 17β-bissulfamates, which are also active against triple negative breast cancer. 8α-Steroid estrogen analogs without estrogenic properties also possess high STS-inhibitory activity and block breast cancer cells growth with the activity comparable to tamoxifen.

Keywords: steroid sulfatase (STS), inhibitors, breast cancer, hormone-dependent diseases

1. Introduction

Breast cancer (BC) is the most common malignant tumor in women (12%) worldwide and is the second leading cause of cancer mortality after lung cancer (26%) [1].

Approximately 95–97% of tumors are estrogen-dependent in the early stages of their development [2, 3] and more than 70% express very high levels of estrogen receptor alpha (ERα) [4]. The fundamental difference of extragonadal estrogen synthesis is its autocrine nature—that an organ producing estrogens is a target organ at the same time. Thus, local concentration of estrogens in such organs may be markedly elevated. Peripheral estrogens formation is increased after menopause, and compensates estrogens deficiency in different organs and tissues [5]. Extragonadal estrogens' production may rise with the aging. Moreover, it was continually emphasized in the literature that the increased level of estrogens in the body is considered as a risk of the BC development [6, 7].

Biologically active hormones, in particular the most active estrogen estradiol (E2), play a critical role in the initiation and development of hormone-dependent breast cancer (HDBC). In premenopausal women, estrogens are mainly (75%) synthesized in the ovaries, and thus, a luteinizing hormone-releasing hormone (LH-RH) agonist [8, 9] is useful to suppress the function of pituitary hormone. In postmenopausal women estrogens are produced in peripheral tissues such as adipose tissues, skin, and mammary glands [10, 11].

Adrenal dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), and adrenal or ovarian androstenedione are also sources of E2 in peripheral tissues. In postmenopausal women, concentrations of DHEAS, DHEA, and androstenedione in plasma are relatively high; approximately 1.8, 6.6, and 1.9 nM, respectively. In contrast, plasma concentrations of estrone (E1) and (E2) are several-fold lower (70 and 30 pM, respectively) [12].

Another important steroid precursor for estrogen formation is E1-sulfate (E1S). It is the most important estrogen in the peripheral blood, with relatively high (0.6 nM) concentrations in postmenopausal women. E1S levels are associated with high body-mass index, which suggest that E1S originates from adipose tissue. Concentrations of E1S in plasma are 10–20 times higher than those of E1 and E2, as well as its half-life in the plasma is longer than the half-life of unconjugated estrogens.

Enzyme steroid sulfatase (STS) converts E1S to E1, followed by the reduction to the biologically active estrogen, E2, by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), which is overexpressed in many breast tumors.

In BC tissues estrogens can be locally produced *de novo* by estrogen synthesis enzymes to promote tumor growth.

The level of estrogens in BC tissues of postmenopausal women can be 10–40 folds higher than in blood circulation and 5–10 times higher than in noncancerous breast tissues [13]. Furthermore, the intratumoral E2/E1 ratio is significantly higher in postmenopausal BC than in premenopausal BC. High concentrations of estrogen in breast tissue increase the risk of BC development [14, 15].

Thus, inhibition of enzymatic synthesis of estrogens is an effective therapeutic strategy for postmenopausal women with estrogen receptor-positive (ER+) tumors [16, 17]. *In situ* transformations of inactive steroids require activity of a series of enzymes that were found in hormone-sensitive cancers.

The scheme of estrogens formation in human body includes: (a) formation of E1 from androstenedione under the action of cytochrome P450 aromatase, (b) reduction of E1 by 17β-HSD1 leads to more active E2. Importantly, almost insoluble in aqueous media E1 is converted into water-soluble E1S under the action of

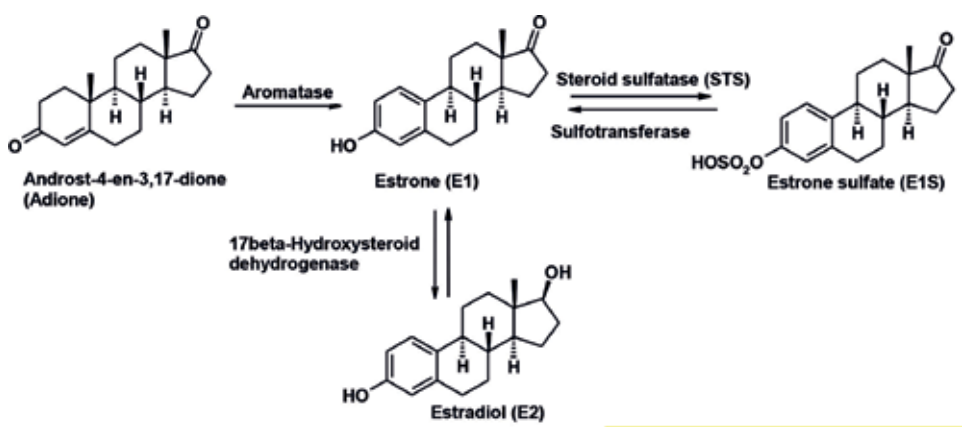


Figure 1.
Estrogens formation in human body.

sulfotransferase (STS). E1S does not possess hormonal activity, however it may be transported into various targets (**Figure 1**) [18, 19]. Several reviews focus on aspects of human steroidogenesis [18, 20–29].

Free hormones are formed from sulfates of estrogen and androgens under action of steroid sulfatase. At high concentrations, androgens compete for binding with ERs. The activation of ERα under the action of androstenediol and DHEA in BC cells has been detected. It is confirmed by the inhibition of cell growth in the presence of antiestrogens. The evaluation of E1S level during diagnostic of various oncological diseases (for example, prostate cancer) is of high importance [30].

2. Approaches for the manipulation of estrogen level in tumors

2.1 Endocrine therapy

Hormonal (endocrine) therapy is effectively used for the treatment of HDBC. Most types of BCs are estrogen-dependent, with approximately 55% in premenopausal women and 75% in postmenopausal women [31–34].

Selective estrogen receptor modulators (SERMs) or down-regulators (SERD), such as tamoxifen, raloxifene, ospemifine, and fulvestrant are compounds that are currently used in clinical practice to treat BC [9, 35]. In breast tissues, SERMs effectively block the activation of ER(α) by endogenous ligands, preventing the transcription of genes mediated by estrogen response elements [36, 37]. SERMs have tissue-specific effects on ERα that results in antagonist activity in breast and uterus tissues as well as agonist activity in bone. Although tamoxifen and raloxifene possess the desired SERM activity, they also increase the risk of venous thromboembolism [38] and exhibit toxicity [22]. Given that resistance (*de novo* or acquired resistance) is a major limiting factor in the use of endocrine therapy, additional endocrine therapies with other mechanisms of action are needed [39, 40].

2.2 Inhibitors of enzymes responsible for the estrogen formation in tumors

The aromatase enzyme is responsible for the conversion of testosterone and androstenedione to E2 and E1, respectively. Thus, inhibition of the aromatase enzyme is one of the approaches for the development of new drugs to treat BC [41–43].

Nonsteroidal third-generation aromatase inhibitors (AIs), such as anastrozole (Arimidex), letrozole (Femara), and exemestane (Aromasin), are often used for postmenopausal hormone-dependent BC treatment in clinical practice. Despite the success of AIs in the clinic, numerous BC patients still progress after AI therapy due to the development of resistance to AIs and side-effects such as osteoporosis caused by whole-body deprivation of estrogen [44, 45]. Mechanisms of AI resistance include ligand-independent activation of the ER and signaling via other growth factor receptors; new insights about resistance are published recently [45].

The overall response rates for AIs (40–50%) suggest the presence of alternative sources of estrogens. The production of E1, DHEA and androstenediol is an important mechanism of resistance to AI treatment [46].

It was demonstrated that AIs used sequentially with tamoxifen had higher efficacy compared to tamoxifen alone, with an improvement in overall survival [47].

There are other factors involved in tumor growth [48]. The enzymes STS and 17β-HSD1 have been identified as essential parts in E2 production and subsequent promotion of cancer growth. Recently it was shown that 17β-HSD7 also plays a key role in increasing the E2/E1 ratio in BC tumors [49]. Very recently, some evaluations of the "sulfatase pathways" in tumor stroma have been carried out [50].

The STS is also responsible for the hydrolysis of DHEAS to DHEA, which is an immediate precursor of androstenediol, a potent estrogenic steroid [51], whose formation is not influenced by AIs. DHEAS stimulates proliferation of MCF-7 cells from BC, which could be blocked by an antiestrogen or STS inhibitor but by an AI. E1S and DHEAS are particularly abundant in blood circulation and could act as a reservoir of steroid precursors, specifically in BC [29, 52]. The formation of DHEA through the STS pathway accounts for the production of 90% of the androgen androstenediol [52], which possesses estrogenic properties, that are 100-times weaker than estradiol [13, 53]. Androstenediol is present at 100-fold higher concentrations than estradiol in the circulation, and may have estrogenic properties that are equal to estradiol [54]. Thus, inhibition of STS has the dual property of reducing local androstenediol biosynthesis [55, 56].

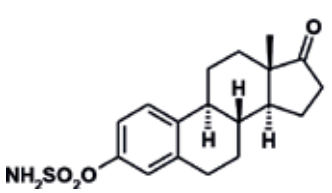
2.3 Steroid sulfatase enzyme (STS)

The STS enzyme (EC 3.1.6.2, aryl sulfatase C, steryl-sulfatase) is widely distributed throughout the body and plays critical role in steroidogenesis [54]. Publications in recent years indicate the role of STS activity in gynecological diseases [57], mentioning diminished endometriosis *in vivo* under the action of STS inhibitors [58, 59]. However, a phase II trial with STS inhibitors in endometrium cancer patients with advanced disease revealed no superior effects as compared to progestin megestrol acetate, and further studies are ongoing [60]. STS inhibitors are also useful for the treatment of ovary cancers and prostate cancer [16, 61].

According to the *in vitro* studies, STS is the main enzyme responsible for estrogen production in hormone-dependent breast tumors, and has several hundred times higher activity in liver and normal/malignant breast tissues than aromatase [13, 53, 62]. STS mRNA expression (74%) in ERα-positive breast tumors is an independent prognostic indicator in predicting relapse-free survival, with higher levels of expression being associated with a poor prognosis [63]. Like aromatase inhibitors, sulfatase inhibition can only be used in postmenopausal women. Probably, the greatest benefit with sulfatase inhibition is in those cases where DHEAS levels are high. To date, STS inhibitors are still in an early stage of development [53, 64, 65].

The human STS is a protein, integrated in microsomal membrane. Its three-dimensional structure has been determined (PDBcode 1P49) [66]. However, knowledge about regulation of its expression as well as activity is limited. The topology of the active site of the steroid sulfatase and the arylsulfatases A and B is similar [66].

Most of the STS inhibitors discovered to date, act as irreversible active-site-directed inhibitors. An aryl sulfamate group ($ArOSO_2NH_2$) is considered as the pharmacophore for irreversible inhibition of the enzyme. One of the first time-, pH-, and concentration-dependent irreversible active-site directed-steroidal inhibitor is estrone-3-O-sulfamate (EMATE), which inhibit STS in MCF-7 cells from BC by 99% at 0.1 μM and has an IC_{50} value of 65 pM (IC_{50} = 80 nM in placental microsomes). EMATE was evaluated in clinical trials [67]. The highest effectiveness of EMATE has been demonstrated in rats (subcutaneous and oral administration). STS activity was also inhibited when EMATE was administered to humans in dose 0.5 mg/kg [68].



estrone-3-O-sulfamate
(EMATE)

Despite the exceptional potency of the EMATE [67, 68], it is not used in clinical practice to treat hormone-dependent BC because metabolic conversion of EMATE by STS releases estrone, which act via estrogen receptors, and can directly promote tumor growth [69]. Nevertheless, EMATE is now the prototypical inhibitor, and used as standard during evaluation of other potential STS inhibitors [19].

2.4 Mechanisms of inactivation of steroid sulfatase

Several research groups made attempts to establish the mechanism(s) of sulfatase inactivation. However, the precise mechanism of inhibition is still uncertain. In 2010, Spillane and Malaubier have established that the hydrolysis of EMATE occurs by two different mechanisms: an SN2 mechanism below pH 9.5 and E1cB mechanisms involving N-sulfonylamines at higher pHs [70]. Detailed presumable mechanisms have been discussed in recent reviews [71–73].

Based on the mechanisms, the result of the hydrolysis is free estrone. Moreover, under per os administration, the activity of EMATE is several times higher than the activity of estrone, due slow metabolism of EMATE in liver [68]. EMATE is not subjected to metabolic inactivation in red blood cells. Thus, consideration of hormonal activity and side-effects of steroids with free phenolic group is important in the modeling of sulfatase inhibitors for therapeutic use [54, 74, 75].

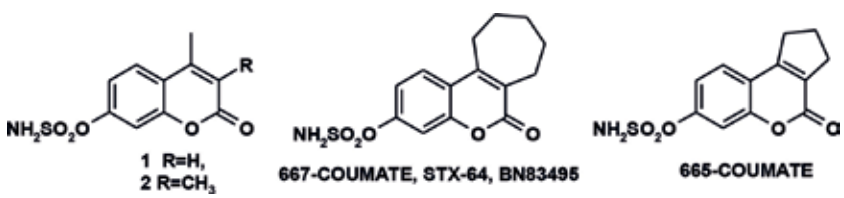
The knowledge of the crystal structure opens the rational drug design of molecules for the inactivation of steroid sulfatase.

2.5 Nonsteroidal STS inhibitors

Many investigations have been carried out to develop nonsteroidal STS inhibitors, because nonsteroidal drugs and their metabolites may have less undesirable effects.

4-Methylcoumarin-7-O-sulfamate (**1**, Coumate) was the first time- and concentration-dependent STS inhibitor (IC_{50} = 380 nM) in oral dose 10 mg/kg/day, and *in vivo* has no estrogenic activity. 3,4-Dimethylcoumarin-7-O-sulfamate (**2**) was a more potent inhibitor (IC_{50} = 30 nM) [76].

A search of an orally active, nonestrogenic, nonsteroidal STS inhibitors among tricyclic compounds based around the coumarin core resulted in the discovery of Irosustat (667-coumate, STX64, BN83495) [77], which is the first-in-class irreversible time- and concentration-dependent STS inhibitor for the treatment of hormone-dependent BC in postmenopausal women that has been clinically evaluated in breast, endometrial, and prostate cancers [77] and there is potential for innovative dual-targeting approaches [78, 79], with an IC_{50} value of 8 nM in placental microsomes. The inhibitor (**2**) does not possess any estrogenic activity in *in vitro* and *in vivo* assays [80].

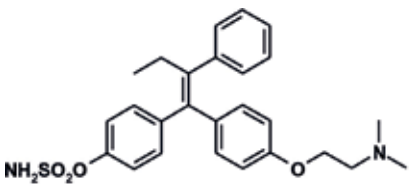


The optimum dose of 40 mg/day was estimated in phase I/II trials [81]. Efficiency of Irosustat has also been demonstrated in a phase II study in (ER+) endometrial cancer in women with advanced or recurrent disease [82]. The high bioavailability of Irosustat is explained by the prevention of degradation by sequestration

inside red blood cells where it, similarly to EMATE, binds to (and inhibits) carbonic anhydrase II (IC_{50} = 22 nM) [83]. The inactivation mechanism suggests that a sulfamate group is transferred to the gem-diol form of formylglycine 75 of steroid sulfatase due to a facile E1cB elimination of sulfamate anion to give the corresponding coumarin, which has a long half-life in blood [84]. However, the further development of Irosustat in monotherapy was stopped in the phase I/II clinical studies, because Irosustat does not possess superior properties to the current standard of care megesterol acetate, and its relative bioavailability decreases with increasing dose. The study of its combination with other hormonal therapies (for example, with the aromatase inhibitor anastrozole) is underway [85]. Metabolism of Irosustat has been investigated [86]. Irosustat also inhibits skin and liver STS [86].

2.5.1 Dual selective estrogen receptor modulators/STS inhibitors

One of the first examples of the dual SERM/STS inhibitor was published by Duquesne University [87]. 4-Hydroxytamoxifen is a metabolite of main drug tamoxifen used as endocrine therapy in (ER+) BCs [88]. This metabolite is a SERM and has antiestrogen effects in breast tissues, however, acts as an estrogen agonist in other tissues such as bone marrow. The sulfamate derivative **3** of 4-hydroxytamoxifen was shown to be an STS inhibitor with Ki = 35.9 μM.



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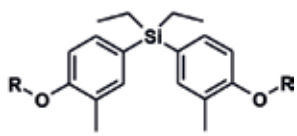
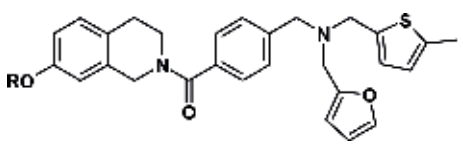


4 R = SO_2NH_2
5 R = H

Surprisingly, among silicon-containing derivatives compound **4** exhibits strong STS-inhibitory activity (IC_{50} = 0.17 μM). Furthermore, its metabolite **5** possesses potent ERα-antagonistic activity (IC_{50} = 29.7 nM) [89].

Poirier with colleagues, among tetrahydroisoquinoline-N-substituted derivatives [90], found second-generation dual-action compounds that inhibit STS and act as a SERM. These compounds are devoid of estrogenic activity and toxicity. Their sulfamate derivatives possess high inhibitory activity toward STS (IC_{50} of 3.9, 8.9, and 16.6 nM). Both phenolic and their sulfamate derivatives show no estrogenic activity and moderate antiestrogenic properties. All compounds significantly stimulate osteoblast-like Saos-2 cell proliferation, thus suggesting a SERM activity. The results of molecular docking experiments suggest that the most active compounds **6** and **7** bind in a competitive manner with E2 [91].



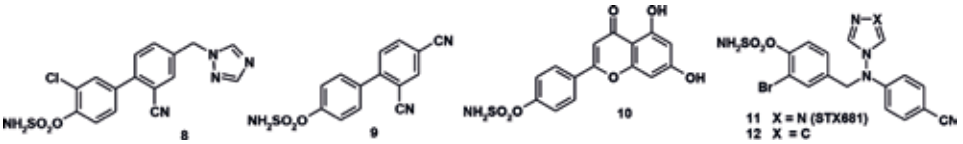
6 R = SO_2NH_2
7 R = H

2.5.2 Dual aromatase/STS inhibitors

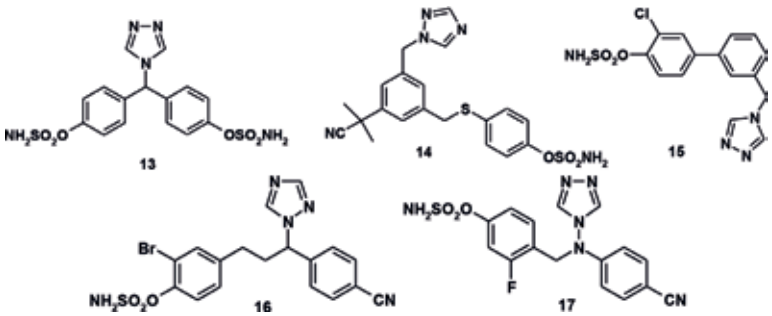
Another approach for the treatment of hormone-dependent BC is the development of DASIs, which may have an additive or synergistic antitumor effect. The potential advantages of a single chemical agent with the ability to interact with

multiple biological targets were highlighted previously [92]. In the case of DASIs, this goal is being pursued by the introduction of the critical sulfamate unit in structures with known aromatase-inhibiting properties [93, 94]. All DASIs are still in preclinical investigations [95].

One of the best dual inhibitors is compound **8** with nonestrogenic properties. 2′,4′-Di-cyanobiphenyl-4-O-sulfamate (TZS8478) (**9**) also shows the best STS inhibition [96].



One of the most potent dual inhibitor is compound **10** with 98 and 85% inhibition of STS and aromatase, respectively, at 10 μM [97]. A series of DASIs have been investigated [98, 99]. Compound **11** (STX681, IC_{50} = 0.82 nM for aromatase and IC_{50} = 39 nM for STS) and similar analog **12** also exhibit an excellent profile against aromatase (IC_{50} = 0.13 nM) and STS (IC_{50} = 3.5 nM) and are not estrogenic [100]. Bissulfamate **13** at a single oral dose of 10 mg/kg inhibits aromatase and rat liver STS by 60 and 88%, respectively. The anastrazole inspired compound **12** is also potent dual inhibitor *in vivo* [101, 102].



Among compounds on letrozole and vorozole templates, the most potent inhibitors were compounds **15** (aromatase IC_{50} = 0.5 nM and STS IC_{50} = 5.5 nM) and **16** (IC_{50} = 0.0001 μM) [103]. When orally dosed, compound **15** reduces plasma estradiol levels and inhibits liver STS activity [103].

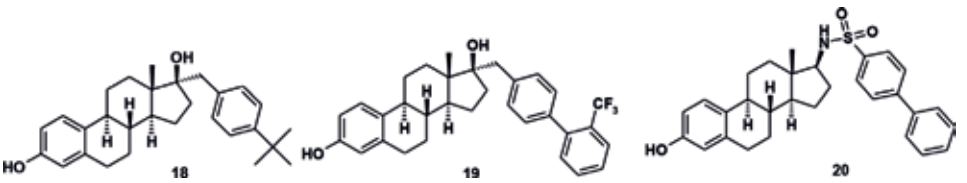
Potter with coauthors published the successful realization of the strategy when the core components of the two leading DASIs resulted in the hybrid structures that exhibit a very high level of dual inhibition against aromatase and STS *in vitro* (IC_{50} = 0.015–0.75 nM). Most active compound is analog **17** (IC_{50} for aromatase = 0.0002 μM, for STS = 0.0025 μM) [104].

The latest achievements in the field of nonsteroidal AIs are presented in recent reviews [105, 106].

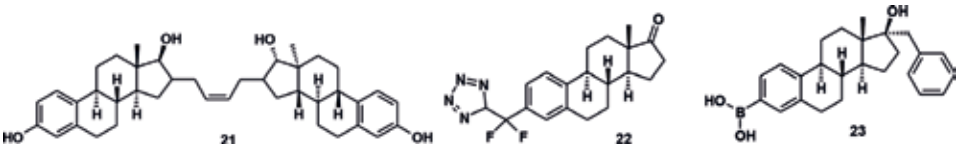
2.6 Steroidal STS inhibitors

2.6.1 Steroid-based STS inhibitors without sulfamate group

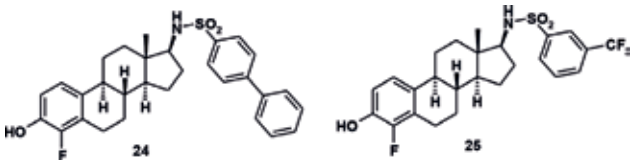
Nonsulfamated STS inhibitors based on estrogens are weaker than EMATE. Most active STS inhibitors without sulfamate group with highest activity are represented by compounds **18**, **19**, and **20** (IC_{50} = 12, 21, and 9, respectively) [107–109].



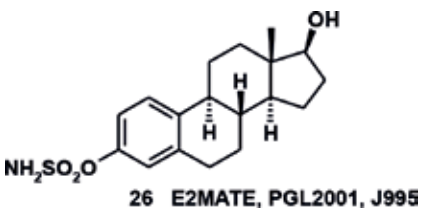
Estradiol dimer **21** also exhibits STS inhibitory activity in nanomolar range [110]. STS inhibitors are exemplified by tetrazole derivative **22** and boronic derivative **23** [111–113].



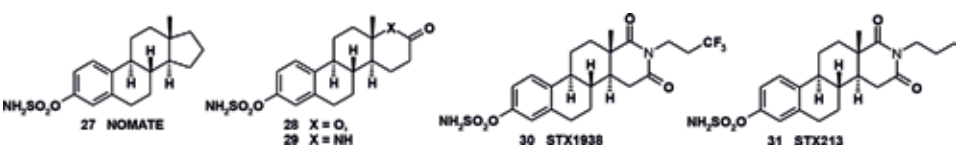
In the series of 4-substituted 17β-arylsulfonamides of 17β-aminoestra-1,3,5(10)-trien-3-ol, compounds **24** and **25** are tight-binding inhibitors with Ki app values of 1 and 2.5 nM [114].



2.6.2 Steroid-based STS inhibitors with sulfamate group



The estrogenicity of EMATE and estradiol-3-O-sulfamate (**26**, E2MATE, PGL2001, J995) is the serious restriction for their development as anticancer agents. E2MATE effectively inhibits STS activity in endometrial tissue *in vitro* and *in vivo* (in doses 1.0 and 0.5 mg/kg) without affecting systemic E2 levels [58, 59, 115, 116], and is introduced into Phase IIa of clinical trials [117]. E2MATE has been also clinically investigated as a pro-drug for hormone-replacement therapy and some limited clinical data are available. EMATE and E2MATE are bound to carbonic anhydrase (for EMATE IC_{50} = 23 nM) within red blood cells, being dual inhibitors of carbonic anhydrases and STS [118].



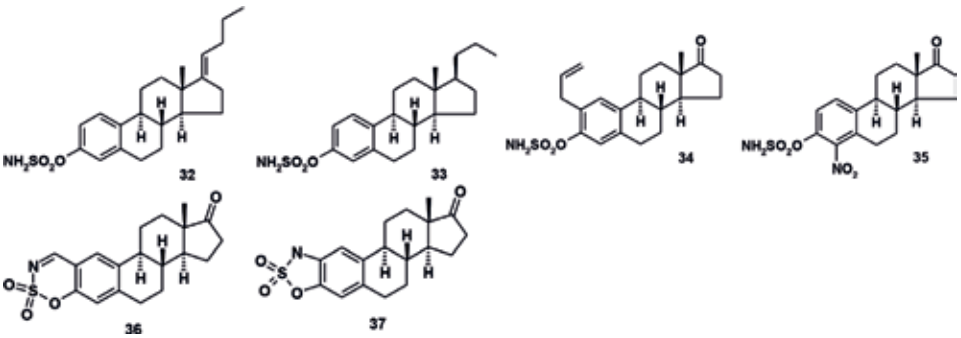
The sulfamate **27** (NOMATE) was evaluated as an STS inhibitor. This steroid without the 17-carbonyl group possesses ablated estrogenicity as well as reduced

STS activity compared to EMATE. NOMATE was shown to exhibit antitumor activity against a range of tumor cell lines [119].

D-ring lactone **28** has been developed as an orally available STS inhibitor [114]. The latest together with related lactam **29** were independently developed by Imperial College and University of Bath [120]. These compounds are potent STS inhibitors (98 and 91% inhibition of STS activity in MCF-7 cells at 0.1 μM, respectively; oral dose of 2 mg/kg/day) without estrogenic effects.

Simple modifications of the D-ring have led to dramatic variations in estrogenicity. Thus, the conversion of EMATE to the oxime results in a super-estrogen. From the other hand, D-ring heterocyclic derivatives exhibit reduced estrogenicity [121, 122].

The replacement of ring D with N-substituted piperidinedione moiety results in the loss of estrogenic properties and greater STS inhibitory activity *in vivo* compared to STX64, as it was shown by the compounds STX213 (**31**) [123] and STX1938 (**30**) [124]. The STX1938 (**30**) and STX213 (**31**) inhibit STS with IC_{50} of 1 nM and 35 pM correspondingly (90- and 18-fold more potent than EMATE, respectively) [125]. STX213 and STX1938 possess superior properties in comparison with STX64 *in vivo* models with once weekly oral dose 1 mg/kg [125, 126]. The docking studies explained the greater potency of STX1938 in comparison with STX213 by the increased lipophility of CF_3 group and the ability of the fluorine atoms to participate in C-F---H-O and C-F --- H-N interactions in the STS binding site. STX213 (**31**) demonstrates a greater effect on tumor growth than Irosustat (oral dose 10 mg/kg/day) [21]. Most active among 17-modified EMATE derivatives as STS inhibitors was steroid **32** (IC_{50} = 11 pM) [126]. The saturated analog **33** possesses similar potency (IC_{50} = 34 pM), and is not estrogenic [126].



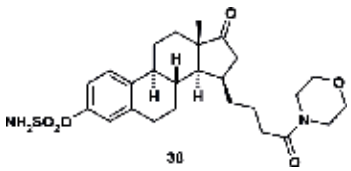
Among various 2- and 4-substituted and 2,4-disubstituted EMATE derivatives, most active compounds are 2-(2-prop-2-enyl)-EMATE (**34**, IC_{50} = 37 nM in MCF-7 cells) [126]; and 4-nitro-EMATE (**35**, IC_{50} = 0.01 nM in MCF-7 cells) (EMATE; IC_{50} = 0.83 nM in MCF-7 cells), and steroid **34** is nonestrogenic [127].

Cyclic sulfamate **36** is an effective STS inhibitor (IC_{50} = 9.3 nM) *in vivo* with dose regime 1 mg/mouse/day for 5 weeks [128]. The derivatives of oxathiazine **36** are claimed as estrogen-ablative agents; however, no data on their activity have been published [129]. Cyclic sulfamates with six-membered ring are time-dependent inactivators [130]. Acyclic mono-alkylated sulfamates are not time-dependent inactivators of sulfatases. Probably, imino compound **36** hydrolyzes to the ortho-formyl sulfamate *in situ* [53]. The five-membered ring compounds such as **37** are not time-dependent inactivators of STS [131].

2.6.3 Dual 17β-HSD1/STS inhibitors

17βHSD converts E1 to E2 and DHEA to androstanediol [132]. Several inhibitors based on steroidal skeleton have been successfully developed [133, 134]. Few dual

inhibitors of 17β-HSD and STS for the treatment of steroid hormone-dependent diseases are patented [135]. The example of such inhibitors is represented by the compound **38**.



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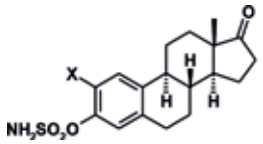
A-ring-modified steroidal sulfamates, for example, series of 2-OMe-estradiol sulfamates and analogs have been investigated as nonestrogenic STS inhibitors [136, 137]. 2-MeO-EMATE **39** demonstrates the excellent inhibitory properties in the relation to STS *in vitro* (IC_{50} = 30 nM) and *in vivo* and is not estrogenic [138]. It strongly shows the antiproliferative effects toward BC cells by inducing apoptosis and cell cycle arresting in the G2/M phase [139].

2-Ethyl-EMATE **40** was identified as a promising multitargeted anticancer agent with strong ability to arrest the cell cycle, inhibit angiogenesis, as well as inhibit tumor growth in a xenograft model [140]. It was found that 2-ethylestrone (desulfamoylated compound **40**) belongs to series of potent superoxide dismutase inhibitors [141].



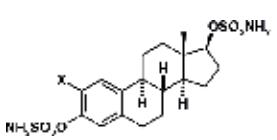
39 X = OMe (2-MeOEMATE)
40 X = Et

It is known that 2-methoxyestradiol, a metabolite of E2, possesses antiangiogenic properties and prevents tumor growth through disrupting tubulin polymerization by binding at the colchicine-binding site [142, 143]. 2-Methoxyestradiol is considered as the perspective compound for the treatment of endometriosis [144].

The anticancer effects of the 2-substituted sulfamate estrogen derivatives arise from disruption of tubulin polymerization, and the compounds also binding at the colchicine site [145]. 3,17β-Bissulfamates of estrogens are other representatives of multitargeted antitumor agents, acting as STS inhibitors with antiproliferative activity (IC_{50} = 18–250 nM) [146]. Such bissulfamates compete with colchicine for tubulin binding and disrupt microtubules resulting into cell cycle arrest just by apoptosis *in vitro* and *in vivo* [147, 148] and inhibit angiogenesis *in vitro* and *in vivo* [149]. The STS inhibitory activity of bissulfamate **41** is comparable to EMATE activity [150]. Bissulfamoylated derivatives with 2-MeO (**42**, STX140) and 2-Et (**44**, STX243) substituents in steroidal skeleton exhibit high STS inhibitory activity (IC_{50} = 39 and 1000 nM, respectively) [151].

STX140 (**42**) and STX243 (**44**) possess *in vivo* activity also against the MDA-MB-435 cell line (at 20 mg/kg oral) [152]. STX140 *in vivo* inhibits MDA-MB-231 breast tumors [152–154].

Coordination of the 17-sulfamate residue to the zinc in active site of the complex of STX140 with human carbonic anhydrase II is revealed [155].

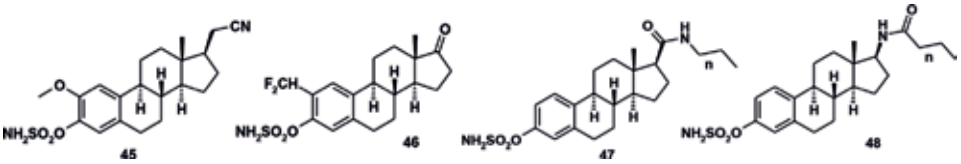


41 X = H
42 X = MeO (STX140)
43 X = MeS
44 X = Et (STX243)

STX140 depolarizes mitochondrial bioenergetics, activates caspase 3/7 causing apoptosis through the intrinsic mitochondrial pathway, and downregulates

the expression of caspase inhibitors [156]. The activity of such compounds is also explained by their ability to disrupt the tubulin-microtubule equilibrium in cells as being central to their antitumor activity. STX140 and STX243 bind with the colchicines binding site of tubulin. 2-(^{11}C)Methoxy-3,17β-OO-bis(sulfamoyl)estradiol has been proposed as a new potential PET agent for imaging of steroid sulfatase in cancers [157].

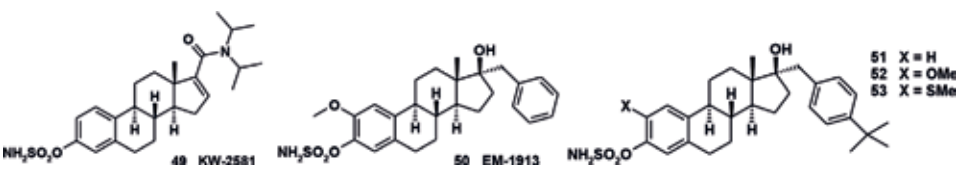
One more example of 2-MeO-derivatives as effective STS inhibitors is illustrated by compound **45** containing cyano group at position C-17 [158].



2-Difluoromethyl-E1-3-O-sulfamate (**46**) is 91-fold more potent inhibitor compared to EMATE (IC_{50} = 0.1 and 9.1 nM, respectively) [159].

The level of STS inhibition for 17β-(N-alkylcarbamoyl)-estra-1,3,5(10)-trien-3-O-sulfamates (**47**) and 17β-(N-alkanoyl)-estra-1,3,5(10)-trien-3-O-sulfamates (**48**) is similar to or exceeded that of EMATE. Some of these compounds are nonestrogenic. 17-(N-alkylcarbamoyl)-estra-1,3,5(10)-triene-3-O-sulfamates and the inverse amides have been patented as good STS inhibitors [129].

Among a series of C17-ketone and amide-modified estrone-derived sulfamates, compound KW-2581 (**49**, 17-diisopropylcarbamoyl-1,3,5(10),16-estratetraen-3-yl-sulfamate) is the most promising, not estrogenic, orally active anticancer agent for the treatment of hormone-dependent BC and endometrial cancer [160]. KW-2581 as STS inhibitor is five times more potent compared to STX-64 (IC_{50} = 4 nM) [161]. It was also demonstrated that the compound inhibits the ability of androstanediol-S to stimulate the *in vivo* growth of MCF-7 cells from BC overexpressing STS. However, KW-2581 is practically insoluble in water (approx. 0.1 ng/mL). The attempts to increase its oral bioavailability showed that the milled powder exhibited poorer properties than the intact sample, including a lower level of crystallinity, higher water content, and increased decomposition rate [162].



Diverse 17α-alkylated estradiol sulfamates as STS inhibitors have been patented [163] and 17α-benzyl-derivatives have been investigated [164].

Compound EM-1913 (**50**) is nonestrogenic steroidal STS inhibitor with IC_{50} = 0.05 nM [165], which also inhibits dehydroepiandosterone sulfate action in androgen-sensitive tissues, being therefore considered as a potential drug for the treatment of prostate cancer [166].

17α-Benzyl substituent yields reversible STS inhibitors in the absence of a sulfamate group, and incorporation of an aryl sulfamate onto the A-ring results in a potent time-dependent irreversible inhibitor. The IC_{50} of the tert-butylbenzyl derivative **51** is low (8.3 nM); however, steroid **51** is estrogenic. A-ring substitution leads to the reduced estrogenicity. 2-Methoxyderivative **52** has an IC_{50} = 0.04 nM. The compound without the tert-butyl group is nonestrogenic and effective STS inhibitor *in vivo* [167].

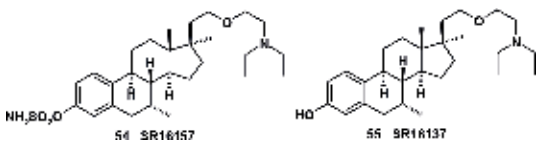
In the series of A-ring thioether-modified sulfamates, the steroid **53** is 50-fold more potent inhibitor of STS than steroid **52**; however, it possesses weak inhibitory activity against MCF-7 cells proliferation (IC_{50} = 10 μM) [168].

2.6.4 Dual STS/SERM inhibitors

Maximum estrogen blockade in the treatment of (ER+) BC may be achieved using dual ERα antagonists and STS inhibitors, which might cause osteoporosis as a side effect [169]. In this case, the compound possessing SERM properties is needed.

Thus, a novel orally available irreversible dual STS/SERM inhibitor SR16157 (NSC 732011) (**60**) (IC_{50} = 0.1 μM) has been developed as a very promising inhibitor with excellent pharmacokinetics and acceptable toxicological profile [170].

Desulfamoylation of SR16157 (**54**) results in SR16137 (**55**), which is a tissue-selective antiestrogen with beneficial effects on bone and cardiovascular system [171]. SR16157 is 10 times more potent as a growth inhibitor of MCF-7 cells than either the antiestrogens tamoxifen or SR1613. Additionally, SR16137 has a 10-fold higher affinity for ERα as compared to tamoxifen. SR16157 was shown to possess minimal genotoxic activity [172]. SR16157 has been recommended in initial phase I of clinical trials with the starting dose of 1.3 mg/kg/day administered as a single dose in humans.



54 SR16157 55 SR16137

We demonstrated that 8-alpha-analogs of steroid estrogens effectively inhibit the growth of BC cells, including triple negative BC [173, 174].

3. Conclusions

Manipulation of hormone biosynthesis in tumors by enzymes inhibitors is a very attractive approach for the treatment of hormone-dependent tumors such as breast, prostate cancer, and endometriosis.

The importance of STS in human body has been underlined by many investigations. Thus, STS-catalyzed hydrolysis of pregnolone-3-sulfate and dehydroepiandro-sterone-3-sulfate in the brain regulates neurosteroid synthesis and influences memory. STS inhibition for the potentiation of memory in sufferers of neurological diseases such as Alzheimer's disease and dementia has been postulated [175]. The role of STS inhibitors as agents to reveal beneficial endogenous glucocorticoid effects was also claimed. The use of STS inhibitors in combination with the immunosuppressive ascomycin for the treatment of acne, seborrhoea, androgenetic alopecia, and hirsutism is patented. The administration of an estrogen (including norgestimate and norelgestromin), in combination with a progestogen in hormone-replacement therapy act by inhibiting STS, thus reduce estrogen production and protect the endometrium and breast from hormone-dependent cancers [176]. STS inhibitors prevent ovarian cycle disturbance, prolonged unopposed secretion of estrogens, and ovarian follicular cyst formation in premenopausal women, as well as prevent premature uterine contractions, particularly for preterm labor [177].

The importance of STS inhibition in endometriosis, prostate cancer, as well as latest discussions about mechanism of inhibition is well considered in the review of Prof. Potter [178]. The significance of steroid sulfatase and sulfotransferases in gynecological diseases are summarized in the review [57].

As far as estrogenic compounds may stimulate tumor cells growth, the main requirement for STS inhibitors and their metabolites is the absence of estrogenicity. Among nonsteroidal STS inhibitors only one nonestrogenic compound-Irosustate was evaluated in clinical trials with excellent properties, however its further development was stopped. Currently, the action of Irosustate in the combination with AIs is investigated.

The discovery of dual (multitargeted) inhibitors is the most promising nowadays. For example, several DASIs based on anastrazole, letrozole, and vorozole templates inhibit both STS and aromatase in nanomolar concentrations, being nonestrogenic; and have a chance to be introduced in clinical trials.

Among STS inhibitors based on steroid skeleton 17α-benzyl-derivatives, 17β-arylsulfonamides, and 17-diisopro-pylcarbomoyl-3-O-sulfamates exhibit the best properties, especially as multitargeted (dual) anticancer potential drugs. The same modifications result in the increased activity against STS in the case of 2-OMe-3-O-sulfamates as well as 2-OMe-3,17β-bissulfamates. The latter also possess activity against most aggressive form—triple negative BC.

Additionally, 8α-steroid estrogen analogs without estrogenic properties possess high STS activity and block BC cells growth with the activity comparable to standard of care for BC treatment tamoxifen.

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Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Abbreviations

AIs	aromatase inhibitors
BC	breast cancer
COMT	catechol-O-methyltransferase
Coumate	4-methylcoumarin-7-O-sulfamate
DASI	dual aromatase-sulfatase inhibitor
DHEAS	dehydroepiandrosterone sulfate
FGly	for-mylglycine
GPER, GPR30	G-protein-coupled estrogen receptor
HDBC	hormone-dependent breast cancer
17βHSD	17β-hydroxysteroid dehydrogenase
E1	estrone
E2	estradiol
E2MATE	estradiol-3-O-sulfamate
EMATE	estrone-3-O-sulfamate
ER	estrogen receptor
LH-RH	luteinizing hormone-releasing hormone
2-OHE1	2-hydroxyestrone
4-OHE1	4-hydroxyestrone

2-OHE2	2-hydroxyestradiol
4-OHE2	4-hydroxyestradiol
STS	steroid sulfatase
SERD	selective estrogen receptor down-regulators
SERM	selective estrogen receptors modulator
UGT	UDP-glucuronosyltransferase

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Chapter 4

Control of Liver Gene Expression by Sex Steroids and Growth Hormone Interplay

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Abstract

Sex steroids have important physiological actions, which are not limited to reproductive organs, in both females and males. They exert important physiological roles, including the regulation of somatotropic-liver axis, intermediate metabolism, or gender dimorphism. This is in part because the liver is a sex steroid-responsive organ where sex steroid- and growth hormone (GH)-dependent signaling pathways connect to regulate complex gene expression networks. Sex steroids can impact liver gene expression by a direct, through hepatic estrogen receptor (ER)α and androgen receptor (AR), or indirect mechanisms, by modulation of pituitary GH secretion and/or interaction with the GHR-STAT5b signaling pathway. Therefore, deficiency of sex steroid- and GH-dependent signaling pathways might cause a dramatic impact on mammalian liver physiology. In this chapter, we will focus our attention on main concepts and paradigms involved in the role and interplay between sex steroid- and GH-dependent signaling to regulate gene expression networks in the mammalian liver. A better understanding of how sex steroids and interactions with GH-STAT5b signaling pathway influence physiological and pathological states in the liver will contribute to improve clinical management of patients with disorders in body growth, development, and metabolism.

Keywords: estrogens, androgens, GH, liver, gene expression

1. Introduction

The liver is as sex steroid-responsive organ [1–13]. The natural estrogen, 17β-estradiol (E2), and androgens, testosterone (T)/dihydrotestosterone (DHT) have physiological actions, which are not limited to reproductive organs, in both females and males. The nonreproductive actions of sex steroids have relevance in liver physiopathology [1–7, 14–18]. The effects of E2 and T/DHT on liver gene expression can be direct, through their hepatic receptors, or indirect, by modulating growth hormone (GH) actions centrally [1, 2], regulating pituitary GH secretion, and, peripherally [5, 7, 19, 20], by modulating growth hormone receptor (GHR)-dependent signaling which can be exemplified by (1) E2 modulating GH actions in the liver through induction of suppressor of cytokine signaling (SOCS) 2 which in turn negatively regulates GHR signal transducer and activator of transcription (STAT)5b signaling pathway and (2) the positive interplay between T/DHT and GH to enhance somatic growth and liver composition in men. Accordingly, deficiency of E2-ERα [4, 18, 19, 21, 22],

androgen/AR [6, 7, 21–25] or GH-GHR [26–32] signaling pathways in adults causes a similar metabolic-like syndrome (i.e., fatty liver, adiposity, insulin resistance), a phenotype that might be ameliorated by E2/T or GH replacement. Therefore, the interplay between sex steroids and GH is clinically relevant because of its importance in the regulation of endocrine, metabolic, and gender-differentiated actions on the mammalian liver [33]. A better understanding of this complex sex steroid-GH interplay in physiological and pathological states will contribute to prevent health damage and improve clinical management of patients with growth, developmental, and metabolic disorders. In this review, we will summarize the role of sex steroid- and GH-dependent signaling pathways on liver gene expression.

2. The liver is as sex steroid-responsive organ

Sex steroids can regulate liver gene transcription through direct and indirect mechanisms.

2.1 Direct regulation of liver gene expression by sex steroids

The liver is a direct target of sex steroids through several receptors and tissue-specific mechanisms [34–36]. The direct interaction between E2 and the transcription factors ERα and ERβ mediates the classical estrogen signaling, which is responsible for most estrogenic effects [35]. ER dimers directly bind DNA, specifically to estrogen-responsive elements located in the ER target gene-regulatory regions, followed by transcription activation. Furthermore, E2 can regulate the expression of its target genes by the interaction between ERs and other transcription factors, such as STAT5. Non-genomic mechanisms, via membrane ERs that activate downstream kinase pathways, have also been described to trigger E2-dependent effects. For instance, the orphan membrane G protein-coupled receptor (GPR)-30 was shown to mediate a rapid estrogen signaling, although conflicting results on this receptor have been reported [37]. Lastly, estrogenic effects are closely related to ER tissue expression. ERα expression has been reported in the bone, reproductive tissues, white adipose tissue, liver, and kidney, whereas the ovary, gastrointestinal tract, lung, bladder, prostate, hematopoietic tissue, and central nervous system are the main ERβ-expressing tissues. This indicates that selective ERα agonists might be used for treating ERα-related liver diseases [38]. Similarly to classical E2 signaling, most of the known androgenic effects are mediated via direct interaction of T/DHT with the DNA-binding transcription factor AR which plays an important role in regulating androgen-dependent gene expression [34, 36]. AR regulates the transcription of a variety of target genes through the interaction with different positive regulators (co-regulators) that provide tissue specificity of androgen actions. In addition, androgen/AR can signal by non-genomic mechanisms. Palmitoylation of AR determines its localization to the membrane, where it can be found in lipid raft membrane [39, 40]. Interestingly, membrane-localized AR can modulate both rapid androgen-mediated G-protein signaling and rapid EGF receptor activation followed by Akt and MAPK signaling pathways and subsequent nuclear AR-mediated effects. T conversion into E2 by aromatase may also play a relevant role to regulate the effects of androgens on body growth and composition.

2.2 Indirect regulation of liver gene expression by sex steroids

Indirect mechanisms, related to the influence of sex steroids on pituitary GH secretory pattern [1, 2] and/or interaction with the GHR-STAT5b signaling pathway in target tissues [5, 7, 19, 20], play a relevant role to regulate the effects of sex steroids on the liver.

2.2.1 Sex steroids regulate the pattern of pituitary GH secretion

Regulation of pituitary GH release depends of two hypothalamic peptides: a positive regulator, GHRH, and the inhibitory hormone somatostatin (SS) [41, 42]. The balance of these peptides is in turn, indirectly, affected by many physiological inhibitors (i.e., GH, IGF-1, glucocorticoids) and stimulators (i.e., sleep, nutrients, exercise, thyroid hormones, sex hormones) of pituitary GH secretion. The final integration of these signals occurs in the hypothalamus. Not only hypothalamic and endocrine factors but also other peripheral elements, mostly metabolic, affect pituitary GH production. These include glucose, fatty acids, amino acids, insulin, leptin, neuropeptide Y, and ghrelin, among others, and are dependent on the metabolic condition of the organism. This is consistent with the GH role in the regulation of somatic growth and composition. A good example to explain the close relationship between GH and the metabolic status is the feedback loop among pituitary and adipose tissues. Adiposity is a powerful negative regulator of pituitary GH secretion. In contrast, GH induces fatty acid mobilization from adipose tissue to reduce adiposity, and circulating fatty acids inhibit pituitary GH secretion. On the contrary, other metabolites such as leptin (also produced in the adipose tissue) [43] and ghrelin (from the stomach) [44] stimulate pituitary GH secretion. Furthermore, sex steroids can also regulate pituitary GH secretion. It has been described that neonatal and postpubertal sex steroids regulate the hypothalamus on its generation of the gender dimorphism of the pituitary GH secretion seen in adulthood. This could explain the gender dimorphism seen in liver physiology [1, 13]. In rodents, gender dimorphism is thought to be controlled by E2 secretion in adult females, whereas it is mediated by T secretion in neonatal and adult males. T neonatal exposure determines adult neuroendocrine control of the pulsatile pituitary GH secretion, which is first seen at puberty, when the GH secretion pattern is perceptible and continues throughout adulthood. In postpubertal rats, the male pituitary GH secretion pattern has been shown to be episodic with peaks every 3–4 hours and no measurable trough levels. As consequence, activation of GHR-STAT5b pathway is episodic as well, and phases with low levels of circulating GH are required to achieve maximal activation of STAT5b-mediated transcription. Conversely, female rat GH secretion is continuous, with higher basal levels and smaller intermittent peaks, and they show reduced STAT5b activation compared with males. Interestingly, depletion of liver-derived IGF-1 (LID mice) [45] or SOCS2 deletion [46] in male mice or exposition of adult male rats to E2 [47] causes liver feminization of some of the GH-regulated biomarkers of gender dimorphism. Therefore, maximal GHR-STAT5b activation occurs at puberty, and suppression occurs during aging or in mutants with defects in GHR signaling. Additionally, xenobiotics (i.e., chemicals, endocrine disruptors) can perturb the hypothalamo-pituitary-liver GH axis and disrupt GHR-dependent activation (masculinization) or suppression (feminization) of STAT5b function in the liver [48, 49]. Other factors that can affect liver STAT5b function include fasting, caloric restriction, and infections. Exposure to DHT and thyroid hormones can cause liver masculinization, whereas glucocorticoids, FGF15, and angiotensin II cause liver feminization [47, 49]. Interestingly, liver feminization has been consistently observed in mouse models of obesity and diabetes. Finally, feminization of the male liver has been also associated with activation of constitutive androstane receptor (CAR) or peroxisome proliferator-activated receptor (PPAR)α, two xenobiotic-responsive receptors, or increased expression of PPARγ but not other lipogenic transcription factors linked to the fatty liver [47–49]. Relevant, GH-activated STAT5b in the liver is also commonly altered by diverse xenobiotics and provides a linkage between chemical exposure and hepatotoxicity.

2.2.2 Sex steroids interact with GHR-STAT5b signaling pathway

E2(ERα)- and T(DHT)/AR-dependent signaling might modulate liver gene expression by interacting with GHR-STAT5b signaling pathway. The level of cell surface GHRs can be influenced by transcriptional, translational, and posttranslational factors (e.g., nutritional status, endocrine context, IGF-1, developmental stage, sex steroids) which, thereby, regulate cell sensitivity to GH actions. In addition, E2 can inhibit GHR-JAK2-STAT5 signaling pathway through induction of SOCS2 and SOCS3 expression which in turn negatively regulates GHR signaling pathway in the liver [19]. Recently, we have shown that subcutaneous administration of nearly physiological doses of E2 to hypothyroid male rats dramatically influenced the hepatic transcriptional program (e.g., genes related to endocrine, metabolic, and gender-differentiated functions) in response to pulsatile (male pattern) GH administration [54]. The effects were associated with increased mRNA expression of several negative regulators of GHR-JAK2-STAT5b signaling pathway (e.g., SOCS2) [54]. It is thought that other negative regulators of JAK/STAT signaling may also contribute to the interaction between E2 and GH in the liver. Indeed, ERα has been shown to stimulate protein inhibitor of activated STAT3 (PIAS3) expression which in turn inhibits STAT3 DNA binding. Intriguingly, as mentioned above, a direct ER-STAT5 interaction might directly control STAT5-dependent transcriptional activity in the liver [50].

Androgen-mediated signaling has shown to be a critical determinant of body composition in adult men, promoting growth of lean mass and suppressing fat deposition [7, 51], a phenotype that is also induced after GH replacement. Interestingly, the GH-IGF-1 axis has been reported to be positively involved in the growth-promoting and metabolic effects mediated by T [5]. Hence, linear growth in children with GH deficiency receiving GH therapy is further stimulated by androgen treatment, and GH is required for reaching whole androgen growth-promoting effect. T increases growth of boys with hypogonadism and those with hypopituitarism under GH prescription. However, T effects on somatic growth are poor in boys with hypopituitarism without concomitant GH replacement. Therefore, it is evident that T-GH interactions are pivotal on body composition, which is clearly exemplified by adult men with GH deficiency, whose lean body mass remains below average even after adequate androgen replacement. Adults with hypopituitarism that are not being treated with GH therapy do not show any effect of T on circulating IGF-1, since both hormones are required to exert an optimal effect on circulating IGF-1. Furthermore, the fact that the effects of GH treatment are more marked in men than in women confirms that T amplifies the anabolic effects of GH in vivo. Although the study is limited to prostate cancer, there are also evidences that T (DHT)/AR signaling interacts with the GHR-STAT5b signaling pathway [20]. In prostate cancer cells, SOCS2 expression was induced by androgens through a mechanism that required STAT5- and AR-dependent transcription. Consequently, SOCS2 inhibited GH activation of JAK2, Src, and STAT5 as well as both cell invasion and cell proliferation in vitro [20]. Thus, in addition of sex steroid regulation of pubertal growth and gender pattern of pituitary GH secretion, induction of negative regulators of JAK2-STAT5b signaling pathway in vivo is a very relevant mechanism that could explain, in part, how sex steroids modulate hepatic transcriptional program. However, further studies are still needed to better understand molecular interactions between sex steroids and GHR-STAT5b-dependent transcription in the liver.

2.3 The GHR-STAT5b signaling pathway

STAT5 proteins are expressed in many tissues and play critical roles in body growth, immune function, cellular differentiation, adipogenesis, oncogenesis, and,

as mentioned above, gender dimorphism [26–32]. Regarding STAT5 tissue distribution, STAT5a is more prevalent in mammary tissue, while STAT5b expression is more enriched in the muscle and liver.

2.3.1 Positive regulation of GHR-STAT5b signaling pathway

GH activates STAT5b via GHR [52–54]. When GH interacts with a preformed dimmer of identical GHR pairs, a conformational change of GHR and the associated tyrosine kinase JAK2 molecules is displayed, exposing the catalytic domain of JAK2 [53]. Thus, through its pseudokinase domain, JAK2 adjacent molecules are activated by transphosphorylation. Activated JAK2 proteins phosphorylate tyrosine residues on the cytoplasmic domain of GHR, activating downstream JAK2-dependent and JAK2-independent intracellular signaling, including, among others, STAT5b-dependent gene transcription. STAT5b phosphorylation by JAK2 results in their dissociation from the receptor, dimerization, and translocation to the nucleus where they modulate a transcriptional network of genes such as IGF-1, SOCS2, CYP2C12, or HNF6 [54–58]. In addition to tyrosine phosphorylation by JAK2, STAT5 activity is also regulated by the Ras/MAPK and the PI3K/Akt pathways. STAT5 proteins have been shown to be regulated by serine phosphorylation which appears to modulate DNA-binding affinity and contributes to STAT5 transcriptional activity in a promoter-dependent manner [59, 60]. STAT5 can physically interact with p85, the regulatory subunit of PI3K and Gab2, which is also involved in the PI3K/Akt pathway [61]. Centrosomal P4.1-associated protein (CPAP) is a cytosolic protein that is normally associated with centrosomes, and it has been shown to physically interact with the unphosphorylated and phosphorylated forms of STAT5A/B [62]. Fyn, a non-receptor tyrosine kinase, and phosphoinositide 3-kinase enhancer A (PIKE-A) also physically interact with STAT5A, and these interactions might be relevant in adipogenesis [63]. It has been shown the adaptor protein [64], CT10 regulator of kinase-like proto-oncogene (CrKL), can form a complex with STAT5 after stimulation with some cytokines (e.g., GH, GM-CSF, EPO) and this complex can translocate to the nucleus and bind DNA to regulate gene expression [65, 66]. Although less known than GHR, there exist several proteins that have been shown to directly associate with STAT5 to enhance its transcriptional activity. Similar to other transcription factors, STAT5 interacts with proteins in the general transcription factor machinery. This is exemplified by CREB-binding protein (CBP) and p300 which are nuclear coactivators that exhibit histone acetyltransferase activity and have been shown to play a positive role in the transcriptional activation of STAT5. There is evidence that p300/CBP binds to the carboxy-terminal transactivation domain of STAT5 and that p300 is responsible for enhancing STAT5 transcriptional activity. The nuclear receptor coactivator 1 (NcoA-1), also known as steroid receptor coactivator 1 (SRC-1) is a nuclear coactivator known to coactivate various nuclear transcription factors such as STAT3, STAT6, progesterone receptor (PR), glucocorticoid receptor (GR), ERα, thyroid hormone receptor (TR), retinoid X receptor (RXR), hepatocyte nuclear factor α (HNFα), and PPARγ. Interestingly, chromatin immunoprecipitation assays have shown that STAT5A/NcoA-1 complex binds to a STAT5 site in the CIS, a negative regulator of cytokine signaling promoter [67].

2.3.2 Negative regulation of GHR-STAT5b signaling

The equilibrium between positive and negative regulators of GHR-dependent activity is of special concern because even slight imbalance may disrupt the GH activity causing serious diseases. Under physiological conditions, activation of GH-induced JAK2-STAT5b is temporary, with a peak of activation achieved within the first 30 min after GH stimulation, followed by an inactivation step [68].

This inactivation period is characterized by an inability of GH to promote maximal JAK2-STAT5 activity in the following 3–4 hours, unless GH is removed from the media. The main post-receptor inhibitors of GHR-JAK2-STAT5 pathway are: the SOCS family, protein phosphatases (PTPs), signal regulatory protein (SIRP)-α1, sirtuin 1 (SIRT1), and protein inhibitors of activated STAT (PIAS). In addition, GH-induced STAT5A phosphorylation and STAT5A-dependent transcription might be negatively regulated by transforming growth factor-β (TGFβ) [69], a cytokine that regulates cell growth, proliferation, differentiation, and death. Furthermore, several proteins that have been shown to directly associate with STAT5 can repress its transcriptional activity. This is exemplified by silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) which is a corepressor for various members of the nuclear receptor family. Although STATs are not member of the nuclear receptor superfamily, SMRT was found to interact with STAT5 and repress STAT5-dependent transcriptional activity [70]. Sac3 domain-containing protein (SHD1) is a protein that has been shown to have a role in mitotic progression and interacts with STAT5, and SHD1 can be induced by various cytokines and hormones, suggesting a potential role in modulating STAT5 transcriptional activity [71].

2.4 The SOCS family

The SOCS protein family is characterized by a specific protein structure as all of them have a SH2 domain and SOCS box domain [72, 73]. From a biological point of view, the SOCS box is an ubiquitination-related domain associated with complexes of elongins C and B, cullin-5, RING-box, and ligase E2, so SOCS proteins may act as ubiquitin E3 ligands that degrade proteins by direct interaction with them. An early step in GH-dependent signaling consists of GHR removal through endocytosis and ubiquitination mechanisms [74–78]. In line with this, SOCS2 has been reported to be essential in GHR-JAK2-STAT5b signaling negative regulation [79]. Regularly, SOCS2 protein levels are constitutively low, but GH rapidly induces its expression, with the subsequent SOCS2 binding to GHR complex, which promotes its ubiquitination and proteasomal degradation. Clinically relevant, SOCS2 negatively regulates GH-dependent control of body growth [26] and glucose and lipid homeostasis [46]. In addition, diverse cytokines, sex hormones (E2 and T), growth factors (e.g., insulin), and xenobiotics (e.g., dioxin, statins), can promote SOCS2 expression, generating a cross-talk mechanism through which multiple endo- and xenobiotics can regulate GHR-dependent activities. SOCS2 is responsible, among others, for regulation of the IGF-1 expression in the liver which is mediated by STAT5b [56, 80–83]. Experiments in mice with SOCS2 disruption also support that STAT5b is critical for GH regulation of somatic growth [72, 82]. In the SOCS2-deleted mice, the difference in body weight after weaning was associated with significant increase in bone length and increase in weight of most organs [80]. This phenotype was also associated with increased levels of IGF-1 mRNA expression in several organs [74, 80]. SOCS proteins can bind directly to tyrosine kinases to deactivate them but can also block docking on cytokine receptors to inhibit the activation of STAT in the JAK/STAT pathway [79]. It has been shown that SOCS7 also interacts directly with STAT5 and inhibits prolactin-, leptin-, and GH-dependent activation of STAT5 [84]. Interestingly, the oncogene PIM-1, a serine-threonine-protein kinase 1, might participate in the mechanism of the negative regulation of STAT5 activity by interacting with SOCS1 and SOCS3 [85].

2.5 Protein phosphatases and signal regulatory proteins (SIRPs)

As expected, tyrosine phosphatases such as PTP-1B and PTP-H1 [86] are negative regulators of the GHR-JAK2-STAT5b pathway. The absence (or inhibition) of

these PTPs produces prolonged activation of STAT5 and STAT3, by GH. Relevant in wild-type fasted mice, the GH resistance state develops, which is manifested by disorders in somatotropic axis at the GHR level, whereas in fasted PTP-1B KO mice, despite starvation, GH resistance state does not develop. PTP-1B KO mice are characterized by increased STAT5b tyrosine phosphorylation and augmented level of IGF-1 [87, 88]. The PTP known as Src homology 2 (SH2) containing protein tyrosine phosphatase (SHP-1) was initially described in the hematopoietic system [89]. GH can activate SHP-1 and induce its translocation into the nucleus, where SHP-1 binds to STAT5b and, subsequently, participates in the termination of GH signaling in the male rat liver [90]. The SHP-2 also plays a critical role in the regulation of GHR-dependent signaling [91]. The absence of SHP-2 binding to GHR results in an increased activation of STAT5b-dependent transcription [92]. The clinical role of the SHP-2 in the regulation of the GH signal transduction is confirmed by Noonan [93] and Leopard [94] syndromes. In addition, dual-specificity phosphatases (DUSPs), a family of type-I cysteine-based protein tyrosine phosphatases that act on both tyrosine and serine/theronine residues on a substrate, are also of interest for its ability to interact with STAT5b [95]. However, further studies are required to understand the mechanism of this interaction. Finally, low molecular weight PTPs (LMW-PTPs) are phosphatases that play a role in controlling cell proliferation via the dephosphorylation of tyrosine kinase receptors and docking proteins. These PTPs are also of interest for their interactions with STAT5 and oncogenesis [96, 97]. Finally, SIRPs are glycoproteins which can bind to the SH2 domains of SHP-2 protein [98]. Particularly, SIRPα-1 decreases GH-induced phosphorylation and activities of STAT5, STAT3, and ERK1/2 and, therefore, acts as a negative regulator of GH-dependent signaling.

2.6 Sirtuins

Human sirtuins are a family (from SIRT1 to SIRT7) of nicotinamide adenine dinucleotide (NAD+)-dependent enzymes that regulate a varied metabolic pathway [99]. SIRT1 plays a critical role in the organization and stabilization of the genome, response to stress, glucose homeostasis or cell differentiation, cell survival, inflammation, mitochondrial biogenesis, and oxidative damage. Interestingly, SIRT1 inhibits GH-induced IGF-1 mRNA expression in the liver, decreases lysine acetylation on STAT5, and inhibits the GH-induced tyrosine phosphorylation [100]. SIRT1 might be involved in GH resistance state. In fasted mice, SIRT1 protein level was increased, and SIRT1 inhibition restored lysine acetylation of STAT5 and STAT5 phosphorylation to basal levels, which reversed the GH resistance state [100]. The inhibitory effect of SIRT1 has been also observed on STAT3 protein activity in the liver. Resveratrol, an estrogenic/antiestrogenic stilbene and stimulator of SIRT1, also caused inhibition of STAT5 and STAT3 activities [101–104].

2.7 Protein inhibitors of activated STAT (PIAS)

PIAS proteins play an important role in the modulation of multiples signaling pathways which include to the AR-mediated transcription [105, 106]. STAT protein may be modulated by PIAS proteins in varied ways: (1) the interaction of PIASs with STATs may be type-dependent (e.g., PIAS1-STAT1, PIAS3-STAT3); (2) the PIASs can inhibit STAT-induced gene expression by DNA-binding inhibition (e.g., PIAS1-STAT1) or without DNA-binding inhibition (e.g., PIAS4-STAT1); and (3) PIAS proteins are expressed in different tissues. Relevant, intranuclear prolactin/cyclophilin B complex might act as a transcriptional inducer by interacting directly with STAT5, resulting in the removal of the PIAS3, thereby enhancing STAT5 DNA-binding

activity and prolactin-induced STAT5-dependent gene expression [107]. However, the role of PIAS proteins in somatotropic-liver axis has not been thoroughly investigated.

2.8 STAT5 interacts with Oct-1 to regulate cell cycle

Cyclin D1 is involved in regulation of the cell cycle and is a STAT5 target gene [108]. Octamer-binding protein 1 (Oct-1) is a transcription factor ubiquitously expressed in the nucleus that contains POU (pituitary-specific, octomer transcription factor, Unc-86) domain, a DNA-binding domain that recognizes the octamer motif. Oct-1 physically interacts with STAT5A in the nucleus, and this interaction is necessary for activating the cyclin D1 promoter and regulating D1 expression.

2.9 STAT5 associates with steroid receptors

PR and GR physically interact with STAT5. PR interacts with STAT5A in the cell nucleus, and STAT5A functions as a coactivator in the regulation of several PR target genes (i.e., RANKL, Wnt4, Areg) [109]. The GR has been shown to physically interact with both STAT5A and STAT5B in a variety of cell types including mammary gland, adipocytes, and hepatocytes. GR acts as a coactivator of STAT5 during mammary gland and somatotropic-liver axis development [110, 111]. Interestingly, GR acts as a positive regulator (coactivator) for STAT5b transcriptional activity in the promotion of body growth and sexual maturation. In fact, mice with inactive GR, specifically in the liver, have impaired body growth, suggesting the importance of GR in hepatocytes for GH-dependent postnatal growth. In addition, genes whose expression was similarly altered by GR and STAT5 deletions in mice included male-predominant genes, GH-responsive genes, steroid dehydrogenases, ribosomal protein genes, or IGF-1 and ASL, two genes which are involved in promoting body growth and gender dimorphism. In addition to GR acting as a positive activator of STAT5 transcriptional regulation, STAT5 has a role in repressing GR-mediated gene transcription [112, 113].

2.10 Epigenetic modulation of STAT5 transcriptional activity: a cross talk with xenobiotics

Finally, STAT5-dependent gene expression might also be regulated by epigenetic mechanisms [114–116]. Lysine-specific demethylase 1 (LSD1) and histone deacetylase 3 (HDAC3) are epigenetic modifiers that are typically associated with the modulation of histone activity. Nevertheless, the biological impact of the LSD1/HDAC3/STAT5A interaction network remains unclear, and further studies are required in order to elucidate the function of these interactions [117]. Enhancer of zeste homolog 2 (EZH2) is a histone-lysine N-methyltransferase enzyme involved in the methylation of DNA, and studies in different tissues have shown that EZH2 can also modulate several activities of STAT5 [118].

3. STAT5b in liver physiology

Target disruption or mutation of the GHR-JAK2-STAT5b signaling pathway together with clinical studies of GH-resistant mutants has shown that this pathway is a key in GH regulation of target genes associated with postnatal body growth, lipid and glucose metabolism, gender dimorphism, and liver pathophysiology (e.g., fatty liver, insulin resistance, fibrosis, hepatocellular carcinoma) [26–32].

3.1 Postnatal body growth

GH modulates postnatal growth [42]. The liver is the main source of circulating IGF-1, and STAT5b directly controls GH-dependent transcription of IGF-1 [26]. How GH treatment is administrated determines GH actions on the liver. In rodents, it has been reported that intermittent (male pattern) GH administration more potently stimulates body growth rate, IGF-1 expression, and STAT5b activity in the liver than continuous (female pattern) GH administration. However, GH is more efficient than IGF-1 since GH triggers additional growth independent of IGF-1. As pointed above, not only STAT5b but also other transcription factors that interact with STAT5b can influence body growth, including GR, a critical coactivator of STAT5b in the liver [119], or ER, which interacts with E2 and STAT5 [50]. Besides endocrine actions, paracrine effects of STAT5 in GH activity on muscle have been described, since a reduction of IGF-1 transcripts in the muscle and a loss of mass in muscle-specific deletion of STAT5a/b were reported [120].

3.2 Lipid and glucose metabolism

Energy/fuel metabolism, and particularly lipid metabolism, is the main metabolic process affected by GH status [26, 47, 121, 122]. GH promotes protein synthesis and inhibits protein degradation in muscle, bone, and other large tissues, thereby blocking glucose and amino acid catabolism and placing lipids as the main source of energy. GH exerts these actions by inhibiting insulin actions and leading fatty acid mobilization from adipose tissue and liver [26, 27]. In adipose tissue, GH poses lipolytic effects and reduces fat mass. This is especially evident in individuals that show an excess of fat accumulated during periods of GH deficiency [26–28]. Furthermore, GH displays triglyceride synthesis and secretion in the liver, and, besides increasing lipogenesis (e.g., SREBP1), GH inhibits PPARα expression and reduces lipid oxidation [47, 123]. In the skeletal muscle, GH drives triglyceride uptake and lipid oxidation, effects that can be reverted by external factors such as nutrition, exercise, or sex steroid hormones. In adulthood, GH can unleash a metabolic syndrome (i.e., increased visceral adiposity, fatty liver, decreased muscle mass, metabolic disturbances) that can be ameliorated by GH replacement therapy. In rodents and humans with fatty liver and adiposity, an ineffective GHR-JAK2-STAT5 signaling has been reported, which is attributed to increased lipogenesis and reduced triglyceride secretion, as well as lowered lipolysis [28, 29, 124]. In fact, it has been shown that STAT5b-deleted male mice become obese in later life [125] and that deletion of STAT5b in a mature human is associated with obesity [126]. In contrast, ablation of SOCS2, with subsequent increased STAT5 signaling, was shown to protect mice from high-fat diet-induced liver steatosis [46]. These evidences highlight two physiological aspects of GHR-STAT5b signaling: (a) STAT5b is essential in the regulation of key enzymes or genes otherwise involved in lipid and energy balance. Clinically relevant is that GH anti-obesity actions increase with the male pattern of pituitary GH secretion because of pulsatile STAT5 signaling and (b) absent GHR signaling, and therefore reduced STAT5 activation, provokes the fatty liver even with normal plasma levels of free fatty acids and minimal adiposity. Interestingly, agonists of liver X receptor (LXR), which cause hepatic steatosis [127], can inhibit GH-STAT5 activation through the induction of sterol regulatory element binding protein 1 (SREBP1) [128]. SREBP1, a LXR target gene, downregulates STAT5b gene transcription and stimulates STAT5b protein degradation. These findings highlight the molecular interactions of LXR with GH-STAT5 signaling in the liver.

GH activates the production of glucose in the liver by promoting glycogenolysis; however, GH can exert either a stimulatory or no effect on gluconeogenesis, due to GH antagonism of insulin action that triggers hepatic/systemic insulin resistance [27]. Furthermore, IGF-1 has an important role on carbohydrate metabolism and may increase insulin sensitivity by suppressing GH release. Therefore, activation of IGF-1 signaling increases the degree of complexity in understanding the molecular mechanisms involved in GH-induced insulin resistance in vivo. GHRKO and GH-deficient mice show improved insulin sensitivity and upregulated hepatic insulin signaling, thereby suggesting that GH locally antagonizes insulin signaling in the liver [129]. However, human GH gene overexpression has been shown to increase basal hepatic glucose uptake and glycogen burden in rats [130]. GH-induced insulin resistance may emerge from the increased mobilization of free fatty acids from peripheral adipose tissue. This can be affecting liver insulin sensitivity, leading to insulin resistance and upregulation of gluconeogenic genes (e.g., glucose-6-phosphatase, phosphoenolpyruvate carboxykinase), essential to glucose homeostasis in the liver. Intriguingly, LID mice have been shown to present a 75% reduction in circulating IGF-1 levels, three- to fourfold increase in circulating GH levels, and insulin resistance, without significant enhanced circulating free fatty acid levels. This suggested a possible local cross talk between GH and insulin signaling systems within the hepatocyte. Additionally, crossbreeding between LID mice and GH transgenic mice resulted in significantly increased serum free fatty acid levels and improved insulin sensitivity due to higher glucose uptake in hepatic, skeletal muscle, and adipose tissues [131]. Besides free fatty acids, the SOCS family of proteins, whose expression is induced by both GH and insulin in the liver, has also been suggested to contribute to insulin resistance [79, 128]. Recently, we have reported that SOCS2 deletion protected mice against the fatty liver, but, paradoxically, worsened insulin resistance was observed in high-fat diet-fed mice [46]. In contrast, SOCS2 deletion was shown to protect adult male mice against streptozotocin-induced type I diabetes [132].

3.3 STAT5b is a master regulator for "liver sexuality"

Gender dimorphism in the mammalian liver contributes to gender differences in body growth, intermediate metabolism, and steroid and xenobiotic compound metabolism. Many sex-dependent liver genes are regulated by sex differences in pituitary GH secretion, with STAT5b, proposed to mediate signaling by the pulsatile, male plasma GH profile. Most of the gender dimorphism in the liver can be explained by the female-specific pattern of pituitary gh secretion, through the induction and suppression of female- and male-predominant transcripts, respectively. The 20–30% of rodent hepatic genes have a sex-specific expression pattern. Genome-wide screens of gene expression have shown that several families of hepatic genes involved in endo- and xenobiotic metabolism and metabolic functions (e.g., lipid metabolism) are dependent on GH- and sex-dependent regulation. Moreover, other hepatic transcripts that encode plasma proteins, enzymes, transcription factors, and receptors and are involved in the metabolism of proteins, carbohydrates, or lipids have been found to be up- and/or downregulated by the different patterns of GH or sex steroid activity [47, 55]. A consensus exists that the response to sex-different pattern of pituitary GH secretion is the major cause of gender dimorphism in the liver. Large-scale gene expression study has been conducted using male and female mice, wild type and STAT5b inactivated, to characterize sex differences in liver gene expression and their dependence on STAT5B [26, 55, 133, 134]. Total disruption of STAT5b triggers loss of sexually dimorphic body growth in mice, as evidenced in affected male mice with reduced size (comparable with female size) and female mice unaffected.

Furthermore, a 30–50% reduction in circulating IGF-1 was found in affected male, but not in female mice. Nevertheless, the combined interruption of STAT5a and STAT5b significantly reduced body weight gain in female mice and repressed body growth in male mice more significantly than in male STAT5b null mice, which resemble both GH- or GHR-deficient mice. These findings confirmed the importance of STAT5b in male-specific body growth while exhibiting that STAT5a equally regulates body growth in both sexes. STAT5b is crucial for sex-dependent liver gene expression, a characteristic of approximately 4% of the genome. In male mice, male-predominant liver gene expressions are positively regulated by STAT5b or STAT5b-dependent factors, whereas female-predominant liver genes are repressed in a STAT5b-dependent manner. Remarkably, a number of the STAT5b-dependent male genes encode transcriptional repressors; these may include direct STAT5b target genes that repress female-predominant genes in the male liver. Several female-predominant repressors show enhanced expression in STAT5b-deficient male mice; these may contribute to the major loss of male gene expression found in the absence of STAT5b. Thus, STAT5b is a key player in this scenario, and it is responsible for the masculinization of the male liver [55, 125]. Conversely, other transcription factors (e.g., HNF6, HNF3β) are more efficiently activated in the female liver or by the continuous GH administration [135, 136]. In addition, SREBP1c induction, as well as hepatic triglyceride synthesis and VLDL secretion, and PPARα inhibition can be observed in the liver after continuous GH administration [47, 123]. However, it is likely that other factors are behind some sex differences in the liver. Potential mechanisms that could contribute to this "liver sexuality" are the pituitary-independent effects of sex steroids through interaction with GH-JAK2-STAT5 signaling pathway in the liver.

4. Sex steroids in liver physiology

The transcriptional program regulated by E2/ERα- and T(DHT)/AR-dependent signaling is linked to body growth and composition, drug-induced hepatotoxicity, liver growth, hepatic carcinogenesis, or even control of fertility [3, 4, 6, 7, 14–17, 23]. However, the specific roles of altered androgen/AR signaling dysfunctions, as well as its influence on GHR-dependent signaling, in the pathophysiology of metabolic phenotypes in the liver remain, in comparison with E2/ERα signaling, largely unknown. Conversely, the influence of JAK2 on ERα/AR-dependent transcription might also play a central role in the regulation of liver physiology and suggests a more complex level of cross talk between E2/ERα- or T/AR-dependent signaling and GHR in the liver [137].

4.1 Body growth and composition

The impact of sex steroids on body growth and composition is complex [4, 5, 19, 138]. Increased pubertal growth velocity associated with enhanced GH secretion has generally been attributed to T secretion in boys and to E2 or adrenal androgen secretion in girls. However, recent evidences support that E2 may be the main hormone promoting pubertal growth spurt in both sexes [139, 140]. Intriguingly, the lack of E2/ERα-dependent signaling, but not of ERβ, mediates key effects of estrogens in the skeleton of male mice during growth and maturation. A similar phenotype to ERα null mice can be found in aromatase-deficient (ArKO) male rats, where T cannot produce estrogens. Remarkably, E2 can retrieve skeletal growth rates in the absence of GHR (i.e., GHRKO mice), which is associated with an elevated hepatic and serum levels of IGF-1. This provides a novel mechanism of hepatic IGF-1 production, independent of GHR [139]. In addition, E2 can induce IGF-1 gene expression in the hypothyroid male rat liver, accompanied by low or

undetectable levels of circulating GH [47]. Gender-related differences in body composition during pubertal growth are thought to be partially mediated by sex steroids through GH-IGF-1 axis modulation. Oral E2 administration to postmenopausal women was shown to decrease circulating IGF-1 levels and increase GH expression, whereas transdermal E2 application was reported to elevate both GH secretion and IGF-1 concentrations [141]. Likewise, oral administration of pharmacological doses of estrogen to hypopituitary patients suppressed GH-regulated endocrine and metabolic effects (i.e., circulating IGF-1 levels, lipid oxidation, and protein synthesis). These effects on metabolism and body composition are attenuated by transdermal administration which suggests that these route-dependent effects are consequence of hepatic first pass effect of oral estrogen leading to direct inhibition of GHR-JAK2-STAT5-IGF-1 signaling pathway. This inhibition might be explained by E2 induction of SOCS2 and SOCS3 which are negative regulators of GHR-JAK2-STAT5b signaling in the liver [19]. E2 modulation of GH signaling is also exemplified by GH treatment inducing a greater increase in lean mass and decrease in fat mass or a greater increase in indices of bone turnover and in bone mass, in GH-deficient male than female patients [142, 143].

4.2 Lipid and glucose metabolism

Gender dimorphism also affects lipid and glucose metabolism [21, 22, 24]. In human and rodents, E2 physiologically mediates lipid and glucose metabolism. In fact, deficiency of E2/ERα signaling can trigger a metabolic syndrome-like phenotype (i.e., fatty liver, adiposity, insulin resistance) [18, 21, 144]. It has been shown that postmenopausal women are more prone to develop metabolic syndrome than premenopausal women. ERα deficiency or reduced levels of aromatase activity have been reported to promote the development of visceral adiposity, insulin resistance, and hyperinsulinemia both in male humans and mice. In ERαKO and ArKO mice, this metabolic syndrome-like phenotype can be reverted by E2 treatment. The favorable effect of E2 in lipid and glucose homeostasis stabilization is also found in ob/ob and high-fat diet-fed mice, models of obesity and type 2 diabetes. Treatment of ob/ob mice with PPT has been shown to improve glucose tolerance and insulin sensitivity, thus confirming the key role that ERα plays in the control of glucose homeostasis. Estrogenic signaling via GPR-30 has also been connected with glucose homeostasis and insulin production. ERα mainly controls antilipogenesis, reduction of adiposity, and improvement of insulin sensitivity, whereas ERβ may be detrimental for the maintenance of normal glucose and lipid homeostasis. In ERαKO mice, insulin resistance, accompanied by increased lipid content and hepatic glucose production, is mainly localized to the liver. Surprisingly, when hepatic ERα was selectively ablated (LERKO mice), mice did not restore the observed ERαKO mice phenotype (i.e., adiposity, glucose intolerance, insulin resistance), even when challenged with a high-fat diet. This suggests that unidentified compensatory mechanisms may be arising or that hepatic insulin resistance occurs as a secondary effect upon ablation of E2 signaling in other cell types. Intriguingly, selective ablations of ERα in the hypothalamic brain region or in hematopoietic/myeloid cells evoke increased body weight and reduced glucose tolerance. The antilipogenic effects of E2 in the liver are partially a result of the activation of PPARα- and the inhibition of LXRα-dependent signaling pathways, with subsequent increased fatty acid oxidation and inhibition of lipogenic genes (e.g., SREPB1c, Apo E) [47]. Activation of LXRα-dependent signaling enhances triglyceride accumulation in the liver. In contrast, E2/ ERα signaling suppresses lipogenic pathway expression and the fatty liver induced by LXR activation [38]. Similar to E2/ERα deficiency, reduced androgen/AR signaling is associated with a metabolic syndrome-like phenotype

(i.e., truncal adiposity, fatty liver, increased triglycerides/cholesterol, reduced HDL, insulin resistance type 2 diabetes), and this is improved after T replacement therapy [6, 25]. Nevertheless, the specific role of the androgen/AR signaling in liver metabolism regulation is still largely understood. Tissue-specific AR signaling has been shown to be involved in the regulation of lipid metabolism (i.e., inhibits lipogenesis, prevents liver steatosis) and promote anabolic growth in peripheral tissues [25]. Deletion of AR (ARKO) causes late-onset obesity in male mice, whereas the liver-specific ARKO (LARKO) exhibits increased insulin resistance and steatosis, with decreased β-oxidation, upon high-fat diet. Clinically relevant, high insulin resistance and impaired glucose tolerance have also been revealed in men with T deficiency [6]. Furthermore, some AR polymorphisms with reduced AR activity are connected to an excess of body fat and fat distribution pattern in both sexes [36]. Remarkably, T treatment diminishes visceral fat and improves nonalcoholic fatty liver disease in mice and human males [6, 23–25]. However, most E2/ERα actions that regulate body weight and lipid/glucose metabolism equally affect both female and male, thus suggesting that T aromatization in E2, via ERα, might also contribute to energy homeostasis in males. In summary, reduced E2/ERα or T/AR signaling is associated with metabolic disorders, including metabolic syndrome-like phenotype with adiposity and hepatic steatosis, which resembles deficiency of GHR-JAK2-STAT5 signaling. Notably, these metabolic disorders can be partially prevented or ameliorated, by E2/T and/or GH replacement therapies, thus suggesting that these hormones control overlapping cellular networks related with physiological control of lipid and glucose homeostasis.

5. Conclusions

Estrogen/ERα-dependent signaling and androgen/AR-dependent signaling are essential components in liver physiology and pathology in both male and female. Both direct and indirect actions of sex steroids in the liver are physiologically and therapeutically relevant. Particularly relevant are sex hormone interactions with GH-regulated endocrine (e.g., IGF-1), metabolic (e.g., lipid and glucose metabolism), and gender dimorphism (e.g., endo- and xenobiotic metabolism) functions in the liver. Therefore, the pituitary (GH)-gonadal (E2 and T)-liver axis is relevant in physiology and pathophysiology in mammals. Additionally, the endocrine and metabolic consequences of long-term exposition to compounds derived from sex hormones and their influence on the pituitary-liver axis need to be further understood. Thus, going in-depth in the study of this complex interaction in both physiological and pathological states may contribute to prevent health damage and ameliorate clinical outcome of patients with growth, developmental, and metabolic disorders.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 5

Role of Androgens in Cardiovascular Diseases in Men: A Comprehensive Review

Dilip Mukherjee, Koushik Sen, Shreyasi Gupta, Piyali Chowdhury, Suravi Majumder and Payel Guha

Abstract

The present knowledge on the androgens role in cardiovascular physiology is not fully completed. It remains unclear whether low serum testosterone concentrations in men are an independent risk factor for cardiovascular diseases (CVDs) or a marker of the presence of CVD. However, we demonstrated that endogenous testosterone levels may be implicated in CVDs. Androgens role in modulating cardiovascular function is one of the highest importances, given that its deficiency is strongly associated with hypertension, atherosclerosis, diabetes, obesity, and cardiac hypertrophy. Although significant and independent association between testosterone levels and cardiovascular events in elderly men have not been confirmed in large prospective studies, cross-sectional studies, however, suggested that low testosterone levels in elderly men are associated with CVDs. The results of androgen therapy are not also conclusive. Perhaps, the effects of testosterone treatment of cardiovascular mortality and morbidity have not been extensively examined in control studies. Data on male animal experimentation of the effect of testosterone replacement therapy are either neutral or beneficial on the development of atherosclerosis. Since circulatory androgen levels modulation is expected to cause many other side effects, it seems to be essential to develop a strategy to target androgen receptor for better treating the CVDs.

Keywords: testosterone, miocardial infarction, men, lipid profile, ROS

1. Introduction

Cardiovascular diseases (CVDs) refers to a class of diseases that involve the heart and/or blood vessels and still the highest leading cause of death in developed and developing countries with earlier onset and possibly of greater mortality risk seen in males compared to females. Approximately 17.5 million people died from CVDs in 2012 representing 31% of all global deaths. It is anticipated that by 2030, the number of death due to CVDs will be reach to more than 23.6 million [1]. Since male gender is one of the risk factors for premature coronary artery disease, stroke, peripheral vascular disease, and heart failure, androgens have often been considered as a cause underlying this male disadvantage [2, 3]. Androgens, mainly

testosterone, may also play in cardiovascular morbidity and mortality by modulating the risk factors of atherosclerosis and vascular functions, lesions to cerebral and peripheral arterial vessel and myocardial infarction leading to heart failure in male [4].

A recent perspective study reveals that testosterone levels in men decline gradually with increasing age and this caused a dramatic increase in the incidence of CVDs [5, 6], but the mechanism of age-related cardiovascular performance remains to be completely understood. However, a protective role of androgen for CVDs in men has been reported and its deficiency may increase the significant risk factor for CVDs. Moreover, controversy also exists whether this age-associated decline in testosterone level is a natural physiologic processes or combination of co-morbidities and life-style choices [7]. With the prospects of much wider therapeutic approaches of testosterone on CVDs, it has become increasingly important to address whether testosterone treatment might increase the risk of severity of CVDs. Considering the importance of therapeutic use of testosterone as have been reflected in several recent studies, it is important to address the issue in a more critical way.

2. Cardiovascular diseases: types and risk factors

CVDs refer to any dysfunctional condition of the heart or the blood vessels (arteries, veins, and capillaries). Coronary heart disease (CHD) and stroke are two fundamental components of CVDs [8]. CVDs can be classified in eight major groups. These are: stroke-disruption of the blood supply to the brain either from blockage or from rupture of blood vessels; CHD-disease of blood vessels, transporting blood to the heart muscle; rheumatic heart disease-caused due to rheumatic fever by streptococcal bacteria when heart muscles and valves are damaged; congenital heart disease-structural malformation of heart; aortic aneurysm-dilation and rupture of aorta; peripheral arterial disease-disease of the arteries that supply blood to arms and legs; deep venous thrombosis and pulmonary embolism-blood clot in leg veins, which can dislodge and move to heart and brain; and other CVDs- tumors of the heart, vascular tumor of the brain, disorder of the heart muscle lining etc.

Risk factors can be categorized as modifiable and non-modifiable risk factors. Modifiable risk factors include; high blood pressure, abnormal blood lipids, tobacco use, physical inactivity, obesity, unhealthy diets, and diabetes mellitus. Non-modifiable risk factors are advancing age, hereditary or family history, gender, and race.

3. Testosterone and its function

Testosterone, a C19 androgen, is the most vital circulating androgens both in male and female. In men, it is mainly synthesized in the testes and a small amount is also derived from adrenal cortex. Testosterone is essential for male sexual differentiation, development and normal function of male reproductive organs, and maintenance of secondary sexual characters. In addition, testosterone promotes many other physiological processes like bone formation, growth of muscle, hair growth, body composition, and erythropoiesis and decreased the risk of osteoporosis [9]. In normal adult men, testosterone concentration ranges between 241 and 827 ng/dl [10]. Secretion of testosterone varies with circadian rhythm.

Circulating testosterone is mainly bound to sex hormone binding globulin (SHBG) and albumin and only 1–2% remains as unbound form.

In target cells, testosterone binds to the intracellular androgen receptors (ARs) or is converted to dihydrotestosterone (DHT) catalyzed by 5α-reductase, which then binds to AR. In some target tissues, testosterone is converted to estrogens by cytochrome P450 aromatase enzyme and estrogens then bind to estrogen receptors. Both androgen and estrogen receptors act as transcription factors and mediate genomic effects [11]. In addition, various *in vitro* and *in vivo* studies have shown that testosterone and its derivatives can affect cellular processes in a non-genomic fashion [12]. Testosterone has been shown to regulate cell to cell ion exchange via gap junction in Sertoli cells and cardiac cells in young rats [13]. Testosterone also promotes vasoconstriction [14, 15] and rapid rise of Ca^{2+} in cultured cardiomyocytes by PLC/IP_3-dependent mechanism [16].

4. Circulatory levels of testosterone and CVDs

Association of blood testosterone levels and incidence of CVDs in men with increasing age is based mainly on observational studies and the main disadvantages of such type of studies are the extremely variable endpoints of CVDs, heterogeneous study groups, and diverse selection criteria. A continuous study for months to several years on a particular study group of CVD patient is very difficult for various reasons. Importantly, patients in these study groups are mostly in medications or modified their life style. Moreover, selection of poorly-matched controls and timing of blood sampling are not always standardized for diurnal variation of hormone levels. All these factors have a serious impact to draw a definite conclusion. However, taking all these into consideration, recently, we investigated the relationship between serum total testosterone levels and lipid profiles as well as fasting blood glucose (FBG) levels in elderly men with angiographically confirmed CVDs from two thickly populated and socio-economically backward districts; Nadia and Murshidabad of West Bengal, India. We observed that relationship between sex hormones, lipid profiles and FBG levels of CVD patients is strikingly different from men with no CVDs of similar age group [17]. Considering the previous observational studies along with our study, we presented a comprehensive idea on the relationship between serum testosterone levels and CVDs globally.

In normal men of developed countries, the overall incidence of testosterone deficiency increases with age and approximately one half a million new cases of testosterone deficiency are expected in men aged 40–90 years old (**Figure 1**) [18]. An independent effect of age on serum testosterone in a study of 890 men has also been demonstrated [19]. Prevalence of testosterone deficiency in men aged >45 years is approximately 38.7% based on total testosterone (T) levels and about 36.3% based on bio-available or free T [20]. They have documented that major risk factors such as obesity, diabetes, hypertension, hyperlipidaemia, prostate disease, and asthma or chronic obstructive pulmonary disease are responsible for low testosterone levels in men compared without such conditions. A schematic representation of the association of testosterone and cardiovascular risk factors is depicted in **Figure 2**. It has been reported that low testosterone levels are associated with increased death from CVDs [21]. Whereas, for a long time prospective studies failed to find significant association between testosterone levels and risk of cardiovascular events in middle aged men [22, 23]. However, a study of osteoporotic fractures in elderly men of Sweden reported that high serum testosterone level is associated with reduced risk of cardiovascular events [24]. This is consistent with the influence of testosterone levels on multiple risk factors such as obesity, diabetes, blood

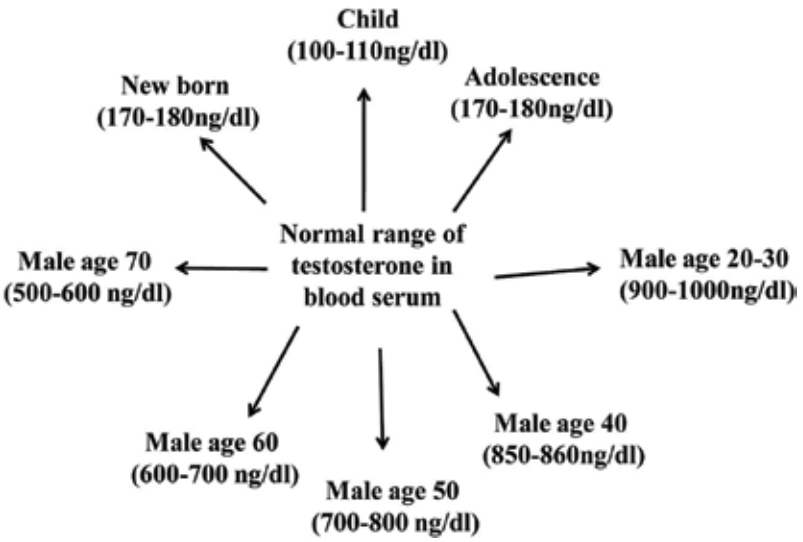

Figure 1.
Testosterone levels in men at different ages of life.

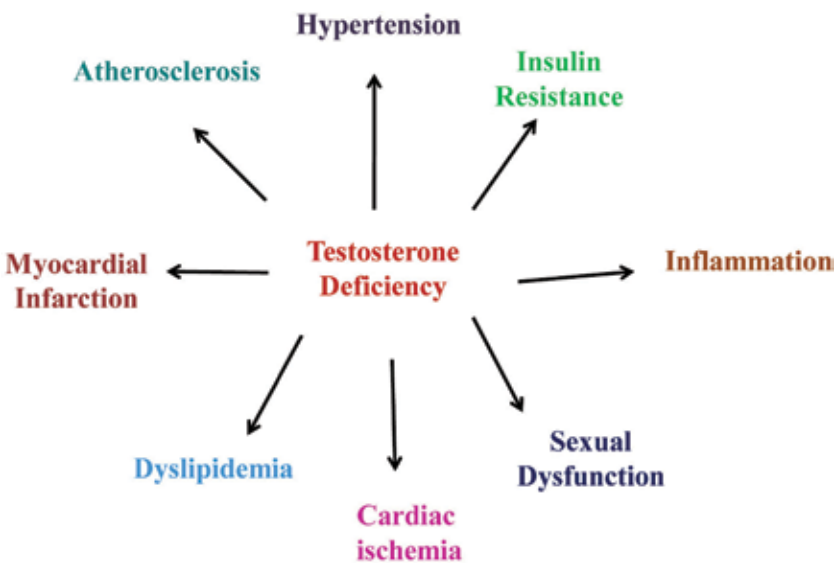

Figure 2.
Association of testosterone deficiency and cardiovascular risk factors.

pressure, and carotid atherosclerosis [25, 26]. A recent meta-analysis showed that low testosterone levels predicted risk for CVDs in elderly men but not middle-aged men [27]. Interestingly, using data from the French Three-City prospective cohort study (3650 men aged >65 years) after adjustment for cardiovascular risk factors, a J-shaped association between plasma total testosterone and incidence of ischemic arterial disease (IAD) in elderly men has been reported [28]. They have suggested that both high and low plasma testosterone levels are associated with an increased risk of arterial ischemic events in elderly men and an optimal range of testosterone levels may confer protection against cardiovascular events. In a recent study, Kelly and Jones [29] observed that testosterone replacement in men diagnosed with hypogonadism shown to be a beneficial effect on several cardiovascular risk factors, cardiac ischemia, functional exercise capacity, and mortality.

5. Association of various risk factors with CVD

5.1 Role of lipids in CVD

It has long been established that lipids play a central role in the initiation and progression of CVDs [30–32]. Dyslipidemia comprises the abnormalities of lipid profiles characterized by high levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) along with low levels of high density lipoprotein (HDL) that contributes to the development of atherosclerosis [33]. In older men, reduced testosterone levels are associated with adverse profiles of lipids. Low testosterone level is associated with high TC, high LDL [34, 35], and high TG [36, 37]. Hypo-gonadal men exhibit abdominal or central adiposity [38, 39]. This finding has led to conclude that all parameters of lipid profile except HDL might be more strongly associated with CVD risk, whereas some investigators reported a negative correlation between HDL and CVD [40, 41]. A strong inverse correlation between body fat and testosterone level is also observed [42]. Higher mass of visceral adipose tissue is inversely correlated with bio-available testosterone [43]. In an epidemiological study from our laboratory, we studied the relationship between serum total testosterone levels and lipid profiles in male patients ranging the age group between 40 and 70 years with angiographically proven CVDs from Nadia and Murshidabad district of West Bengal, India and compared the data with normal men with no CVD history. We observed a significantly low serum total testosterone levels in CVD patient group compared to normal group and further demonstrated a significant negative association between serum total testosterone and TC, TG, LDL, and VLDL among CVDs patients. However, a significant positive correlation between serum total testosterone and HDL was observed [17]. Thus, in these two districts of West Bengal, low levels of serum total testosterone in elderly men are associated with CVD that appear together with an atherogenic lipid milieu that may be involved in pathogenesis of CVD. The molecular mechanism of sex hormone-induced changes in the serum lipid profile is incompletely understood [33]. However, there are evidence from animals, cell, and clinical studies that testosterone controls the expression of important regulatory protein involved in lipid and cholesterol metabolism namely, apolipoprotein A-1

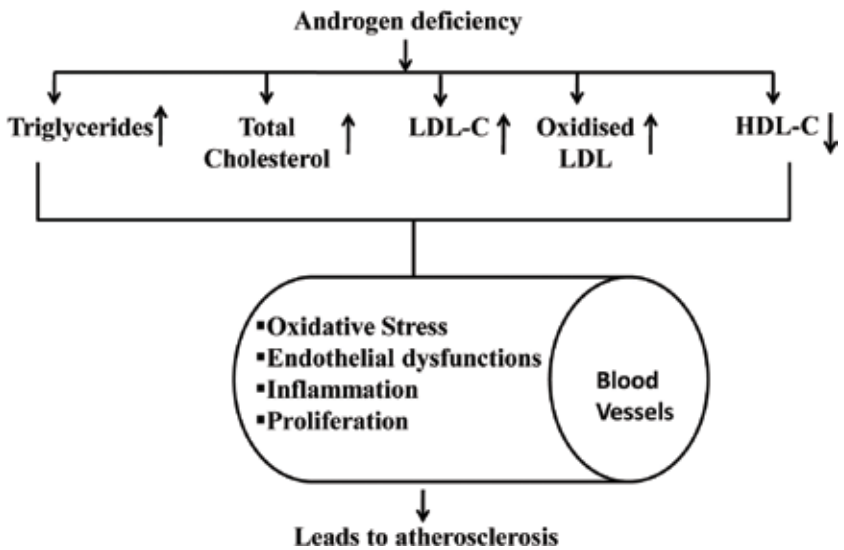

Figure 3.
Changes in lipid profile due to androgen deficiency, leading to atherosclerosis.

(apoA1) [44, 45], and scavenger receptor class B type 1 (SRB1) [46–48]. The major component of HDL is apoA1, which is secreted by the liver in lipid free or minimally lipidated form [44]. The interaction between apoA1 and lipid transfer ABCA1 present in the peripheral tissues results in the formation of minimally lipidated apoA1, which through a series of steps is converted to discoid shaped pre-HDL. This does not possess atheroprotective properties [45]. In addition to apoA1 and SRB1, lipoprotein modifying enzymes are also critical in maintenance of serum lipid homeostasis. One of the most important lipoprotein modifying enzymes is lipoprotein lipase (LPL), present on the endothelial cell surface [49]. Other enzymes are lecithin-cholesterol-acyl-transferase (LCAT) which esterifies the free cholesterol of HDL and cholesterol ester transferase protein (CETP), which mediates the exchange of cholesterol ester between HDL and LDL [44]. Testosterone might promote the expression of SRB1 receptor and facilitate the selective uptake of HDL, thereby exerting an antiatherogenic role [50]. A schematic association of testosterone deficiency and atherogenic lipid profile is depicted in **Figure 3**.

6. Relationship between low testosterone levels and cardiovascular risk factors

6.1 Role of androgens in hypertension

Hypertension is one of the major risk factors for developing CVDs leading to atherosclerosis and sudden cardiac death. Studies with human reveal that hypertension is more prevalent and occurs earlier in men than in women [51, 52]. Sexual dimorphism in blood pressure develops and is maintained until the age of 60 years [53–55]. Epidemiological data further indicate that women older than 60 years, show gradual increase in systolic blood pressure over a period of 5–20 years, until hypertension is highly prevalent in women as in men [55–57]. In hypertensive patients, treatment with antihypertensive drugs can reduce sexual activity and blood concentrations of testosterone [58, 59]. However, treatment of androgen to such patients found to exacerbate hypertension and increase the risk of CVDs [60–62]. There is also higher incidence of hypertension in individual with reduced free testosterone [63].

In animal studies, all major mouse and rat models (noncastrated, castrated, and anti-androgen treated) potential role for androgen in the pathogenesis of hypertension have been documented [55, 64]. In mice, castration and subsequent treatment with testosterone at high dose produce the onset of hypertension and further observed that this effect is mediated by androgen receptor [65]. Long back, it was found that *tfm* X chromosome (including a mutated non-functional AR) rats and castrated rats have lower blood pressure than intact control rats, suggesting that androgen/AR signaling pathway might be involved in hypertension [66]. Thus, androgen-AR signaling pathway appears to be involved in the regulation of hypertension in men and as androgen level reduce with increasing age this might have a deleterious effect on the development of hypertension. Antiandrogen treatment might be able to suppress hypertension. Moreover, some recent studies using AR knockout mice in selective cells suggest that AR in individual cell types may have independent role in the development of hypertension [67, 68].

6.2 Testosterone association in type 2 diabetes and insulin resistance, a risk factor of CVD

Low level of testosterone is associated with type 2 diabetes mellitus (T2DM) irrespective of age, race, and obesity [69–72]. High plasma testosterone level is

associated with reduced risk of developing T2DM [73]. Insulin resistance is the most common hyperglycemic condition and hallmark of T2DM [74]. It is a state where target cells are not responding to normal levels of circulating insulin leading to development of T2DM [75, 76]. An inverse relationship between total testosterone concentration and insulin resistance has also been reported in men [77, 78]. Clinical trials have demonstrated that testosterone administration improved insulin sensitivity, reduced glycaemia, and heart failure progression in men [79].

Cohort studies from Farmingham, Heart Study, EMAS, and Osteoporotic Fractures in Men study [80] and Western Australian Health in Men Study [81] reported that men with T2DM have lower testosterone levels compared with men without T2DM. In fact, different earlier studies showed that men with T2DM have 30–40% lower circulatory testosterone levels than that of healthy men [82–84]. In a study of 3156 men from various ethnic backgrounds, aged 45–84 years and after adjusting for age, ethnicity, BMI, it has been shown that T2DM and FBG levels are inversely associated with total testosterone concentration [70]. In a recent study with elderly male patients (40–70 years of age) of two district of West Bengal, we observed a highly significant negative correlation between serum total testosterone and FBG levels in CVD patients compared with non-CVD patients of same locality [17]. Our results further indicate that low levels of serum total testosterone might have role in the development of hyperglycemia as evidenced from high FBG levels in elderly men. Moreover, a recent study demonstrated that insulin resistance, hyperinsulinemia, and associated hyperglycemia can promote the development of specific form of cardio-morphopathy, which is independent of coronary artery disease and hypertension and a major cause of morbidity and mortality in developed countries [85]. It is characterized by myocardial insulin signaling, mitochondrial dysfunction, activation of sympathetic nervous system, activation of renin-angiotensin-aldosterone system, and male adaptive immune responses [86]. These patho-physiological changes result in oxidative stress, fibrosis, hypertrophy, cardiac diastolic dysfunction, and eventually systemic heart failure [87].

Association of testosterone deficiency with hyperglycemia has also been observed in animal model [88]. It has been demonstrated that castration-induced testosterone deficiency not only enhanced the hepatic gluconeogenesis but also decreased extra-hepatic insulin sensitivity in aged male rats [89]. Unpublished data from our laboratory also demonstrate that castration in adult male mice is followed by an increase in FBG level compared to sham operated control group and this increase in serum FBG levels was reversed after treatment with testosterone.

The mechanism linking androgen with T2DM and insulin receptor is not fully understood. Testosterone administration up-regulate the expression of GLUT-4, insulin receptor substrate-1 (IRS-1) in cultured adipocytes, and skeletal muscle cells [90]. Another study showed that testosterone promotes AKT and PKC phosphorylation, the major mediator of insulin receptor signaling, which regulate GLUT-4 translocation (**Figure 4**) [91]. The beneficial effects of testosterone on diabetes through increasing the metabolic rate in muscle promoting gain of energy from adipose tissue resulting decreased fat mass concentration has also been reported [92]. *In vitro* study of murine model also demonstrated that testosterone administration reduces β cell apoptosis [93], whereas, testosterone deficiency promote elevation of the expression of RBP4, which increases insulin resistance [94]. On the contrary, several studies demonstrate a non-positive correlation between testosterone supplementation and heart failure. Several clinical trials led to propose that testosterone supplementation at physiological doses could be a treatment for men with metabolic syndrome and heart failure [95].

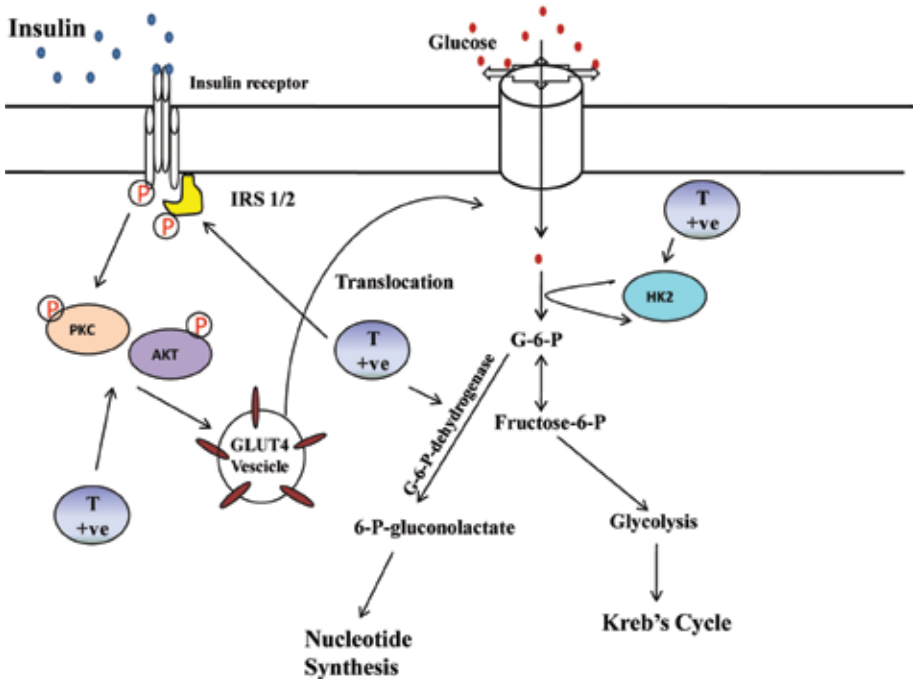

Figure 4.
Proposed mechanism of action of testosterone on cellular IRS activation leading to glucose homeostasis. Testosterone increases GLUT4 expression and membrane translocation which increases cellular uptake and utilization of glucose. Abbreviations: GLUT4, glucose transporter 4; G6P, glucose-6-phosphate; HK2, hexokinase 2; IRS, insulin receptor substrate; T +ve indicates targets or activity increased by testosterone PKC and AKT.

6.3 Testosterone and vascular inflammation

It is now well accepted that atherosclerosis is a chronic inflammatory disease. Individuals with hyperlipidaemia and signs of systemic inflammation develop atherosclerosis, with specific defects in lipid processing and immune activity consequentially occurring at the vessel wall. It is known that the activation of endothelial cells promotes the adhesion of leukocytes to the blood vessel wall as an early atherogenic event leading to increased vascular permeability for not only the inflammatory leukocytes, but also the circulating lipid components, such as LDL [96]. It has been suggested from observational studies that many pro-inflammatory cytokines like interleukin 1β (IL-1β), IL 6, TNF-α, C-reactive protein (CRP), and serum testosterone levels are inversely related in patients with CVDs and T2DM [97–99]. These inflammatory cytokines are known to modulate lipid metabolism, endothelial functions, and atherosclerosis [100]. Testosterone has been reported to reduce the levels of TNF-α and elevated circulating anti-inflammatory IL-10 [101, 102] and circulating CRP [102] in hypogonadal men with CVDs. *In vitro* studies also support the protective effect of testosterone supplementation on atherosclerosis, but the mechanism is not fully known [103, 104].

7. Testosterone deficiency and vascular functions

A negative correlation between testosterone and hypertension has already been discussed. In a subpopulation study of 206 aged males, it was shown that serum testosterone level is an independent negative predictor for developing arterial

stiffness and this association remained after adjusting for the other risk factors [105]. Carotid-intima media thickness (IMT) is a marker for CVDs [106]. The relationship among the progression of carotid-IMT, atherosclerotic plaque formation, and total testosterone was investigated and an inverse relationship between this hormone and atherosclerotic plaque formation was observed. This study also reported for a positive co-relation between carotid-IMT and atherosclerosis [107]. Men with low serum testosterone level exhibit higher IMT compared to normal control [108–110]. Long term testosterone administration reduced carotid-IMT in men with CVDs [111, 112]. Animal models also demonstrated that castration or hypogonadism in mice or rabbits fed a pro-artherogenic diet results in increased atherosclerosis and testosterone supplementation inhibits plaque formation [113]. The cellular and molecular mechanism by which testosterone induced IMT is little understood. Other studies, however, have shown that testosterone may reduce IMT by down regulating the inflammatory response or acting as a regulator of apoptosis or increasing vascular smooth muscle cell stability [7].

Endothelial cells play an important role in atherosclerosis, regulation of vascular tone and forming a barrier that regulates the uptake of cells and macromolecules into the vessel wall [114]. Clinical evidence suggests a link between testosterone deficiency and endothelial dysfunction [115–117]. Flow-mediated dilation (FMD), which represents endothelial dysfunction is decreased in men with testosterone deficiency and increased after exogenous administration of the steroid [118, 119]. Testosterone can exert direct effects on various cells of vascular wall by activation of androgen receptor or by non-genomic effects on plasma-membrane receptors and channels [114]. Testosterone can modulate calcium flux by mechanism that is independent of androgen and estrogen receptors in macrophages and endothelial cells [120]. Androgen receptors are expressed in endothelial cells, smooth muscle cells, and cardiomyocytes and all of these are relevant to atherosclerosis and heart failure [121]. It has also been demonstrated that testosterone may improve endothelial function through modulation of nitric oxide (NO) release. Endothelium-produced NO plays a variety of roles in vascular function maintenance like vasodilatation, inhibition of cell death, and platelet aggregation [96, 122].

8. Role of androgens in cardiac hypertrophy

Cardiac growth can be divided into two categories: normal growth in the developmental process and cardiac hypertrophy induced by hemodynamic overload. Since cardiomyocytes are terminally differentiated and lost their ability to multiply soon after birth, they respond to increased workload by an increase in cell size (hypertrophy), not by an increase in cell number (hyperplasia). Cardiac hypertrophy is prevalent in men with hypertension and recognized as an independent risk factor for congestive heart failure and sudden cardiac death [123]. The most impressive evidence of the effect of androgens on heart is the case of highly conditioned athletes, who died by sudden cardiac death. Examination of such death indicated anatomical abnormalities in heart, known as hypertrophy-cardiac myopathy [124]. Since, the net weight of heart is increased as a result of individual cardio-myocyte, the cardiac hypertrophy is assessed as heart weight to body weight ratio and left ventricular hypertrophy (LVH). LVH is the most potent predictor of adverse cardiovascular outcomes in hypertensive populations and is independent risk factors for coronary heart disease, sudden death, heart failure, and stroke. Clinically LVH is diagnosed by evaluating ventricular functions, such as left ventricular ejection fraction, left ventricular shortening fraction, end-systolic, and end-diastolic volume by electro physiological studies. Although directly related to

systolic blood pressure, other factors including age, sex, race, body mass index, and stimulation of renin-angiotensin-aldosterone system and sympathetic nervous system play an important role of pathogenesis of LVH. LVH is associated both with hypertension and increased cardiovascular morbidity and mortality [125], and it has been suggested that testosterone could be influential in modulating left ventricular mass [126]. Low level of testosterone in male is associated with high blood pressure and left ventricular mass [127]. Interestingly, this association is mediated through obesity. Very recently, it has also been suggested that testosterone can induce hypertrophy in rat heart, which is independent of exposure duration [128].

A central link for the development of skeletal muscle hypertrophy is the activation of mammalian target of rapamycin (mTOR) [129, 130], which also have been reported in testosterone-induced cardiomyocyte hypertrophy [131]. Both type I and type II skeletal muscle fibers have shown to respond in testosterone treatment increasing muscle mass, cross-sectional areas (CSA), and satellite cell number after hormone administration [132]. Testosterone and its synthetic cognates have been used both clinically and illicitly to increase muscle mass [133]. However, the cellular mechanism explaining these effects is not completely understood. Different cellular and molecular mechanisms are shown to be involved in skeletal muscle hypertrophy induced by testosterone, including promotion of nuclear accretion, entry of satellite cells into cell cycle [132–134], and activation of intracellular androgen receptor [135]. Besides regulating gene expression via AR, testosterone also produces fast, non-transcriptional responses involving membrane-linked signal transduction pathways [12]. A rapid non-genomic action exerted via G-protein coupled receptor, intracellular calcium increases, and extracellular signal regulated kinase ½ (ERK 1/2) activation has been described for the action of testosterone in skeletal myotubes [136]. Recently, a cellular lineage of myoblast, which lack the classical AR (L6 myoblast), testosterone has shown to promote the proliferation and differentiation of L6 cell via G-protein coupled receptor [137]. Altogether, these data suggest that in men testosterone, increased cardiac hypertrophy and aside from classical mechanism of action of testosterone, non-classical actions are also implicated in development of cardiac hypertrophy.

9. Testosterone replacement therapy in CVD patients

Testosterone replacement therapy (TRT) is increasingly promoted and suggested to be a possible curative way for the adverse effect of low testosterone on CVDs in elderly men. Whereas, the effectiveness of TRT in hypogonadal men has been shown to be effective in alleviating the symptoms of fatigue, sexual dysfunction, depression, decreased bone density, decreased muscle mass, among others [138–141], uncertainties remain with respect to cardiovascular safety for its use. In 2004, a committee on assessing the need for clinical trial of testosterone replacement therapy by Institute of Medicine (IOM) in a review concluded largely based on placebo control trials and show that there is no clear evidence on benefit of the heath outcome examined. In fact, no positive effect of TRT on cardiovascular events was observed [142–144]. Observational studies evaluating the cardiovascular safety of TRT in men have also generated inconsistent results [145, 146]. An independent review conducted by European Medicines Agency (EMA) also found a lack of consistent evidence for TRT increasing cardiovascular risks (European Medicines Agency (EMA)), 2015 [147]. Very recently, in systematically review and meta-analysis by various authors did not find any significant association between exogeneous testosterone treatment and myocardial infarction, stroke or morbidity of randomized control trials [148, 149]. But, a recent study demonstrated that testosterone treatment in men with low

endogenous testosterone shows improved survival rate in CVD patients [150]. Physiological replacement of testosterone has been shown to decrease cholesterol level and LDL concentration in men [101, 151]. Studies on the effect of TRT on HDL concentration yielded conflicting result with either a decrease [152] or no changes [102, 153]. Other investigators observed an increase in concentration of HDL level after testosterone administration [154]. In a recent study on the effects of TRT on lipid metabolism in hypogonadal men with T2DM, it has been hypothesized that because the relationship between lipid metabolism and artherosclerosis are unequivocal, TRT, which ameliorates lipid metabolism, may decrease the morbidity and mortality of CVD in hypogonadal men with T2DM by preventing atherogenesis [155]. Data from randomized placebo-controlled trials (RCTs) suggest that treatment with testosterone is not effective in reducing CV risk; however, when TRT is correctly applied, it is not associated with an increase in CV risk and it may have beneficial effects in sub-population [156]. On the contrary, available reports indicated that TRT is positively correlated with increased cardiovascular risk [157]. It has been reported that those who are under TRT showed increased risk of CVDs [158, 159]. A systemic review and meta-analysis of the effect of testosterone therapy on cardiovascular events showed that testosterone increases cardiovascular related events among men. The risk of TRT was particularly marked in trials [160].

10. Effects of testosterone on myocardial infarction

The myocardial cells undergo a dynamic repair process after myocardial infarction (MI), which is also regulated by hormonal factors and characterized by removal of necrotic tissue and chamber dilatation for so-called "cardiac remodeling" [161]. Recent cohort studies and meta-analyses of randomized clinical trials reported that testosterone therapy is associated with an increased risk of MI, ischemic stroke, and overall mortality [157, 159]. Supplemental testosterone treatment dramatically increased cardiac rupture and mortality in female mice with or without ovariectomy, whereas castration significantly decreased both the events in males [160]. This indicates role of testosterone is sex specific and even hypotesteronemic condition is good for MI associated cardiac remodeling. Findings suggest that testosterone may adversely affect myocardial healing and early remodeling during the acute phase of MI, causing the observed "gender difference." However, this is highly controversial and association between testosterone therapies and cardiovascular disease is complex and need more dose-specific and time-specific statistical analyses and molecular studies to conclude that whether TRT is beneficial or not.

11. Conclusion

For last two decades, androgens have attracted significant interest in explaining the gender difference in CVDs. Although, strong evidences show that testosterone is associated with prevalence of CVDs and affects several key cardiovascular risk factors and increase the risk of cardiovascular mortality, significant independent association between androgen levels and cardiovascular events in men have not been confirmed in large prospective studies. Effects of testosterone therapy on cardiovascular mortality have not also been definitely confirmed in prospective controlled studies. Testosterone administration in men and animal induces both beneficial and deleterious effects on cardiovascular risk factors. Further research in this field is necessary to know the real cardiovascular effects of androgen and to understand the role of androgen in therapeutic applications in CVDs.

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Chapter 6

Steroid-Based Supramolecular Systems and their Biomedical Applications: Biomolecular Recognition and Transportation

Ruilong Sheng

Abstract

In this chapter, the biomedical application of steroid-based compounds at "beyond the molecule"—supramolecular level—is reviewed. The renewable and economic natural steroid compounds could be employed as building blocks in the design and construction of steroid-based supramolecular systems. The specific physicochemical features (size, shape, topology, hydrophobicity, chemical modifiability, etc.) and biological properties (biocompatibility, biodegradability, bioaffinity, etc.) could be integrated into functional supramolecular systems by chemical synthesis, modification and intermolecular interactions (such as hydrogen bonding, π-π stacking, van der Waals forces, inclusion interactions, chiral interactions, electrostatic interactions, and so on). The steroid-based (supra)molecules could be employed for molecular recognition and/or be self-assembled into various functional supramolecular assemblies for biomedical applications. The specific physicochemical and biological properties, good biocompatibility, and biological activity endow the steroid-based supramolecular systems good feasibility to be employed in biomolecular recognition/sensing and biomolecular transportation (gene/drug delivery). The examples in this chapter are exemplificative of the transformation of natural steroid-based compounds into functional steroid-based supramolecular systems through molecular and supramolecular engineering technology, moreover, which may inspire the systematic study of natural product-based supramolecular (nano)materials toward future pharmaceutical and biomedical industry.

Keywords: steroid, supramolecular, biomolecular recognition, biomolecular transportation, gene delivery, drug delivery

1. Introduction

Transformation of renewable and biocompatible natural products [1] into a variety of molecular building blocks to construct functional molecular systems and then following the molecular assembly processes to create new functional materials has been highly focused for nurturing the sustainable development. Steroids, a large natural lipid family known as "keys of life," played vital roles including membrane formation, hormone metabolism, and cell signal transduction in organelles. Some steroidal compounds possess special physicochemical features

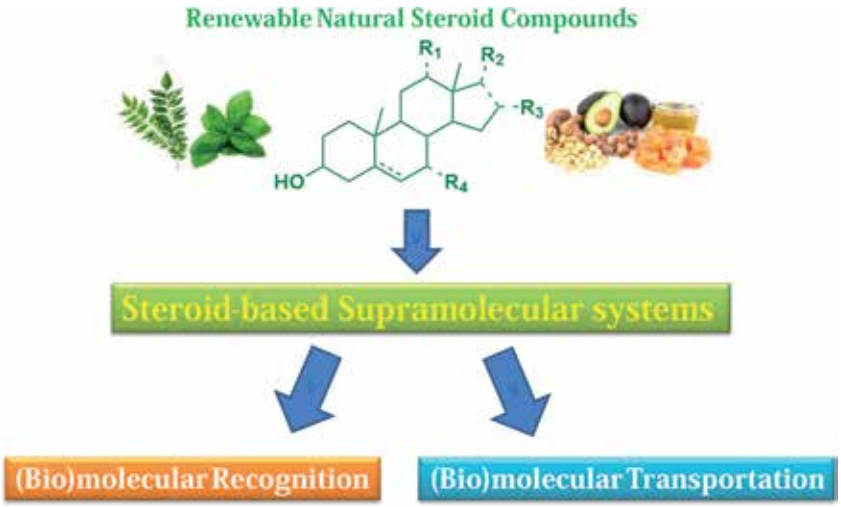

Figure 1.
Steroid-based supramolecular systems for biomolecular recognition and biomolecular transportation.

such as hydrophobicity, rigidity, mesogenic behaviors, and so on, which made them the functional building blocks for the construction of supramolecular architectures [2] and soft nanomatters toward biomaterial application [3].

In general, the functions of supramolecules mainly cover molecular recognition, molecular transportation, and molecular catalysis [4]. Molecular recognition is a fundamental process that integrates molecular information (size, shape, charge, etc.) by interacting (host) molecules with certain (guest) molecular species [5]. Molecular transportation is the use of supramolecules to translocate bounded/loaded molecular species (such as anions [6]) through membranes (especially cell membranes [7]), which could be coupled with chemical potentials [8]. Biomolecular recognition (detecting/sensing of certain biomolecules) and biomolecular transportation (administration/delivery of bioactive molecules into the cells/organs) have been regarded as two important fields in biomedical-orientated supramolecular (medicinal) chemistry [9]. The steroid-based supramolecular systems could be divided into two groups according to their function: (1) steroid-based supramolecular system for biomolecular recognition and (2) steroid-based supramolecular system for biomolecular transportation (**Figure 1**).

2. Steroid-based supramolecular system for biomolecular recognition

Recognition/sensing of biomedically important substances such as specific ions (cations/anions), nucleic acids, peptides, proteins/enzymes, volatile bioorganic molecules, biometabolites, as well as tumor biomarkers is very essential for the deep understanding of biochemical mechanisms. Earlier analytical tools, including chemiluminescence, amperometry, electrochemistry, spectrophotometry, high-performance liquid chromatography, etc., have been developed for the detection of biomedically important substances. However, these traditional methods have some drawbacks such as requirement of expensive instruments and complicated pre-treatment processes, which largely restricted their practical application. Rapid development of artificial molecular receptors or molecular sensors may provide powerful tools for the recognition/sensing of chemical species/analytes, which can be attributed to their advantages of easy-to-manipulate, high-sensitivity, fast-response, high-temporal, and spatial resolution [5].

2.1 Steroid-based macrocyclic molecular receptors

Artificial/synthetic macrocyclic molecular receptors are important supramolecular architectures, which can be used as a host molecule to recognition-specific guest molecules [10]. They can also be used to mimic complex biological host-guest systems, e.g., cell surface receptors, nuclear receptors, as well as enzymes for substrate recognition. Typical macrocyclic molecular receptors bind guest molecules inside their designated cavity. During the past decades, many steroids were developed to construct molecular receptors. Among them, bile acids, a family of molecules with facial amphiphilicity, specific molecular chirality, and multiple reactive sites (hydroxyl and carboxylic acid groups), are often employed as molecular skeletons/scaffolds in the construction of supramolecular architectures for molecular recognition [11, 12].

In an early work, Davis et al. synthesized a neutral and lipophilic system from the steroid cholic acid (**Figure 2**). It forms 1:1 complexes with fluoride, chloride, and bromide ions and shows good discrimination of $Cl^- > Br^- > I^-$ [13]. In this work, the anion recognition process was carried out in organic solvents.

Also for anion recognition, more recently Peng et al. synthesized cholate-based cage amphiphilic systems with combination of structural rigidity and flexibility. These cage compounds with extending and bridging three polar chains were prepared by click reaction. The connecting chains composed of oligo(ethylene glycol) units or chains containing 1,2,3-triazole units to present flexibility, for example, a model compound (triazole **21a**), could recognize halide anions with a binding sequence of $Cl^- > Br^- > I^- \sim F^-$, which makes them potential anions receptors/sensors [14].

Recently, steroid-based macrocyclic molecular receptors with the combination of multifunctions (e.g., chiral recognition-optical properties) emerged as a new trend of research. In this context, Wu et al. synthesized a deoxycholic acid-based macrocycle receptor **CDTB**, which selectively recognized Hg^{2+} involving 1,2,3-triazole motifs as binding sites. The as-formed [**CDTB**·Hg^{2+}] complex could be used to perform enantioselective recognition of amino acids (especially cysteine) in aqueous solution (**Figure 3**), leading to difference in fluorescence enhancement of the chiral BINOL macrocyclic structure at ~358 nm. This research provided cascade recognition of chiral amino acids and bestows the future design of steroid-based dual-functional macrocyclic molecular receptor models for chiral natural product discrimination/recognition [15].

Although some progresses had been made in this field, the synthesis of steroid-based macrocyclic molecular receptors is still mainly focused on the mono steroid-containing macrocyclics and C_2-symmetric macrocyclics; the facile and low-cost

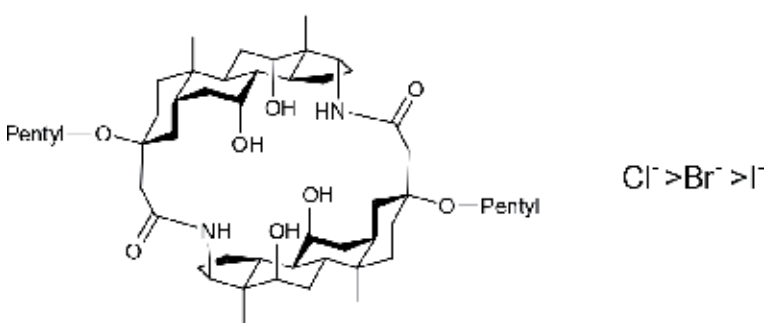

Figure 2.
Cholic acid-based macrocyclic receptor for halides Cl^-, Br^-, and I^- recognition.

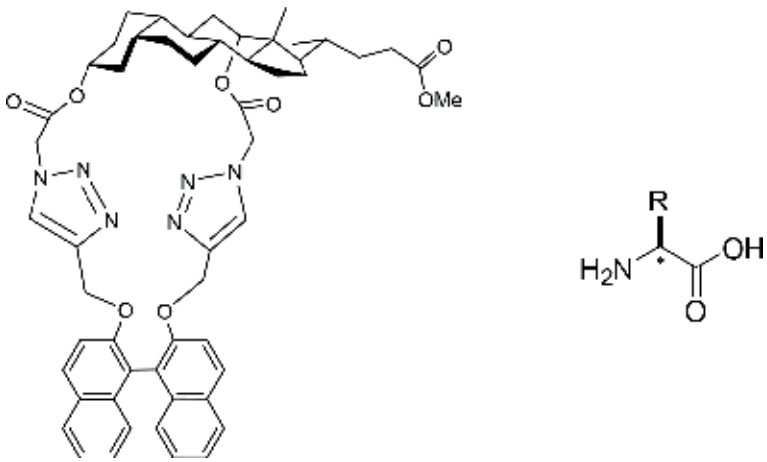

Figure 3.
Cholic acid-BINOL-based fluorescent macrocyclic receptor for chiral amino acid recognition.

preparation of macrocyclics need to be developed. Notably, the steroid-based macrocyclics with higher-order symmetric elements (such as C_3, C_4, D_{xh}, etc.), modifiable and derivable sites, various topological diversities [16], as well as chiral/asymmetric features (giant chiral macrocyclics) are rare. Moreover, for practical application, the functionalities (such as optical, radioactive, paramagnetic, etc.) of the steroid-based macrocyclic molecular receptors need to be largely expanded.

2.2 Steroid-based molecular clefts/tweezers

Another type of artificial/synthetic molecular receptors is open-structured molecular clefts/tweezers, which can recognize guest molecules by forming a sandwich-type structure through π-π stacking, hydrogen bonding, and/or ionic and electrostatic interactions. For the recognition of aromatic molecules, the arms of the molecular clefts/tweezers were generally designated to be aromatic and with special geometrical arrangements. Taking the advantages of low cost, head-tail-modifiable molecular groups, rigidity, chemically different hydroxyl groups, unique amphiphilicity, and natural chiral microenvironment, bile acids and their derivatives are mostly employed to construct steroid-based molecular clefts/tweezers [17].

For the steroid-based molecular clefts/tweezers toward anion recognition, acidic amide groups (such as NH in ureas or thioureas) were always used to achieve higher affinities [17]. In this context, Davis et al. constructed anion receptor by placing squaramide groups in axial positions at the hydroxyl groups of steroid (cholic acid) skeleton, which could fix the NH groups on squaramide at certain locations for cooperatively bind anions (**Figure 4**). By using the steroid-squaramide receptor, anions Cl^- and AcO^- could be transferred from water to organic solvent by liquid-phase extraction. The binding constants of the steroid-squaramide receptor to Cl^- and AcO^- of tetraethylammonium salts exceeding $10^{14}\,M^{-1}$ in chloroform solution have been measured. The results indicated that these anion receptors might serve as transmembrane anion carriers or artificial cell surface receptors for biomedical application [18].

The synthesized molecular tweezers for small biomolecule recognition mainly have charge-bearing moieties/groups such as carboxylic acids and amine/guanine groups. As an example, Rao et al. have designed and synthesized a bile acid-based molecular tweezer with two carboxylic acid groups attached to the C-3 and C-12

hydroxyl groups, which could complex 9-*N*-butyladenine and biotin methyl ester [19] by π-π and electrostatic interactions along with restricted rotation effects (**Figure 5**). Notably, the sensitivity and selectivity of this kind of receptors are not high enough to distinguish biomolecules with similar structures. To design highly selective molecular tweezers, a possible strategy is to mimic the microchemical environment of protein (or sugar) domains responsible for enzyme-substrate recognition or cell receptor-ligand interactions [4].

For chiral amino acid recognition, Davis et al. [20] prepared guanidinium-bearing steroidal molecular tweezers, which could recognize and extract *N*-acetyl-amino acids (**Figure 6**) from aqueous solution into the organic phase ($CHCl_3$) by electrostatic interactions between guanidinium moiety and carboxylic acid groups, with enantiomeric excesses (ee%) of about 80% [21]. In general, the association constant for these acceptors should be around ~10^{-4}–10^{-5}.

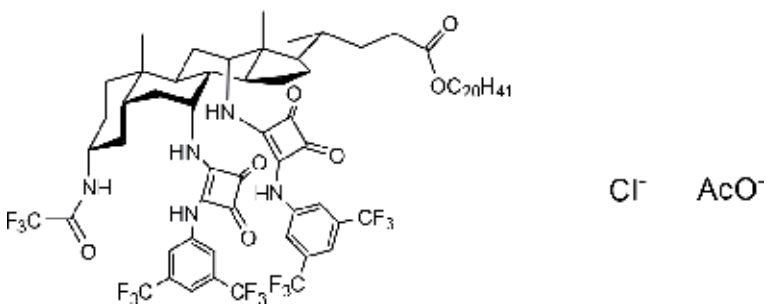

Figure 4.
Cholic acid-squaramide conjugates as a molecular tweezer for anions Cl^- and AcO^- recognition.

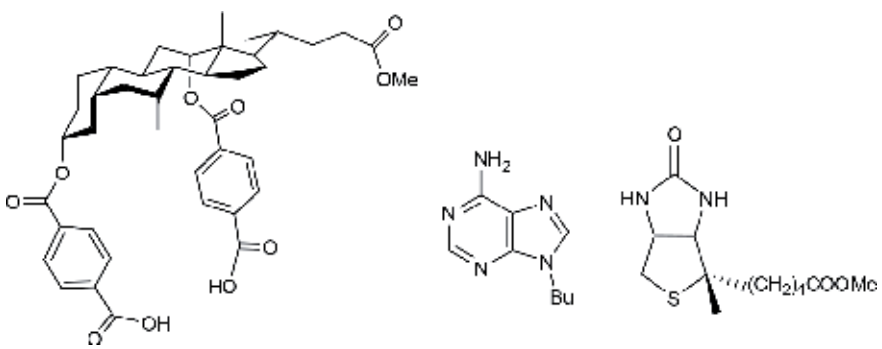

Figure 5.
Cholic acid-based molecular tweezer for N-butyladenine and biotin methyl ester recognition.

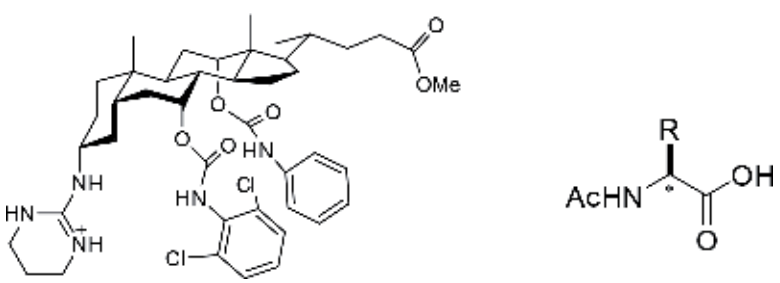

Figure 6.
Cholic acid-guanidinium molecular tweezer for N-acetyl-amino acid recognition.

Bile acid-based receptors containing 2,6-diaminopyridine and the dioctylamide of 2,6-diaminopyridine were also used to bind 7,8-dimethyl flavin analogues. The association constants increased with increasing electron-donating capacity of the substituents at the 7 and 8 positions of the flavin analogues [22].

To our knowledge, up to date, the molecular recognition of the steroid-based molecular tweezers mainly focuses on several simple molecules including anions, nucleosides, and amino acids. Their recognition properties toward more biomolecular analytes/substrates (such as oligosaccharides, peptides, biometabolites, as well as pharmaceuticals) need to be continuously explored. Further improvements on the sensitivity and selectivity, possibility to perform quantitative detection/recognition, increasing signal-noise ratios, as well as developing portable in situ test kit/membrane also need to be taken into consideration. Notably, the cell biological behaviors such as uptake, metabolism, and pharmacological applications of these steroid molecular tweezers are far from being understood. Moreover, the emergence of natural compound such as coumarin [23–25]-based fluorescent molecular receptors/sensors may inspire further development of steroid-based multichannel molecular receptors [4].

3. Steroid-based supramolecular system for biomolecular transportation

Transportation/delivery technology of biomolecular species (especially therapeutic agents) across cell membranes and other biological barriers emerged and rapidly developed as a pivotal area in pharmaceutical and clinical biomedicine, since many biological barriers prevent the implementation of clinically effective therapeutic agents (e.g., genes, antitumor drugs, cell signal inhibitors, neuron modulators, etc.). Therefore, developing functional therapeutic (gene/drug) transportation/delivery systems with the merit of low cost, facile-to-prepare, high storage stability, low cytotoxicity, high gene/drug-loading/delivery capacity, as well as controllable releasing/targeting features has attracted much attention in recent years [26–32].

3.1 Steroid-based supramolecular system for gene delivery

Using renewable and biocompatible natural-based resources to construct supramolecular biomaterials has attracted great attentions in recent years. As a hot spot in biomaterial research, developing new cationic lipids as non-viral gene (DNA, oligo DNA, SiRNA, etc.) carriers toward gene therapy has been achieved increasing attentions in the past few decades [33, 34]. An ideal lipid gene carrier should be highly biocompatible [35] and could efficiently load and release therapeutic gene substances [36] into target cells. In this context, recent researches revealed that the introduction of some steroidal hydrophobic molecules in gene carriers could enhance gene loading capacity and delivery efficiency [37], improve estrogen receptor (ER) affinity [38], lower cytotoxicity and membrane disruption [39], and so on, making the steroid-based cationic amphiphiles/lipids promising candidates for gene delivery/transfection (**Figure 7**).

Among the steroid compounds, cholesterol was the most commonly used steroidal compounds in the construction of functional gene/drug [40] carriers. As an example, Bhattacharya and Bajaj developed a series of cholesterol cationic lipids [41] and gemini-lipids [42–46] with remarkably high gene transfection efficiency and transfected p53-EGFP-C3 plasmid DNA to induce tumor apoptosis [47]. In another example, Rana et al. [48] prepared some cholesterol-hybridized cationic lipids with enhanced SiRNA delivery efficiencies and lower cytotoxicity.

In addition, Zenkova et al. [49–51] disclosed a series of cholesterol cationic lipids modified with heterocyclic (pyridine, methylimidazole, etc.) or polyamine headgroups having low cytotoxicity and high transfection efficiency, and some cholesterol-based cationic glucosidal lipids also have similar properties [52].

In our earlier work, we prepared a series of bioreduction-responsive cholesterol disulfide cationic (CHOSS) lipids [53], which possessed low cytotoxicity, high pDNA transfection efficiency, as well as perinucleic localization effect (**Figure 8**). Afterward, we studied the structure-gene transfection relationship of some cholesterol-based cationic lipids bearing versatile amino acid headgroups and chemical linkage bonds [54], and it was found that the physicochemical features and gene transfection-related properties of the cholesterol-based lipids relied greatly on the cationic headgroups [54].

Besides cholesterol, some other steroidal compounds such as diosgenin (a phytosteroid sapogenin used in the preparation of different steroids, e.g., cortisone), bile acids, etc. were employed to construct lipid gene carriers. As an example, Regen et al. developed a series of "molecular umbrella" amphiphiles [55] and disulfide-containing bile acid-SiRNA conjugates [56] for intracellular SiRNA delivery. In addition, Yi et al. [57–59] synthesized some diosgenin-based cyclen cationic lipids with the merit of low cytotoxicity and high transfection efficiency. In a previous work, we also synthesized some cholesterol and lithocholate-derived cationic lipids via CuAAC "click" approach and disclosed that their gene transfection efficiency relied greatly on the steroid structures [60].

It has been known that the endocytosis mechanism greatly affects the intracellular gene transfection efficacy and subcellular distribution of gene carriers [61]. For the endocytosis pathways of steroid-containing gene carriers, only a few cases were

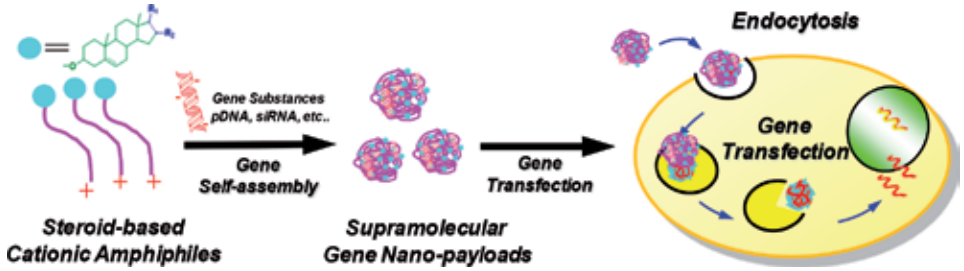

Figure 7.
Steroid-based cationic amphiphiles/lipids for gene delivery/transfection.

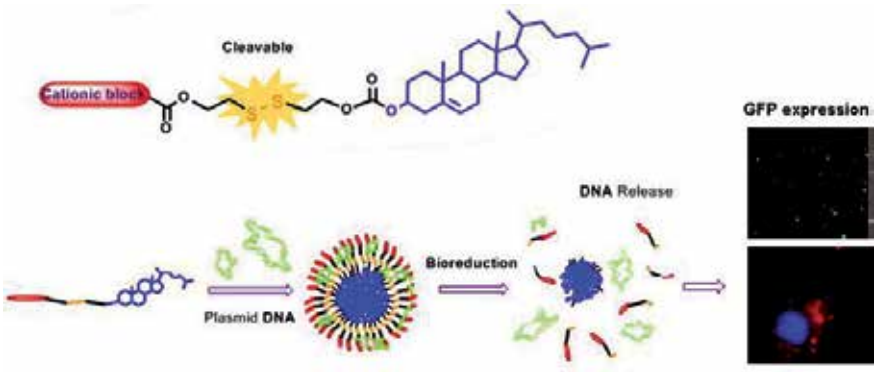

Figure 8.
Bioreduction-responsive cholesterol-based disulfide cationic lipids/pDNA supramolecular payloads as efficient gene delivery carriers.

investigated. In this context, Bae et al. [62] found that clathrin-mediated endocytosis is the dominant pathway for cholesterol-based (CHOL-E) liposomes. On the other hand, Pozzi et al. [63] disclosed that macropinocytosis is the only endocytosis pathway of a cholesterol cationic lipid (DC-Chol) containing multicomponent envelope-type nanoparticle system (MENS). Besides, Jeong et al. [64] disclosed that clathrin, caveolae, and pinocytosis pathways are involved in the cellular uptake mechanism of hydrophobic 5β-cholanic acid containing glycol chitosan (HGC) nanoparticles.

In a recent work, our research team successfully prepared a series of steroid-based cationic lipids by integrating various hydrophobic steroid skeletons with (*l*-)-arginine headgroups via a facile and efficient synthetic approach. We found that the plasmid DNA (pDNA)-binding affinity of the steroid-based cationic lipids, average particle sizes, surface potentials, morphologies, as well as stability of the steroid-based cationic lipids/pDNA lipoplexes depend largely on the steroid skeletons. Cellular evaluation results revealed that cytotoxicity and gene transfection efficiency of the steroid-based cationic lipids in H1299 and HeLa cells strongly relied on the steroid. Interestingly, the steroid lipids/pDNA lipoplexes seemed to enter H1299 cells mainly through caveolae- and lipid-raft-mediated endocytosis pathways, and an intracellular trafficking route of "lipid-raft-mediated endocytosis→lysosome→cell nucleic localization" was accordingly proposed (**Figure 9**). The study provided possible approach for developing high-performance steroid-based lipid gene carriers, in which the cytotoxicity, gene transfection capability, endocytosis pathways, as well as intracellular trafficking/localization manners could be tuned/controlled by introducing proper steroid skeletons/hydrophobes. Noteworthy, among the lipids, Cho-Arg showed remarkably high gene transfection efficacy even under high serum concentration (50% FBS), making it an efficient gene transfection agent for practical application [65].

Although many remarkable achievements have been made in the steroid-based gene delivery systems, the working performance such as biocompatibility, gene transfection efficiency, serum compatibility, cell membrane permeability, as well as the in vivo transfection of the most of steroid-based gene carriers were still far from their maximum value, especially far below from their natural virus (adenovirus, SV40, etc.) counterparts. The correlation between steroid-based molecular structures and their transfection efficiency is not well known, and, notably, the correlation between molecular structures and endocytosis pathways, endonucleasis gateways, and intracellular trafficking and subcellular targeting/localization for the most of steroid-based gene delivery systems still remains unclear. Elucidating these correlations may offer new routes to further design steroid-based supramolecular systems with "endocytosis pathway selection" and "subcellular organelle targeting/localization" features.

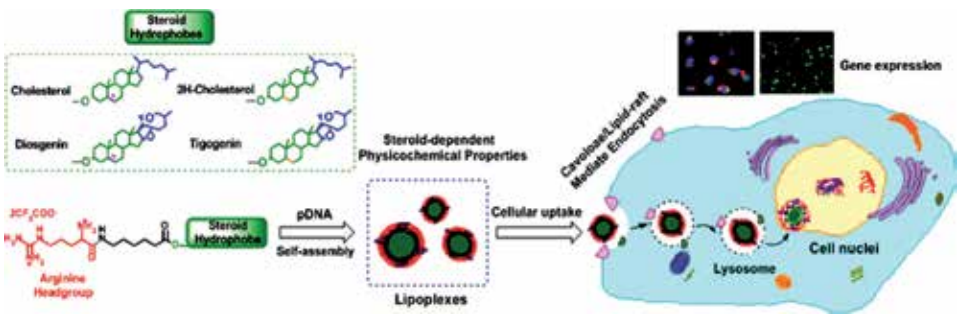

Figure 9.
Steroid-based cationic lipids/pDNA supramolecular payloads as efficient gene delivery carriers and the caveolae/lipid-raft-mediated cellular uptake pathway.

Moreover, to achieve combo-chemotherapy and high theranostic performance, remote [66] factors (e.g., near-infrared light, ultrasonic, X-ray, or γ-ray)—induced controllable gene releasing and (optical and radioactive) imaging agents—which incorporated steroid-based supramolecular gene carriers need to be taken into consideration. For future research, we envisioned that "smart" features such as enzyme-responsive [67], self-programmable [68], self-replicable, as well as self-evolution technology could be implemented on the steroid-based supramolecular gene carriers by designing/optimizing the steroid-based molecular structures or supramolecular architectures through molecular or supramolecular engineering approaches.

3.2 Steroid-based supramolecular system for small molecule/drug delivery

Similar to gene delivery, controllable delivering of small molecules, including drugs and other bioactive compounds by steroid-based supramolecular systems, is another important field. Some steroids such as bile acids and diosgenin were utilized to prepare drug delivery carriers. In an early study, Regen et al. developed some cholic acid-based molecular umbrellas, which were utilized to transport small biomolecules such as adenosine 5-triphosphate (ATP) [69], glutathione (GSH) [70], as well as an oligonucleotide (S-dT16) [71] across phospholipid bilayer membranes prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol.

To improve the hydrophilic, long-retention/stealth effect, and biocompatibility, polyethylene glycol (PEG) was often introduced to steroid scaffolds [72]. In fact, PEGylated bile acids were synthesized to further prepare self-emulsifying drug delivery systems (SEDDSs), which could enhance the solubility and absorption of poor water-soluble antitumor agent (doxorubicin [73]) or antibiotics (itraconazole [74]), thus providing a significant enhancement of solubility and bioavailability of these small molecular drugs. The emulsions consisted of spherical micelles with a mean hydrodynamic diameter around 100–220 nm, with good biocompatibility (low cytotoxic and hemolytic effect).

Taking advantage of organotropism effect of certain steroid compounds (such as cholesterol and cholic acid), steroid-drug conjugates enable enhanced active targeting of drug delivery into certain organelles to improve their bioavailability. Some bile acid-based prodrugs are prepared by conjugating drugs through degradable bonds, either direct or via spacer molecules to the carboxylic group or to the chemically different (C-3, C-7, and C-12) hydroxyl groups [75]. Tolle-Sander et al. found that cholic acid-acyclovir conjugated prodrugs could target human apical sodium-dependent bile acid transporter (ASBT) to enhance acyclovir bioavailability. In this case, a valine linker between cholic acid and acyclovir could be cleaved upon esterase hydrolysis and release acyclovir [76]. Later, other bile acid-based prodrugs such as cholic acid-cytarabine conjugates [77], cholic acid-5-fluorouracil (FU) conjugates [78], and bile acid-tamoxifen conjugates [79] were developed. The bile acid-based prodrug transport systems showed improved drug absorption, membrane permeation, as well as the "trojan horse" effect [80] that largely increased the bioavailability of the antitumor drugs. In 2009, Regen et al. reported molecular umbrella-hydrophobic drug conjugates, which exhibit enhanced uptake capability to enter living (such as HeLa) cells and increased drug activity, suggesting the conjugates could be used as drug carriers [81]. Besides, the organ-specific targeting properties, especially the liver and small intestine distribution effect, were making the bile acid-based prodrug transport systems efficient candidates for the delivery of low-bioavailability molecular pharmaceutics [82]. The bile acid-based prodrugs provide efficient building blocks for constructing and developing supramolecular prodrug drug delivery systems (SPDDS), which also inspired the extensive R&D of other steroid-based

SPDDS [83]. It could be envisioned that, by choosing certain functional moieties to construct steroid-based prodrugs and followed by self-assembly, efficient SPDDS toward controllable chemotherapy could be achieved (**Figure 10**).

Recently Wei et al. designed and prepared a novel diosgenin-PEG (derivative)-based prodrug nanocarrier for inhibiting thrombosis. The steroid diosgenin was conjugated to PEG by means of a pH-sensitive Schiff base bond to prepare the prodrug, then which was self-assembled into nanomicelles in aqueous solution. Under acidic condition (around thrombosis places), the diosgenin-PEG-containing micelles could be cleaved and released and could improve the blood diosgenin concentration to efficiently inhibit thrombosis. Moreover, the diosgenin-PEG micelles without bleeding risk prevented thrombosis by inhibiting activation and apoptosis of platelet. In this study, the observed efficiency of diosgenin-PEG was better than that of the nonsteroid antithrombotic agent aspirin [84].

Multicomponent nanotherapeutic (by combining two or more drugs/prodrugs into a single system) drug delivery systems (MCNDDS) and related formulations have attracted more and more attention. With the merit of easy-to-manipulate, good storage stability, high drug-loading capacity, low cytotoxicity, as well as controllable drug-releasing features, R&D on MCNDDS could be expected to serve as a promising field in nanopharmaceutics and clinical medicine [85]. As mentioned above, cholesterol has been known to play important roles in membrane property regulation, cell adhesion, and signal transduction, regulating lipid bilayer interaction and intracellular trafficking of nanoparticles, thus bringing new potential applications in biomedical engineering. In one case, cholesterol-based adenosine triphosphate has been prepared, which could be efficiently transported across bilayer membranes of liposomes [86]. In recent studies, we prepared a series of combo-nanotherapeutics by controllable incorporation of cholesterol-based/−conjugated doxorubicin prodrug (**Chol-LK-Dox**) with tocopherol polyethylene glycol succinate (**TPGS**), a helper lipid in the construction of functional liposomes or solid lipid nanoparticles, using a thin-film hydration method (**Figure 11**). Among them, we found that a series of **Chol-Dox/TPGS** assemblies (molar ratios 2:1, 1:1, and 1:2) were able to form nanoscaled particles with the average hydrodynamic particle diameter of 100–250 nm and remarkable solution stability (in 0.1 M PBS, 30 days). Notably, the doxorubicin loading and releasing properties could be adjusted by changing the molar ratio of **Chol-Dox** and **TPGS**, thus leading to controllable tumor cell inhibition properties to breast cancer (MCF-7 and MDA-231) cells. Likewise, the physicochemical properties and bioactivity of another cholesterol-based nanodelivery system (**Chol-LK-Dox/TPGS**) could also be tuned by changing the (bioresponsive) linkers and molar ratio of **Chol-LK-Dox** and **TPGS**. The cellular biological properties of **Chol-LK-Dox/TPGS** systems in other cancer cell lines and in vivo therapeutic properties in xenograft mice models will be deeply investigated (project ongoing in our lab).

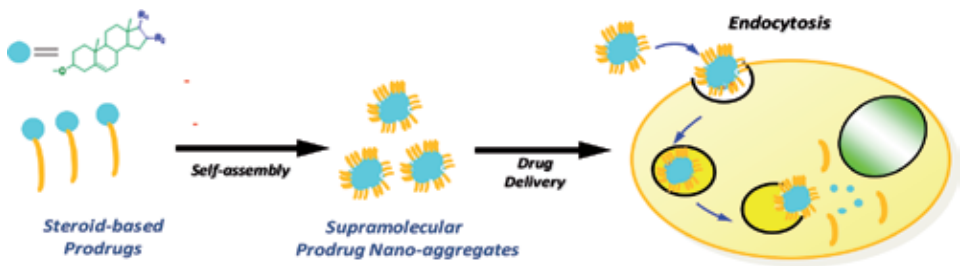

Figure 10.
Self-assembly of steroid-based prodrugs into supramolecular payloads for drug delivery application.

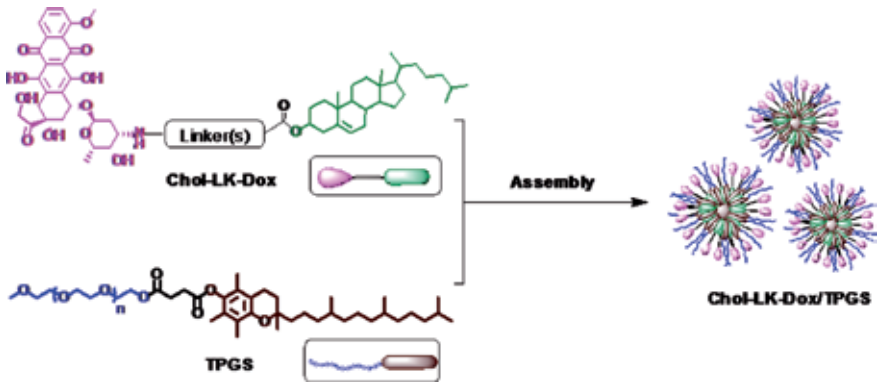

Figure 11.
Self-assembly of steroid (cholesterol)-doxorubicin prodrug (Chol-LK-Dox) with TPGS to prepare MCNDDS for combo-chemotherapy.

Nowadays, for the requirement of "precise biomedical treatment," the steroid-based supramolecular prodrug systems with smart manners such as stimuli-sensitive (temperature, ultrasound, light, electric, pH, redox, biomolecules, and enzyme) features and targeting (cell membrane, subcellular organelles, and cell nuclei) properties need to be further developed.

4. Conclusions

In this chapter, we reviewed the main biomedical application of steroid-based compounds "beyond the molecule"—supramolecular level. The renewable, economic natural steroid compounds could be employed as building blocks in the design and construction of steroid-based supramolecular systems. Based on the specific physicochemical features (size, shape, topology, hydrophobicity, chemical modifiability, etc.) and biological properties (biocompatibility, biodegradability, bioaffinity, etc.), through chemical synthesis, modification, and by means of intermolecular weak interactions (such as hydrogen bonding, π-π stacking, van der Waals forces, inclusion interactions, chiral interactions, electrostatic interactions, and so on), the steroid-based functional molecules could be organized to supramolecules for molecular recognition/sensing and/or be self-assembled into various functional supramolecular assemblies for biomedical applications. The specific physicochemical and biological properties, good biocompatibility, and biological activity endow the steroid-based supramolecular systems good feasibility to be employed in biomolecular recognition/sensing and biomolecular transportation (gene/drug delivery). The examples in this chapter illustrated the transformation of natural steroid-based compounds into functional steroid-based supramolecular systems through molecular and supramolecular engineering technology, which may inspire the systematic study of natural product-based supramolecular (nano) materials toward the future pharmaceutical and biomedical industry.

Although many natural steroid-based supramolecular/nano-systems have been developed and studied, there are still many problems which need to be solved and vast spaces that need to be filled in further extensive research: (1) At molecular level, apart from the natural steroid-based supramolecular shown above, the steroid-based compounds with unique structures (molecular symmetry, geometry and topology, polarity, amphiphilicity, multivalency, etc.), physicochemical (thermal, optical, magnetic, acoustic, radioactive, etc.), properties and biofunctions (biorecognition, targeting, endocytosis, cell signaling, etc.), as well as green synthesis

techniques of the building blocks/units that need to be further developed. (2) At supramolecular level, the self–/forced assembly properties of many natural steroid-based supramolecular/nano-systems were still not well studied; especially their structure–property relationships need to be further explored, realizing the control/adjustment of the steroid-based nanoassemblies with specific physicochemical and/or biological functions. (3) For biomedical application, we need to continue exploring the related biological functions (such as biocompatibility, biometabolic activity, biomimicking manners, etc.) of the steroid-based supramolecular systems and reveal the relationship between the molecular/supramolecular structure and their biological behaviors. Moreover, we anticipated that molecular-level properties of the steroid-based molecules/building blocks would be transferred, enhanced, and/or magnified into supramolecular-level properties, providing a "bottom-up" method to create new renewable resource-derived nanostructures and nanomaterials.

Finally, we need to notice that the steroid-based supramolecular system as aforementioned in this chapter is mostly restricted in low-dimensional 0D and 1D level and, therefore, for real practical application toward complexity systems, higher-ordered steroid-based supramolecular systems (such as 2D and 3D) are needed to be further developed; especially, as for the emergence of natural-based tissue engineering materials and rapid development of 3D bioprinting technology, steroid-based supramolecular system for cell culture and regenerative medicine needs to be taken into consideration and systematically developed in the near future.

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Conflict of interest

No "conflict of interest."

Notes/thanks/other declarations

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The steroid scaffold continues to be the structural basis of new drugs for a variety of targets and diseases. Indeed, steroids interact with enzymes and receptors in a strikingly specific manner. Chemistry and Biological Activity of Steroids aims to provide an updated overview of recent advances in the medicinal chemistry of steroids. Novel synthetic methods in the steroids field, including steroid biotransformations, new steroids able to tackle steroid receptors, and steroid enzymes with clinical relevance, are critically reviewed in this book. Furthermore, the diverse physiopathological roles of oxysterols and their therapeutic value are also discussed.

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