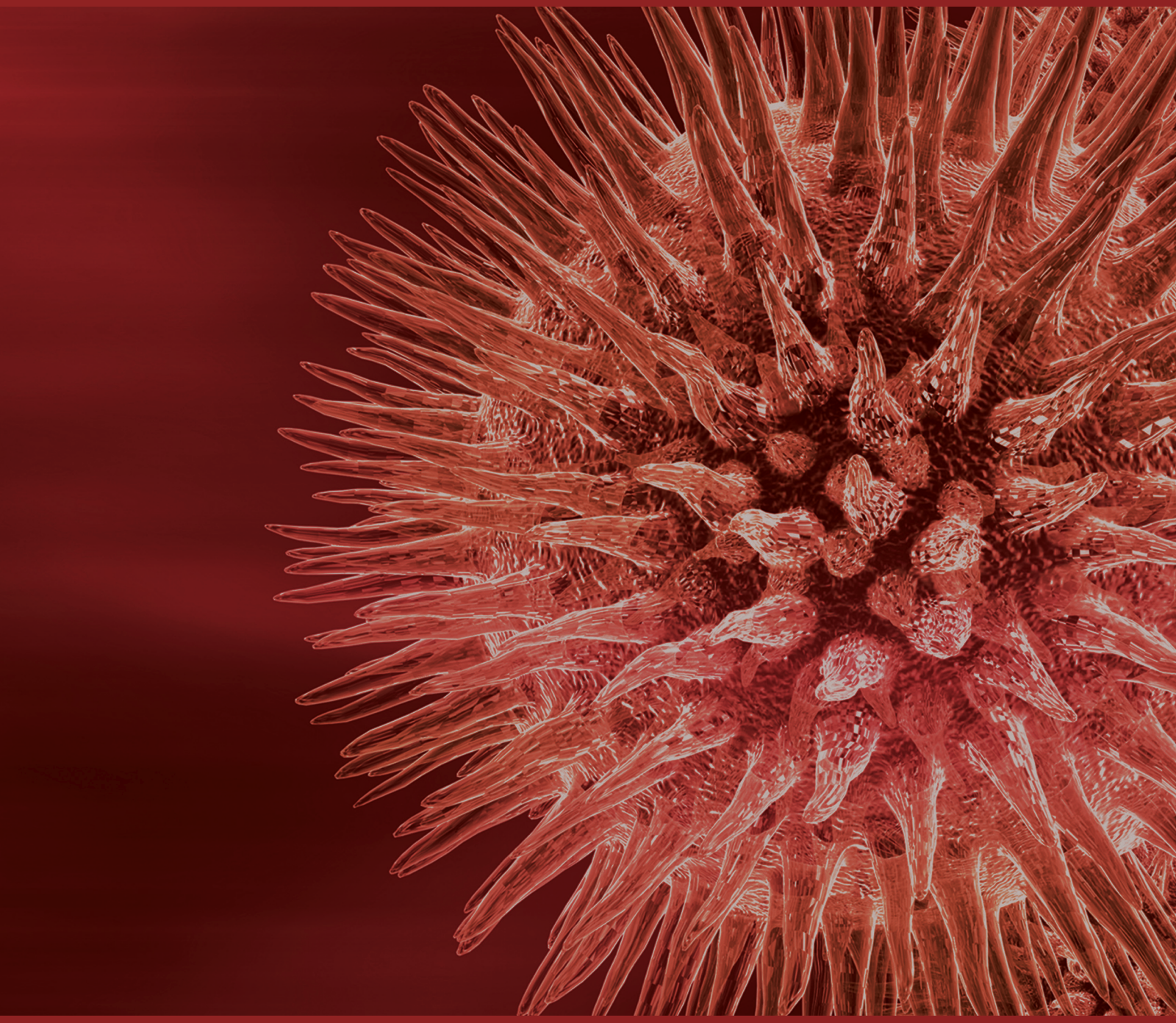
BioMed Research International

Steroids and Related Compounds: Basic and Clinical Aspects

Guest Editors: Fátima Regina Mena Barreto Silva, Leila Zanatta, Rozangela Curi Pedrosa, and Ming-Zhu Fang



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Editorial

Steroids and Related Compounds: Basic and Clinical Aspects

Fátima Regina Mena Barreto Silva,[1] Leila Zanatta,[2] Rozangela Curi Pedrosa,[1] and Ming-Zhu Fang[3]

[1] *Departamento de Bioquímica, Universidade Federal de Santa Catarina, Caixa Postal 5069, 88040-970 Florianópolis, SC, Brazil*
[2] *Health Sciences Area, Universidade Comunitária da Região de Chapecó, Avenida Senador Attílio Fontana 591E, 89809-000 Chapecó, SC, Brazil*
[3] *Department of Environmental and Occupational Medicine, Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, 170 Frelinghuysen Road, Piscataway, NJ 08854, USA*

Correspondence should be addressed to Fátima Regina Mena Barreto Silva; mena@mbox1.ufsc.br

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The steroid hormones generate myriad effects through several well-known mechanisms of action. Beyond the physiological function of steroid hormones, the steroid-like effects of some natural and synthetic compounds have led to the promising field of alternative therapies. However, to these new compounds, the molecular, subcellular, and cellular signal transductions need to be elucidated. Although new questions about the relevance of multiple targets of action for exogenous compounds are deeply in discussion, the original contributions that depict novel insights into basic and clinical aspects with perspectives on medical application are welcome.

This special issue compiles papers from renowned research groups in the world that cover the frontiers of the latest findings on steroids and related compounds effect, mechanism of action and its relevance on carcinogenesis, immune suppression, and osteoporosis, as well as novel steroidal glycosides characterization.

The papers on sex steroids hormones review the role of progesterone (precursor for androgens and estrogens produced by the gonadal and adrenal cortical tissues) and related progestins compounds in hepatocellular carcinoma. The authors highlight that the higher incidence of hepatocellular carcinoma in men than women might have resulted from the stimulatory effects of androgen and the protective effects of estrogen and also eventually suggest a new insight into the associations of progesterone and related compounds with hepatocellular carcinoma development and treatment. Also, the sex steroid hormones in the modulation of bacterial-host interactions were revised since the dimorphic sex difference (low immune responses presented in males as compared to females) is mainly due to the differential modulation of the immune system by sex steroid hormones through the control of proinflammatory and anti-inflammatory cytokines expression, as well as Toll-like receptors expression and antibody production.

Some interesting studieson humans addressed the effect of chronic glucocorticoid therapy on osteoporosis in children with 21-hydroxylase deficiency as much to replace congenital deficits in cortisol synthesis as to reduce androgen secretion by adrenal cortex. As consequence, a secondary osteoporosis is formed. It results in an early, transient increase in bone resorption accompanied by decrease in bone formation, maintained for the duration of glucocorticoid therapy. Based on conflicting results from the literature about the bone status on glucocorticoid-treated patients with 21-hydroxylase deficiency, the authors point that the monitoring of the bone status of these patients, checking bone mineral density and bone turnover markers, and studying the expression of regulators of bone resorption should be useful in order to avoid glucocorticoid-induced osteoporosis in adulthood. Also, based on many epidemiological studies concerning the inverse relationship between isoflavone intake and bone loss and fracture rate, some substances on serum levels after food intake indicated for patients with osteopenia/osteoporosis were analyzed. Concerning genistein bioavailability, it was

deeply discussed that, beyond the intestinal bacteria, solubility and permeability, glucosidase activity, viscosity induced by food additives, and a multitude of transporters on luminal intestinal cells for absorption, several factors from the diet composition influence the net absorption of single entity and also the effectiveness of the bone build. So, with these data in mind, the bioavailability of genistein depends on specific ingredients and excipients in each formulation which can interfere with absorption and could have clinical implications on efficacy.

The ongoing investigations of some groups in the world have characterized new steroidal compounds from plants with medicinal interest. Three new steroidal glycosides, named as stauntosides L, M, and N, along with one known C_{21} steroidal glycoside, anhydrohirundigenin monothevetoside, were isolated from the roots of *Cynanchum stauntonii* (Decne.) and extensively evaluated by spectroscopic analyses, mainly 1D and 2D NMR, HRESI-MS and chemical methods. It is known that C_{21} steroids and their glycosides are of considerable bioactivities, such as hypolipidemic and antitumor activity. So the enriched information about *C. stauntonii* as a significant source of steroidal glycosides deserves careful phytochemistry investigation as well as the classification of bioactive compounds to be proposed as nutraceutical agents interesting both to academy and industry and also to specific therapy option.

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Fátima Regina Mena Barreto Silva
Leila Zanatta
Rozangela Curi Pedrosa
Ming-Zhu Fang

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Review Article

Progesterone and Related Compounds in Hepatocellular Carcinoma: Basic and Clinical Aspects

Yao-Tsung Yeh,[1] Chien-Wei Chang,[1,2] Ren-Jie Wei,[1,3] and Shen-Nien Wang[4,5]

[1] *Department of Medical Laboratory Sciences and Biotechnology, Fooyin University, Kaohsiung 83102, Taiwan*
[2] *Cancer Center and Division of General & Gastroenterological Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung 80756, Taiwan*
[3] *Department of Pathology, Kaohsiung Armed Forces General Hospital, Kaohsiung 80284, Taiwan*
[4] *Department of Surgery, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 80756, Taiwan*
[5] *Division of Hepato-Pancreatico-Biliary Surgery, Kaohsiung Medical University Hospital, Kaohsiung 80756, Taiwan*

Correspondence should be addressed to Shen-Nien Wang; snwang@cc.kmu.edu.tw

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Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers. Major risk factors for HCC include infection with HBV or HCV, alcoholic liver disease, and most probably nonalcoholic fatty liver disease. In general, men are two to four times more often associated with HCC than women. It can be suggested that sex hormones including progesterone may play some roles in HCC. Rather, very limited information discusses its potential involvement in HCC. This paper thus collects some recent studies of the potential involvement of progesterone and related compounds in HCC from basic and clinical aspects. In addition, two synthetic progestins, megestrol acetate (MA) and medroxyprogesterone acetate (MPA), will be discussed thoroughly. It is noted that progesterone can also serve as the precursor for androgens and estrogens produced by the gonadal and adrenal cortical tissues, while men have a higher incidence of HCC than women might be due to the stimulatory effects of androgen and the protective effects of estrogen. Eventually, this paper suggests a new insight on the associations of progesterone and related compounds with HCC development and treatment.

1. Introduction

Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality [1]. Hepatocellular carcinoma (HCC) accounts for 85% to 90% of primary liver cancers. HCC has several interesting epidemiologic features including dynamic temporal trends; marked variations among geographic regions, racial and ethnic groups, and between men and women; and the presence of several well-documented environmental potentially preventable risk factors. Most HCC cases (80%) occur in either sub-Saharan Africa or in Eastern Asia. China alone accounts for more than 50% of the world's cases. Other high-rate areas include Senegal, Gambia, and South Korea [2].

Major risk factors for HCC include infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic liver disease, and most probably nonalcoholic fatty liver disease [3]. In general, men are two to four times more often associated with HCC than women. Epidemiological reports indicate that, regardless of etiologies, the incidence of HCC is higher in males than in females with the male : female ratio usually averaging between 2 : 1 and 4 : 1 [2]. The ratio of men to women is more pronounced in areas with a high HCC incidence [4]. A part of this increased risk among men is explained by their higher frequency of viral hepatitis and alcoholic cirrhosis. A statistical analysis indicated that age at menopause is an important and significant predictor, increasing HCC risk 24% for each later year of menopause (odd ratio = 1.24, $P < 0.001$) [5], implicating that female sex hormones may be associated with HCC risk or development. However, the reason(s) for this residual difference in HCC risk between men and women is unknown and

might be related to the carcinogenic effect of testosterone [6]. In a rat model, testosterone appears to be a growth factor for Morris hepatoma 7787 [7]. Additionally, epidemiologic and animal studies have suggested that men have a higher incidence of HCC than women which might be due to the stimulatory effects of androgen and the protective effects of estrogen [8]. Substituted androgens have been associated with the development of HCC in patients with Fanconi's anemia [9] and aplastic anemia [10]. These findings suggest that androgens may be implicated in the etiology of HCC [11]. In an animal experiment, exogenous and endogenous estradiol/active estrogen can suppress chemical-agent induced hepatocarcinogenesis in a rat, suggesting that estrogen receptors (ERs) may be involved in the inhibition of malignant transformation of preneoplastic liver cells [11]. Pregnancy, which increases serum estrogen levels about 100-fold, was found to exert a protective effect against HCC, and the protection increased with the number of FTP (full-term pregnancies) [12].

Based on available clinical information, chronic hepatitis C appears to progress more rapidly in men than in women, and cirrhosis is predominately a disease of men and postmenopausal women [13]. A larger number of women with advanced fibrosis (cirrhosis) were identified among menopausal women in chronic hepatitis C virus infection [14]. Cirrhosis frequently associates with HCC and hence can be considered a premalignant condition. Indeed, the majority of patients worldwide with HCC have underlying cirrhosis [15]. Both HBV and HCV promote cirrhosis, which is found in 80%–90% of patients with HCC. The 5-year cumulative risk of developing HCC for patients with cirrhosis ranges between 5% and 30%, depending on etiology, region or ethnicity, and stage of cirrhosis [16]. Interestingly, cirrhotic patients with HCC have significantly lower plasma concentrations of testosterone, dihydrotestosterone, and dehydroepiandrosterone than patients with cirrhosis alone [17]. Low levels of testosterone in male HCC patients and high levels of progesterone in cirrhosis patients have been observed [18]. It is controversial that high levels of progesterone are associated with premalignant cirrhosis. Do the higher progesterone levels contribute to HCC development? It is noted that the HCC risk was inversely related to the age at natural menopause. Oophorectomy performed at age 50 or younger during premenopausal years was also a risk factor for HCC [12], suggesting that at least female sex hormones including progesterone or estrogen may be protective against HCC.

2. Potential Involvement of Sex Hormones in Hepatocellular Carcinoma

Sex hormones such as estrogens, progestins, and androgens are hydrophobic ligands, which bind to transcription factors belonging to the superfamily of intracellular receptors. These receptors can be activated by the cognate ligand or in its absence, by posttranslational modifications elicited through the intracellular signaling of membrane receptors, also called nongenomic actions [19, 20]. Upon ligand binding, receptor activation occurs via diversified pathways involving genomic or nongenomic actions [21]; that is, the activated receptor may directly bind to the DNA-responsive elements in the regulatory regions of these genes (genomic actions) or may influence other pathways involved in cell proliferation by interfering with specific proteins in the cytoplasm or in the nucleus (non-genomic actions). Regarding the actions of sex hormones in HCC, their corresponding receptors should be always considered.

A novel cancer phenotype in which mice lacking hepatic androgen receptor (AR) developed more undifferentiated tumors and larger tumor size at the metastatic stage, which AR could orchestrate intrahepatic signaling hierarchies and cellular behaviors, consequently affect HCC progression [22]. Rather, higher androgen levels are frequently associated with HCC development. On the other hand, the incidence of ER content is highly variable according to the different authors, but study groups are not large enough. For the largest study group containing 66 HCC cases, ER content was found in 26 cases [23]. Rather, the presence or absence of progesterone receptor (PR), ER, and AR in HCC and their titers did not have any correlation with alcohol abuse, serum alpha-fetoprotein (AFP) levels, hepatitis B virus markers, or histopathologic types of the tumor [24].

Tamoxifen, a selective estrogen receptor modulator, is one of the most hormonal therapies used in breast cancer that can induce cell apoptosis through protein kinase C, MAPK, c-Myc, and so forth [25]. Interestingly, tamoxifen could also induce apoptosis of HepG2 cells in a dose-dependent fashion and reduced survivin transcript and mTOR activity of these cells [26]. A clinical study used tamoxifen to treat patients with expression of wild-type ER in HCC that has revealed a benefit to reduce tumor size [27] (Table 1). Furthermore, tamoxifen can also independently act without expression of ER in HCC [28]. However, a clinical trial using high-dose tamoxifen (120 mg per day) to treat HCC patients did not improve their survival length [29], questioning the relevance of ER-mediated signaling in HCC. A possible explanation for the negative result may be the lack of proper patient selection according to ER expression. Rather, tamoxifen may also act in HCC via an ER-independent pathway. On the other hand, tamoxifen could be effective only in a selected subgroup of HCC patients with the presence of variant estrogen receptors (vER-) [30]. Tamoxifen could not be effective in tumors with vER-, because of its inability to bind the receptor, and this could contribute to justify tamoxifen lack of efficacy, considering that a relevant proportion of HCC patients have predominant vERs [30]. To date, there is no robust evidence to consider tamoxifen a part of the current managements of HCC.

3. Progesterone and Related Compounds

3.1. Progesterone. Progesterone is a 21-carbon hormone formed from steroid precursors in the ovary, testes, adrenal gland, placenta, and glial cells in the central nervous system [41]. It is present in highest concentrations in the ovarian corpus luteum. In nonpregnant women, the main sites of progesterone biosynthesis are the ovaries and the adrenal cortices [42]. The synthesis of progesterone is stimulated by

Table 1: *In vitro*, *in vivo*, and clinical effects of progesterone and its related compounds in HCC.

Progestin	Bioeffect and physical response	Reference
Progesterone	Activation of Src and downstream MAPK induced Elk-1. Transactivation that was nearly as efficient as Elk-1 activation by EGF increase in the % of cells in G2M+ S phase	[31]
MA	Significant decreased tumor growth and improved survival in treated patients than the placebo group	[32]
	Inhibition of the growth of HepG2 in dose- and time-dependent manner, and HepG2 transplanted tumor *in vivo*	[33]
	HCC patients who received MA treatment would have longer median survival (18 months) compared to untreated patients (7 months)	[34]
	MA improves HCC patients' appetite, bodyweight, and a feeling of well-being with minimal side effects. And a minor reduction of tumour size and a prolonged survival	[35]
	Efficiency of MA treatment can be determined by expression of variant ER in HCC, but MA shows only a temporary inhibition of tumor growth	[27]
	MA has no role in prolonging OS in advanced treatment-naive HCC	[36]
MPA	Increased migration and invasion	[37]
	No significant curative effects were observed in MPA-treated HCC rat	[38]
	Expression level of leptin predicts postoperative treatment efficiency of MPA in HCC patients	[39]
	Tamoxifen- and MPA-combined chemotherapy may not prolong the survival of patients with HCC, although it improves their quality of life	[40]

luteinizing hormone (LH), which primarily acts to regulate the conversion of cholesterol to pregnenolone, a progesterone precursor.

Although the administration of progesterone to human beings gives rise to the excretion of pregnanediol in the urine, the course and sites of the metabolism of progesterone have not been established. When progesterone is administered orally, it first undergoes metabolism in the gut, then the intestinal wall, and the liver to form its hydroxylated metabolites and their sulfate and glucuronide derivatives [43, 44]. The uterus and ovaries are not essential for the reduction of progesterone, since a rise in urinary pregnanediol has been demonstrated in men and in hysterectomized women who were injected with progesterone. However, the liver would appear to be important in metabolizing the hormone. It has been shown in animals that when progesterone is implanted in the spleen, mesentery, or stomach or injected into the portal vein, its biological potency is much lower than when administered subcutaneously. The data presented indicate that progesterone is metabolized by an enzyme system in the liver tissue under the conditions used in these experiments [45]. The urinary progesterone derivatives were assumed to result from metabolism in the liver and included 5β-pregnanes such as pregnanediol (5β-pregnane-3α,20α-diol) and pregnanolone (5β-pregnan-3α-ol-20-one) as well as the 5α-pregnanes, 5α-pregnane-3,20-dione (5αP), 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, and 5α-pregnan-3-3α(β), 20α-diols [46]. The rapid metabolism of intravenously administered [14C] progesterone by eviscerated rats [47, 48], in which tissues such as liver, spleen, gut, and adrenals had been removed, showed that progesterone conversion was also occurring extrahepatically. It then soon became apparent that progesterone serves as the precursor for the major steroid hormones (androgens, estrogens, and corticosteroids) produced by the gonadal and adrenal cortical tissues. These progesterone-metabolizing enzymes included 5α-reductase, 5β-reductase, 3α-hydroxysteroid oxidoreductase (3α-HSO), 3β-HSO, 20α-HSO, 20β-HSO, 6α(β)-, 11β-, 17-, and 21-hydroxylase, and C17–20-lyase [49].

The biological activity of natural progesterone and its binding of AR are controversial. There are reports showing that progesterone have relative binding activity of dihydrotestosterone (DHT), an androgen hormone, with agonist and antagonist activity [50, 51]. On the other hand, it has been reported that progesterone binds the AR with very low affinity or does not bind the AR at all, displaying no androgenic effects but weak antiandrogenic effects in animal models [52, 53]. The antiandrogenic effect is considered as a competitor in inhibition of 5α-reductase activity thereby decreasing the conversion of testosterone to the more active DHT but not the binding of androgen receptor [52]. However, until now, there is no clinical evidence for AR-mediated androgenic and antiandrogenic activity of progesterone [54].

3.2. Megestrol Acetate. Megestrol acetate (MA) is a 17α-acetoxy-6-dehydro-6-methylprogesterone and sometimes abbreviated as MGA or MA, which is a steroidal progestin and progesterone derivative (specifically, 17α-hydroxylated progesterone) with predominantly progestational and antigonadotropic effects [55]. It has been suggested that the remarkably enhanced hormonal activity of progesterone when substituted at C-6 and C-17 in the steroid nucleus is due to increased resistance to metabolizing enzymes. Preliminary experiments with MA, a potent orally active ovulation inhibitor, indicated that it was very resistant to metabolism *in vitro* by rat liver as compared with progesterone [56].

MA acts predominantly as a potent agonist of the PR to exert its effects [57]. In addition, MA can suppress hormone-dependent tumoral cells, though the biological mechanisms underlying its antitumoral activity are not well understood. The growth-inhibitory effects on the cell cycle are not phase-specific, but its activity appears to reach a peak in the G1 phase of cell division [58]. As a potent antiestrogen agent that acts at the postreceptor level and thus independent of ER, MA is used in the second-line management of carcinoma of the breast. However, Fu et al. has revealed that the motility and invasiveness of breast cancer cells (T47D) was increased under MPA stimulation via recruiting extranuclear signaling to actin, which leads to rearrangement of the cytoskeleton and the formation of pseudopodia and membrane ruffles [37] (Table 1). It has been reported to cause minor reduction of tumor size and prolonged survival time in HCC [35] (Table 1). In experimental animal models, however, it has been shown that MA could only inhibit the growth of PR-positive tumors but not PR-negative tumors [59–61].

Furthermore, it produces detectable androgenic effects in animals only at a dose that is the equivalent of approximately 200 times that typically used for the treatment of prostate cancer in men [62].

3.3. Medroxyprogesterone Acetate. Medroxyprogesterone acetate (MPA) is a 17α-hydroxy-6α-methylprogesterone acetate, and commonly abbreviated as MPA, which is a steroidal progestin, a synthetic variant of the human hormone progesterone [55]. MPA is commonly used in contraception and hormone replacement therapy [63]. MPA is a potent full agonist of the AR. Its activation of the AR has been shown to play an important and major role in its antigonadotropic effects and in its beneficial effects against breast cancer [64–66]. In fact, likely due to its suppressive actions on androgen levels, it has been reported that MPA is highly effective in improving preexisting symptoms of hirsutism in women with the condition [67, 68]. Moreover, MPA rarely causes any androgenic effects in children with precocious puberty, even at very high doses [69]. The reason for the general lack of virilizing effects with MPA, despite its binding to and activating the AR with a high affinity and this action playing a crucial role in many of its physiological and therapeutic effects, is not entirely clear. However, MPA has been found to interact with the AR in a fundamentally different way than other agonists of the receptor such as dihydrotestosterone (DHT) [51]. The result of this difference is that MPA binds to the AR with a similar affinity and intrinsic activity to that of DHT but requires about 100-fold higher concentrations for a comparable induction of gene transcription, while at the same time not antagonizing the transcriptional activity of normal androgens like DHT at any concentration [51]. This may explain the low propensity of MPA for producing androgenic side effects.

The intrinsic activities of MPA in activating the PR and the AR have been reported to be at least equivalent to those of progesterone and dihydrotestosterone (DHT), respectively, indicating that it is a full agonist of these receptors [51].

4. Progesterone Signaling

PR is a member of the nuclear receptor family of ligand-dependent transcription activators and is expressed as two different sized proteins from a single gene by alternate promoter usage. The two PR isoforms, PR-A and PR-B, are identical in their DNA binding domains (DBD) and C-terminal ligand binding domains (LBD), differing only in the N-terminal domain that is truncated in PR-A [70, 71]. Notably, PRs are found in the uterus, central nervous system, mammary gland, and pituitary gland.

The general pathway of progesterone-inducible PR-mediated gene transcription has been well characterized. Progesterone binding induces a conformational change(s) in PR that promote dissociation from a multiprotein chaperone complex, homodimerization, and binding to specific progesterone response elements (PREs) within the promoter of target genes [72, 73]. In cancer cells, kinase signaling initiated by extracellular progesterone modulates transcriptional events in the nucleus, which in turn regulate proliferation, migration, and invasion [74]. The major biological response to progesterone is mediated by PR-A and PR-B through distinct signaling pathways [75, 76]. In general, PR-B is a stronger transcriptional activator, whereas PR-A can function as a ligand-dependent repressor of other steroid hormone receptors including PR-B and ER [77]. In addition to direct transcriptional effects mediated by nuclear PR, other authors have shown that progestins can rapidly activate the Src/Ras/MAPK, PI3 kinase/Akt, and JAK2/Stat3 signaling pathway in breast cancer and mammary epithelial cells [31, 78–86] (Table 1). Many of them have been demonstrated in HCC [87]. However, their relation to progesterone signaling in HCC has not been explored so far. Progesterone also exerted a stimulatory effect through the PR on the induction of reactive oxygen species (ROS) generation processes and intracellular pathways, resulting in TGF-beta1 expression, rat hepatic stellate cells (HSCs) activation, and fibrogenic effects [88]. This may raise the possibility that progesterone could establish a tumor-favorable microenvironment and thus contributes to hepatocarcinogenesis. Further investigations are required. These effects of progestins on cell signaling pathways in the absence of transcription are dependent on conventional PR, suggesting that PR has dual functions as a nuclear transcription factor and as a modulator of cell signaling pathways. Human PR contains a polyproline SH3 domain interaction motif within the NTD in a position (aa 421–428) that is shared by PR-A and PR-B [79]. Therefore, the ability of PR to interact with Src appears to be a function of the receptor distinct from its transcriptional activity and is dissociable by point mutations in the SH3 domain interaction motif [89]. Notably, progestin activation of Src/MAPK signaling can regulate selected target genes such as cyclin D1 (CCND1) that lack direct PR binding response elements (PREs) [89]. Furthermore, progestin induction of CCND1 was observed in cells expressing PR-B but not PR-BΔSH3 or PR-A. In contrast progestin induction of Sgk (serum and glucocorticoid regulated kinase) gene, which contains a classical PRE, was observed with both PR isoforms as well as PR-BΔSH3 and was unaffected by Src and MAPK

inhibitors. It is suggested the importance of PR activation of extranuclear signaling pathways in regulating selected target genes and cell cycle progression. The previous study provided evidence that c-Src is often activated in the early stage of human HCC, especially in low proliferating activity, but not in noncancerous liver tissues regardless of their histological types. More interestingly, activated c-Src was not detected in 12 atypical adenomatous hyperplasia occurring in liver cirrhosis, which has been thought to be a representative precursor for HCC [90].

The two putative progesterone membrane receptors PGRMC (progesterone receptor membrane component) 1 and 2 are indentified in various human tissues including liver [91]. PGRMC1 and PR are likely to be continuously active in high presence of serum progesterone [92]. Interestingly, PGRMC1 is regarded as a biomarker for tumor cell proliferation [93] and is strongly expressed in different kinds of cancer [92]. In granulosa cells, PGRMC1 mediated the antiapoptotic action of progesterone [94]. Recent publications describe an interaction of PGRMC1 with a wide range of cytochrome P450 proteins [95]. This is remarkable as PGRMC1 was proposed to be involved in chemotherapy resistance, a well-known characteristic in HCC.

Drug-induced liver injury (DILI) is a major safety concern in drug development and clinical drug therapy. It is generally believed that women exhibit worse outcomes from DILI than men. Intriguingly, evidence showed that progesterone exacerbated the immunomediated hepatotoxic responses in DILI via the Kupffer cells and extracellular signal-regulated kinase (ERK) pathway [96]. Progesterone pretreatment dramatically activated ERK in HAL-induced liver injury, and U0126 (ERK inhibitor) significantly suppressed the exacerbating effect of progesterone and the expression of inflammatory mediators. The study seemed to provide a link between progesterone and some inflammatory mediators including tumor necrosis factor (TNF) α, interleukin (IL)-1β, and IL-6, which have been associated with HCC development.

5. Clinical Application of Progesterone Compounds in Hepatocellular Carcinoma

MA has powerful antiandrogenic and antiestrogenic effects in humans at sufficient doses, capable of decreasing circulating androgen and estrogen concentrations to castrate levels in both sexes and significantly lowering the expression of the AR and the ER in the body [97–99]. Rather, MA is a high-affinity, weak partial agonist/antagonist of the AR [100–102], where it binds with very similar but slightly less affinity relative to the PR [57]. At clinical doses in humans, it appears to behave purely as an antiandrogen. No androgenic side effects have been observed with the use of MA in patients of either sex at doses up to as high as 1,600 mg per day [103]. A report of a phase II study of MA (160 mg/day, orally) in the treatment of HCC showed there were no complete responders or partial responders. Twelve patients (38%) of the enrolled 56 patients had stable disease and seven of these patients had a minor response with a median size reduction in the tumor of 18%. Twenty patients (62%) had progressive disease. Five of 24 (21%) patients had a median reduction in alpha-fetoprotein levels of 59 ng/mL. The overall median survival was 4 months (range 1 week to 27 months). Twenty of 32 (62%) patients had an increased appetite and a feeling of well-being. Fourteen of 22 (64%) patients had a median lean bodyweight gain of 5 kg (range 1–14 kg) [35] (Table 1). Rather, MA was able to favorably influence such severe course, significantly improving survival, which increased from 7 to 18 months, and slowing down tumor growth in inoperable HCC [32] (Table 1). In contrast, Chow et al. indicated that MA has no role in prolonging OS (overall survival) in advanced treatment-naive HCC [36] (Table 1).

MPA commonly is used in contraception and hormone replacement therapy [63]. Liver metastases from breast cancer are present in about 20% of patients at the time of the diagnosis of metastatic disease [104]. Faced with patients with liver metastases in whom the tumor shows positive ER and/or PR, hormonal therapy can have an important therapeutic contribution, if combined with chemotherapy and, in selected cases, even as a single therapy [104]. MPA acts as an agonist of the progesterone, androgen, and glucocorticoid receptors (PR, AR, and GR, resp.) [105]. However, few of these may include faulty patient subset selection criteria, no monitoring of tumor ER and AR expression at the time of recruitment and also during treatment of these patients and lastly the type of hormonal treatment given to the patient. Therefore, the debatable potential of hormone therapy in HCC may finally be attributed to the lack of complete understanding of ER and AR expression and hormonal responsiveness in the liver and their involvement in development of HCC [106]. Some clinical studies found that the use of MPA after hepatectomy was a strong predictor of the overall survival of patients with HCC, although the use of MPA was imperfectly associated with a better overall survival of patients with HCC [39] (Table 1). The leptin expression may intensify the curative effect of MPA in patients with HCC and may serve as a predictor for response to treatment with MPA. Nevertheless, this finding requires further investigation [39] (Table 1). Both MA and MPA are belonging to 17alpha-hydroxyprogesterone derivatives, they share a similar chemical structure and almost have the same enzymatic activity including progestogenic, antigonadotropic, antiestrogenic, androgenic, and glucocorticoid. A major difference is that MA have antiandrogenic activity but not MPA [105]. Previous study has shown that MA inhibits the growth of HepG2 cells *in vitro* in dose- and time-dependent manners. The growth of HepG2 cell-transplanted tumors in nude mice was also inhibited by i.p. injection of MA. Rather, expression of PR was not detected at protein and mRNA levels in HepG2 [33] (Table 1). MA can also exert its action on ER pathways at postreceptorial level. In HCC patients with variant ER expression, MA can temporarily suppress tumor size and increase again after three month during the follows up time [27] (Table 1). Additionally, out of 133 patients diagnosed with HCC and screened for eligibility, 45 patients (33.3%) had variant ER transcripts demonstrated in the tumor and were enrolled in the study. Twenty-four patients were randomized to no treatment and 21 to MA at the daily dose of 160 mg. Median survival in

untreated patients was 7 mo (95% CI, 3.01–10.99 mo) versus 18 mo (95% CI, 13.47–22.53 mo) in patients treated with MA ($P = 0.009$) [34] (Table 1), suggesting that MA improves HCC progression may via other hormone receptors, such as androgen and glucocorticoid receptor [64, 102]. However, an animal model experiment in rat HCC showed that in the group treated with MPA no significant curative effects were observed [38] (Table 1). Tamoxifen- (TAM-) and MPA-combined chemotherapy may not prolong the survival of patients with HCC, although it improves their quality of life [40] (Table 1). Notably, AR, ER, and PR, members of steroid hormone receptor, are known to exist in human HCC [34, 38] (Table 1).

6. Conclusion

Epidemiological reports have indicated that, regardless of etiologies, the incidence of HCC is higher in males than in females with the male : female ratio usually averaging between 2 : 1 and 4 : 1 [2]. It is presumably possible that sex hormones may play roles in HCC risk or development. Rather, rare information regards the potential involvement of progesterone in HCC. We introduce these studies and hope that one can notice the role of progesterone in HCC. It is noted that high levels of progesterone are observed in patients with cirrhosis, one of premalignant lesion [18] and is likely due to major metabolism of progesterone performed in the liver. In addition, progesterone can serve as the precursor for the major steroid hormones (androgens, estrogens, corticosteroids) produced by the gonadal and adrenal cortical tissues, while men have a higher incidence of HCC than women which might be resulted from the stimulatory effects of androgen and the protective effects of estrogen.

The biological activity of natural progesterone on the HCC is controversial and lacks clear investigation. The presence or absence of PR in HCC also seemed not to contribute to clinical features [24]. Rather, progesterone can rapidly activate the Src/Ras/MAPK, PI3 kinase/Akt, and JAK2/Stat3 signaling pathway in breast cancer [78–86]. Many of them have been demonstrated in HCC [87]. Intriguingly, a synthetic progestin, MA, caused minor reduction of tumor size and prolonged survival time in HCC [35] (Table 1). The growth of HepG2 cell-transplanted tumors in nude mice was also inhibited by i.p. injection of MA [33] (Table 1). A major difference is that MA have antiandrogenic activity but not MPA [105]. However, an animal model experiment in rat HCC showed that in the group treated with MPA no significant curative effects were observed [38] (Table 1). Some clinical studies found that the use of MPA after hepatectomy was imperfectly associated with a better overall survival of patients with HCC. Rather, the leptin expression may intensify the curative effect of MPA in patients with HCC and may serve as a predictor for response to treatment with MPA. Nevertheless, this finding requires further investigation [39] (Table 1).

Taken together, we believe that progesterone may have roles in HCC risk and development. Further investigations are required. In addition, monitoring of tumor PR, ER, and AR expression at the time of recruitment will be important.

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Research Article

The Steady-State Serum Concentration of Genistein Aglycone Is Affected by Formulation: A Bioequivalence Study of Bone Products

Alessandra Bitto,[1] Bruce P. Burnett,[2] Francesca Polito,[3] Silvia Russo,[4] Rosario D'Anna,[4] Lakshmi Pillai,[2] Francesco Squadrito,[1] Domenica Altavilla,[1] and Robert M. Levy[5]

[1] *Department of Clinical and Experimental Medicine and Pharmacology, University of Messina, 98125 Messina, Italy*
[2] *Department of Medical Education and Scientific Affairs, Primus Pharmaceuticals, Inc., Scottsdale, AZ, USA*
[3] *Section of Physiology and Human Nutrition, Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy*
[4] *Department of Obstetrical and Gynecological Sciences, University of Messina, Messina, Italy*
[5] *Department of Clinical Affairs, Primus Pharmaceuticals, Inc., Scottsdale, AZ, USA*

Correspondence should be addressed to Alessandra Bitto; abitto@unime.it

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An FDA-regulated, prescription medical food (Fosteum; 27 mg natural genistein, 200 IU cholecalciferol, 20 mg citrated zinc bisglycinate (4 mg elemental zinc) per capsule) and an over-the-counter (OTC) supplement (Citracal Plus Bone Density Builder; 27 mg synthetic genistein, 600 mg elemental calcium (calcium citrate), 400 IU vitamin D_3, 50 mg magnesium, 7.5 mg zinc, 1 mg copper, 75 μg molybdenum, 250 μg boron per two tablets) were compared to a clinically proven bone formulation (27 mg natural genistein, 400 IU cholecalciferol, 500 mg elemental calcium (calcium carbonate) per tablet; the Squadrito formulation) in an 8-day steady-state pharmacokinetic (PK) study of healthy postmenopausal women ($n = 30$) randomized to receive 54 mg of genistein per day. Trough serum samples were obtained before the final dose on the morning of the ninth day followed by sampling at 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, and 96 hrs. Total serum genistein, after β-glucuronidase/sulfatase digestion, was measured by time-resolved fluorometric assay. Maximal time (T_{max}), concentration (C_{max}), half-life ($T_{1/2}$), and area under the curve (AUC) were determined for genistein in each formulation. Fosteum and the Squadrito study formulation were equivalent for genistein T_{max} (2 hrs), C_{max} (0.7 μM), $T_{1/2}$ (18 ± 6.9 versus 21 ± 4.9 hrs), and AUC (9221 ± 413 versus 9818 ± 1370 ng·hr/mL). The OTC supplement's synthetically derived genistein, however, showed altered T_{max} (6 hrs), C_{max} (0.57 μM), $T_{1/2}$ (8.3 ± 1.9 hrs), and AUC (6474 ± 287 ng·hr/mL). Differences in uptake may be due to multiple ingredients in the OTC supplement which interfere with genistein absorption.

1. Introduction

Asian populations consume ~25–50 mg of isoflavones daily with 10% consuming more than 100 mg per day [1]. Americans, on the contrary, consume ~0.15–3 mg per day [2, 3]. Much of the isoflavone consumed by Asian populations is in the form of aglycone from fermented soy product rather than glycoside forms consumed in mostly processed food in the USA. Many epidemiological studies of Asian women support an inverse relationship between isoflavone intake and bone loss as well as fracture rate. A large prospective study of 24,403 Chinese postmenopausal women, for example, demonstrated that ≥21 mg daily soy isoflavone consumption dramatically reduced subsequent fracture incidence over a 4.5-year period [4]. Most clinical trials, especially in the USA, are performed on extracted glucoside isoflavones from soy rather than aglycones forms which are found in fermented foods such as tofu, miso, and natto in the Asian diet. Recent clinical trials of 120 mg/day mixed glycoside isoflavones given to healthy postmenopausal women for 2 and 3 yrs, however, showed

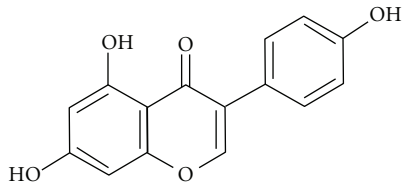

Figure 1: Genistein aglycone.

only modest effects on bone metabolism [5–7]. In a well-controlled dietary trial, natto, a fermented soy product containing 35 mg aglycone isoflavones enriched with 3.6 mg zinc given twice daily, showed statistically significant increases bone formation and decreases bone resorption markers over natto alone [8]. To date, only genistein (aglycone), as a single entity, has been tested in well-controlled clinical trials for its effectiveness on building bone (Figure 1) though studies have begun on S-equol, the intestinal bacterial conversion product of daidzein, for its effect on bone makers [9].

In a 12-month randomized, placebo-controlled clinical trial ($n = 90$), 54 mg of genistein administered daily showed equivalent increases in femoral neck and lumbar spine bone mineral density (BMD) (+3%) compared to the group given 1 mg of 17β-estradiol/0.5 mg noresthisterone acetate per day while the placebo group BMD declined [10]. All groups also received calcium carbonate (1000 mg) and cholecalciferol (800 IU) per day. This pilot study result was replicated in a larger ($n = 389$), long-term (24 months) study using the same amount of genistein compared to placebo [10]. A subcohort ($n = 138$) of this initial study extended to 36 months showed a similar rate of BMD increase (~3%/yr to ~9% over 3 yrs) at femoral neck and lumbar spine while the placebo BMD decreased by ~10-11% at the femoral neck and lumbar spine. [11]. Markers of bone formation increased substantially while markers of bone resorption decreased significantly for the genistein groups in these studies [10–13]. Bone quality assessed by quantitative ultrasound from the subcohort had statistically increased speed of sound, bone transmission time, and stiffness indices versus placebo [14]. In addition, a bone structural study in ovariectomized rats with established osteoporosis in which genistein was compared to alendronate, raloxifene, estradiol, and placebo showed superiority of genistein for all bone formation indices, fracture resistance, and histology (both trabecular and cortical bone) compared to all other therapies [15]. These results have spawned the development of products for bone loss containing pure genistein but no comparative studies have been performed between these new commercial products.

Bioavailability comparisons can predict whether certain active ingredients in product formulations will show the same effect in clinical trials. It has been established that glycoside isoflavones are poorly absorbed in the intestine and that hydrolysis of the glycosidic bond by β-glucosidases activates the aglycone for rapid absorption across the intestinal wall [16–18]. Most isoflavone bioavailability studies are performed in a food matrix using fermented or nonfermented products. Pure genistein and its glucoside, genistin, have been compared for uptake and the appearance in plasma as well as excretion of phase II metabolites in urine of healthy young women after multiple doses [19]. This study showed that there were differences in total genistein C_{max} and AUC as well as several genistein metabolites. The addition of purified components in combination with genistein or genistin is not well studied. One recent study showed that the continuous administration of fructooligosaccharide, a prebiotic, dramatically changed genistein and daidzein C_{max} and AUC obtained from a soy powder containing primarily genistin and daidzin [20]. With these data in mind, it is important to perform bioavailability comparisons for formulations containing purified active ingredients and excipients which surround isoflavones before testing them clinically.

The FDA-regulated, prescription medical food [21], Fosteum, for the clinical dietary management of osteopenia and osteoporosis under physician supervision was formulated in collaboration with Squadrito and coworkers as previously described [11, 12]. The OTC bone supplement, Citracal Plus Bone Density Builder, is based on a bone support formula already on the market (Citracal) and uses literature support to justify the addition of genistein [11, 12]. Since the Squadrito formulation is the only mixture which contains genistein that has been clinically proven to build bone, the first step in determining whether Fosteum and/or Citracal Plus Bone Density Builder are bioequivalent is to test the bioavailability of genistein. Therefore, the steady-state pharmacokinetics of 54 mg of genistein per day was compared for the Squadrito formulation to that of the Fosteum and Citracal Plus Bone Density Builder.

2. Materials and Methods

2.1. Materials. The genistein in the prescription medical food (27 mg natural genistein, 200 IU cholecalciferol, 20 mg citrated zinc bisglycinate (4 mg elemental zinc) per capsule) (Fosteum, Primus Pharmaceuticals, Inc.) and the Squadrito study formulation (27 mg natural genistein, 400 IU cholecalciferol, 500 mg calcium (carbonate salt) per tablet) are obtained from natural sources, whereas in OTC supplement (13.5 mg synthetic genistein, 300 mg calcium (as citrate and carbonate salts), 200 IU vitamin D_3, 25 mg magnesium (as stearate, oxide, and silicate salts) 3.75 mg zinc (oxide salt), 0.5 mg copper (gluconate salt), 1 mg manganese (gluconate salt), 37.5 μg molybdenum (amino acid chelate), 125 μg sodium borate per tablet) (Citracal Plus Bone Density Builder, Bayer HealthCare LLC) genistein is synthetically produced. All mineral levels are expressed in elemental mass units. All three products purport genistein purity of ~99%. The compositions and daily dosages of each formulation tested in the PK study are shown in Table 1.

2.2. Analysis of Genistein Content and Purity in Study Products. In order to compare the purity of genistein and minor isoflavones in each product, HPLC analysis was performed [22]. Briefly, samples were pulverized and then vortexed for 1 min in 2.5 mL of 1 : 1 : 1, hexane to methyl tert-butyl ether to methylene chloride extraction solvent. The samples

Table 1: Composition of three formulations for bone, a medical food indicated for osteopenia/osteoporosis, the Squadrito study formulation, and the OTC bone supplement. All minerals are given as elemental mass.

Constituent	Medical food	Squadrito study formulation	OTC bone supplement
Dosage form	Capsule	Tablet	Tablet
Daily dose	2	2	4
Genistein aglycone	54 mg	54 mg	54 mg
Vitamin D_3	400 IU	800 IU	800 IU
Calcium (elemental)	120 mg	1000 mg	1200 mg
Magnesium (elemental)			100 mg
Zinc (elemental)	8 mg		15 mg
Copper (elemental)			2 mg
Manganese (elemental)			4 mg
Molybdenum (elemental)			150 μg
Boron (elemental)			500 μg

IU: international units.
OTC: over-the-counter.

were then vortexed gently for 15 min followed by a 10 min centrifugation at 3000 rpm to separate the aqueous and organic layers. The aqueous layer of each sample was then frozen at −80°C and the organic layer poured into a 10 mL glass conical screw cap tube where the sample was dried with nitrogen gas at 40°C.

The dried extracts, as well as separate controls (genistein, daidzein, glycitein, and their glycosides), were reconstituted with 0.2 mL of 1 : 1, mobile phase buffer A (0.05% formic acid and 5 mM ammonium formate in distilled water) to mobile phase buffer B (0.05% formic acid and 5 mM ammonium formate in an 80 : 10 : 10 ratio, acetonitrile to methanol to distilled water). Samples were vigorously vortexed for 5 min and then centrifuged for 2 min at 1500 rpm to remove any insoluble material. The supernatants were removed and transferred to 0.25 mL polypropylene injection vials with caps for each chromatography run. Areas under curves were compared to standards to obtain purities.

2.3. Subjects. After the Ethical Committee approved the study, a total of 30 participants were recruited among those reporting to the Center for Menopause in the Department of Obstetrical and Gynaecological Sciences at the University of Messina (Messina, Italy). All participants gave informed consent. All women were 50–65 yrs old, had been post-menopausal for at least 12 months at baseline and were in good general health. At the start of the study, a complete medical and family history was obtained. Exclusion criteria were the same of our previously published reports [12].

2.4. Diet. The intake of soy products, legumes, or other nutrient supplements which could contain isoflavones was prohibited for the 2 weeks before and during the study. The isoflavone intake before randomization as assessed by a food-frequency questionnaire was 1 to 2 mg/day. This intake has been shown to be typical of Western populations.

2.5. Treatment Protocol. The PK study was carried out at the laboratory of the Section of Pharmacology, Department of Clinical and Experimental Medicine and Pharmacology, University of Messina. Participants were randomly assigned to receive one of the following products for orally 8 days: 1 capsule twice daily (BID) of the medical food ($n = 10$); 1 tablet BID of the Squadrito study formulation ($n = 10$); or 2 tablets BID of the OTC supplement ($n = 10$). On the morning of the ninth day, trough serum samples (basal, 0 hr) were obtained following which subjects were given their final dose of study product. Blood samples were then collected using an intravenous cannula at 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, and 96 hrs after final dosing. All other forms of calcium or vitamin D_3 were proscribed before and during the study.

The maximal plasma concentration (C_{max}, nmol/L) and time to maximal plasma concentration (T_{max}, hr) were obtained directly by the visual inspection of each subject's plasma concentration-time profile. The areas under the plasma concentration-time curve (AUC, ng·hr/mL) as well as half-life ($T_{1/2}$, hrs) were determined for each formulation by using the PK Solutions 2.0 software.

2.6. Plasma Genistein Levels. Total genistein levels were measured in plasma samples by a time-resolved fluorometric assay following the manufacturer's instructions (TR-FIA test; Labmaster, Turku, Finland). Briefly, 200 μL of 100 mM acetate buffer (pH 5.0) containing 0.2 U/mL β-glucuronidase and 2 U/mL sulfatase was added to 200 μL serum. Samples were then incubated overnight at 37°C. After incubation, free genistein was extracted twice with 1.5 mL diethyl ether by mixing for 3 min. The water phase is frozen in dry ice-ethanol mixture, and the ether phase was transferred into a disposable glass tube. After thawing, the water phase was reextracted with ether, and the ether phases are combined and evaporated to dryness at 45°C water bath. Then, 200 μL assay buffer was added to each sample. A 20 μL aliquot of this solution was used for time-resolved fluoroimmunoassay. The fluorescent signal was read using a Perkin-Elmer (Norwalk, CT) Victor 1420 multilabel counter.

2.7. Statistical Analysis. Total plasma genistein concentrations were obtained at each time point in duplicate for each subject and PK analyses were performed. The primary variables of interest were C_{max} (the maximum observed concentration of total genistein), T_{max} (the elapsed time at which C_{max} was observed), $T_{1/2}$ (the elapsed time at which genistein concentration was half of C_{max}), and the imputed area under the curve (AUC) estimating the total body exposure to genistein over time. Area under the curve was computed by interpolating the concentrations of total genistein in the intervals between recordings using trapezoid calculations. Imputation was performed by using cubic spline estimation. Each of these variables was computed for each participant, and mean values and standard deviations were computed for the sample. Any value exhibiting $a > 3$ standard deviations ($n = 3$) from the mean were removed from each analysis. A student's t-test was conducted for each measure to see if the observed difference in means was

significant. Descriptive statistics were presented for each of the primary outcome variables.

3. Results

3.1. Genistein Content and Purity in Study Products. The genistein in both the prescription medical food and the Squadrito study formulation are from natural sources, whereas the genistein in the OTC supplement is produced synthetically. The mineral content for all products was confirmed by nutritional analysis (data not shown). HPLC analysis shows that the genistein molecules extracted from each formulation have equivalent purity with relative minor impurities of other isoflavone(s) amounting to <1% (Figure 2). When the chromatograms are aligned and enlarged to compare the very small differences in genistein content between the two natural sources in the medical food (Figure 2(a)) and the Squadrito study formulation (Figure 2(b)) to that of the synthetic source in the supplement (Figure 2(c)), there are only small differences in the contaminating isoflavones of all products. Fosteum is contaminated by a small amount of glycitin, and daidzein, the Squadrito study formulation contains daidzin and genistin and the Citracal Plus Bone Builder supplement has a small amount of glycitin. No appreciable difference is seen in genistein purity in any of the three products with other aglycone impurities being less than 1%. Since the genistein levels are equivalent in all three products, a PK study of genistein should reveal any differences in uptake or excretion based on the surrounding vitamin, mineral, and excipient content in each formulation. Thus a bioavailability analysis can determine if genistein is bioequivalent in Fosteum and/or Citracal Plus Bone Density Builder compared to the Squadrito formulation which has been tested in clinical trials on bone.

3.2. Pharmacokinetic Comparison of Plasma Genistein in Each Treatment Group. The PK profile for genistein obtained during the first 24 hours after the last dose of each study product standardized to 54 mg per day after 8 days intake is shown in Figure 3. Genistein from the medical food and the Squadrito study formulation were absorbed and excreted at approximately equal rates with statistically significant higher concentrations at 1, 2, 5, and 12 hrs. The genistein contained in the supplement showed a much lower overall uptake by comparison. The PK analysis reinforced in this plot showed that the $T_{\max}$ of genistein for both the medical food and the Squadrito study formulation occurred 4 hrs earlier than that found in Citracal Plus Bone Density Builder supplement and the genistein $C_{\max}$ was also ~23% higher at this point (Table 2).

The medical food and the Squadrito study formulation genistein peak serum concentrations are very similar with only nonstatistical differences in concentration at each time point over the course of the terminal half-lives for the products. This would represent the normal PK profile before a subsequent dose was consumed. The absorption and depletion profiles of genistein from the medical food and the Squadrito study formulation exactly overlapped during the initial phase lasting approximately 5 hrs. When compared to OTC supplement, the medical food had a 42% greater AUC while the Squadrito study formulation had a 52% greater AUC for genistein over the entire 96 hr time course suggesting dramatic differences in steady-state genistein absorption. Even after the 1 and 2 hr time points, the steady state amount of genistein found in the serum was significantly lower from the supplement compared to the medical food and the Squadrito study formulation suggesting interfering ingredients within the supplement.

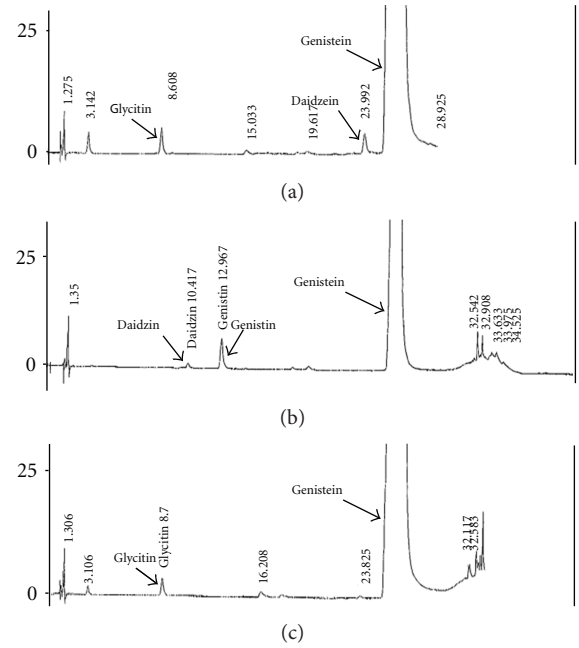

Figure 2: High performance liquid chromatography (HPLC) comparison of genistein purity extracted from the medical food product indicated for osteopenia/osteoporosis (a), the Squadrito study formulation (b), and the OTC bone supplement (c).

4. Discussion

Health benefits of isoflavones are directly related to their bioavailability. Bioavailability is dependent upon an individual's state of health, intestinal bacterial flora, sex, age, food matrix in which isoflavones are consumed, the mix of isoflavones in products as well as host genetics [23]. The results of this PK analysis of three different bone formulations show genistein absorption is affected by specific ingredients formulated with the isoflavone which could have clinical implications on efficacy (Table 2; Figure 3). There are a multitude of factors which could account for this difference.

Normally, genistein is freely absorbed from the intestine and a large fraction is converted to 7β-O-glucuronide as it crosses the brush border and ultimately enters the portal vein [24]. Intestinal bacteria are known to influence glucuronidation and may also drive sulfonation [25, 26]. Only a small percentage of the parent molecule remains as free genistein once it reaches the liver. Once in the liver, genistein undergoes additional biotransformation via CYP450-mediated

Table 2: The maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), areas under curve (AUC) and half-life ($T_{1/2}$) after steady administration of the medical food indicated for osteopenia/osteoporosis, the Squadrito study formulation, and the OTC bone supplement.

Study parameters	Medical food	Squadrito study formulation	OTC bone supplement
T_{max} (hrs)	2	2	6
C_{max} (ng/mL ± StDev)	188.4 ± 2.5	187.1 ± 3.5	153.3 ± 3.5
AUC (ng·hr/mL)	9221 ± 413	9818 ± 1370	6474 ± 287
$T_{1/2}$ (hrs ± StDev)	18.0 ± 6.9	20.9 ± 4.9	8.3 ± 1.9

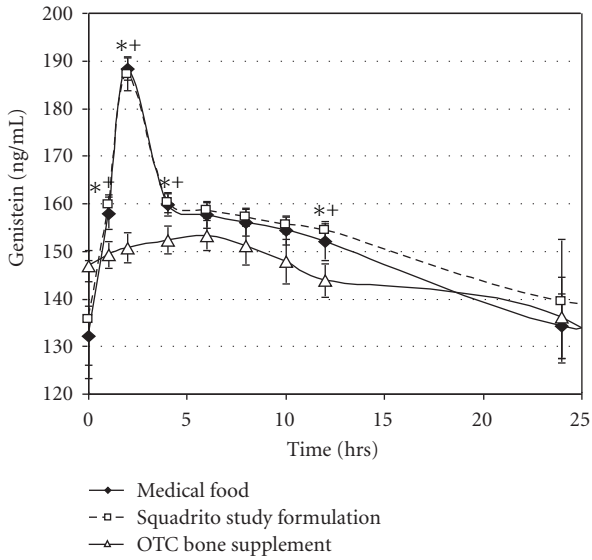

Figure 3: The pharmacokinetic profile for the first 24 hours after the last dose of each study product obtained after 8 days 54 mg per day intake of the medical food indicated for osteopenia/osteoporosis (♦), the Squadrito study formulation (□) and the OTC bone supplement (Δ). $^{*}P < 0.05$ for the medical food versus the OTC bone supplement, $^{+}P < 0.05$ for the Squadrito study formulation versus the OTC bone supplement.

hydroxylation [27] followed by glucuronidation and sulfation by UDP-glucuronosyl transferase and sulphotransferases, respectively [24]. A large majority of glucuronidated genistein undergoes efficient enterohepatic recirculation following biliary excretion. The preponderance of circulating genistein in serum has been found to be in the form of glucuronidate and sulfate conjugates [28].

Food, isolated nutrient molecules and binders, when coadministered with drugs, are known to affect their absorption, distribution in the body, metabolism in the lumen, liver and cells, and elimination [29]. This issue is so important that the FDA has issued guidance on oral administration of drugs with food, their bioavailability and need for bioequivalence studies to assure proper guidance for administration of therapeutic compounds [30]. Genistein is considered a class 2 compound with low solubility and high permeability by the FDA's Biopharmaceutics Classification System. Though there are no formal requirements for this type of analysis of medical foods or supplements, it is important that fasting and fed PK and bioequivalence studies be performed, especially since medical foods have a statutory requirement to be indicated for a specific disease and must be administered under the direction of a physician [21]. Indeed, a fasting and fed PK study of genistein has been performed on the medical food Fosteum indicated for osteopenia/osteoporosis suggesting a minor, nonstatistical effect of food on absorption [31]. There is no published data on genistein bioavailability from the OTC Citracal Plus Bone Density Builder supplement. This steady-state PK study demonstrates that the medical food product for osteopenia/osteoporosis is equivalent to the clinically proven Squadrito study formulation for absorption and bioavailability of genistein, whereas the OTC supplement formulation dramatically and statistically affects the isoflavone absorption (Table 2; Figure 3).

Absorption of bioactive substances is influenced by several different factors such as the intestinal solubility and permeability [32]. Another factor that can affect absorption of bioactive molecules is viscosity induced by food additives, such as guar gum [33]. Citrate, an approved food additive, is also known to increase viscosity in the presence of collagen and fibrous material [34] as well as change the water absorption profile in different parts of the small intestine [35]. It has been added to different oral rehydration formulations to modulate acidosis and glycemic index as a viscosity-promoting agent [36–38]. Based on the above data, it is possible that normal dietary fiber in those randomized to the bone supplement group had increased gastrointestinal viscosity during the time of dosing which affected genistein uptake due to the dissociation of the citrate and calcium ions in the stomach. Though Fosteum contains citrated zinc bisglycinate, citrate along with glycine tightly coordinate zinc and is not ionized in the stomach. Preclinical studies have shown that the zinc from chelates is dissociated from the coordinating molecules on the lumen of the intestine [39]. Hence, the chance of the citrate portion of the chelate interacting with dietary fiber is minimal. Other mechanisms may also account of the lower level of genistein absorption from the supplement.

ATP-binding cassette (ABC) transport proteins are responsible, in part, for the transport of flavonoids, including isoflavones, into luminal intestinal cells for absorption [40]. Genistein specifically interacts with the ABCG2 receptor in a variety of cells including those in the intestinal lumen [41]. Calcium and magnesium ions are typically actively absorbed via transient receptor potential channel proteins (TRP) in the duodenum [42]. Vitamin D_3 is needed for calcium uptake through these channels while magnesium

serves as a cofactor for ABC transport proteins in the uptake of flavonoid molecules. There is no reported evidence that calcium, magnesium or other ions inhibit ABC receptors. Isoflavones, such as genistein, are also transferred from the intestine into the epithelial lumen by organic anion transport proteins (OATs) [43]. The OAT receptor family also serves to maintain anion balance throughout the body, including in the intestinal lumen [44] and is a subclass of a superfamily of proteins termed major facilitator superfamily (MFS) transporters [45, 46]. Citrate is known to interact with both OAT receptors [44] as well as with members of the MFS called citrate-H+ symporter (CitA) [47] and Na+/citrate transporters [48]. Another OAT receptor, Mrp2, also known as canalicular multispecific organic anion transporter (cMOAT) and ABCC2 binds organic anions like citrate and gluconate [49]. Both ABCC2 and ABCG2 have extensive homology and exist together in the intestinal lumen having a broad range of nutrient transport capabilities. These include the transport of organic anions, glucuronidated and sulfonated molecules, and a number of drugs [50]. These receptors have been shown to have specific functional overlap in absorption of various molecules [51]. Therefore, organic anions such as citrate, silicate, gluconate, and stearate present as counterions in the OTC Citracal Plus Bone Density Builder supplement formulation may directly compete with genistein for absorption on these receptors and transporters. This and the possibility that citrate increases viscosity, and hence slows gastric emptying, might explain the difference in uptake resulting in a lower C_{max}, lengthened T_{max} and decreased AUC compared to the medical food product and the Squadrito study formulation. The carbonate anion in the Squadrito study formulation is known to interact with the solute carrier family (SLC) of receptors [52], rather than ABC or OAT receptors. This may explain why calcium carbonate does not affect genistein absorption while the calcium citrate supplement formulation appears to do so.

5. Conclusion

The medical food for osteopenia/osteoporosis, Fosteum, and Squadrito study formulation tested for bone building in clinical trials are bioequivalent for absorption of genistein compared to that from bone supplement Citracal Plus Bone Density Builder which inhibits genistein uptake. Even with the 10% difference in AUC between the medical food and the Squadrito study formulation over the 96 hr period, one could expect similar genistein pharmacokinetic behavior from both products under usual conditions of use. The steady-state genistein concentration attained by dosing with the OTC Citracal Plus Bone Density Builder supplement, however, would presumably be significantly lower compared to Fosteum and the Squadrito study formulation even over long periods of time. This difference could adversely affect overall efficacy on bone metabolism. Based on this evidence, care must be taken when combining bioactive substances like genistein with specific salts to prevent changes in viscosity or competition for receptors or transport proteins during intestinal absorption.

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Review Article

Glucocorticoid-Induced Osteoporosis in Children with 21-Hydroxylase Deficiency

Annamaria Ventura,[1] Giacomina Brunetti,[2] Silvia Colucci,[2] Angela Oranger,[2] Filomena Ladisa,[1] Luciano Cavallo,[1] Maria Grano,[2] and Maria Felicia Faienza[1]

[1] *Department of Biomedical Sciences and Human Oncology, University of Bari, Piazza G. Cesare 11, 70124 Bari, Italy*
[2] *Section of Human Anatomy and Histology, Department of SMB-NOS, University of Bari, 70124 Bari, Italy*

Correspondence should be addressed to Annamaria Ventura; annav81@hotmail.it

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21-Hydroxylase deficiency (21-OHD) is the most common cause of congenital adrenal hyperplasia (CAH), resulting from deletions or mutations of the P450 21-hydroxylase gene (*CYP21A2*). Children with 21-OHD need chronic glucocorticoid (cGC) therapy, both to replace congenital deficit in cortisol synthesis and to reduce androgen secretion by adrenal cortex. GC-induced osteoporosis (GIO) is the most common form of secondary osteoporosis that results in an early, transient increase in bone resorption accompanied by a decrease in bone formation, maintained for the duration of GC therapy. Despite the conflicting results in the literature about the bone status on GC-treated patients with 21-OHD, many reports consider these subjects to be at risk for osteoporosis and fractures. In bone cells, at the molecular level, GCs regulate various functions including osteoblastogenesis, osteoclastogenesis, and the apoptosis of osteoblasts and osteocytes. In this paper, we focus on the physiology and biosynthesis of endogenous steroid hormones as well as on the effects of GCs on bone cells, highlighting the pathogenetic mechanism of GIO in children with 21-OHD.

1. Introduction

21-Hydroxylase deficiency (21-OHD) is the most common cause of congenital adrenal hyperplasia (CAH), caused by sequence variants in the 21-hydroxylase gene (*CYP21A2*) [1]. This disorder is characterized by accumulation of the precursors immediately proximal to the 21-hydroxylation step along the pathway of cortisol synthesis, which are shunted into the androgen pathway. Children with 21-OHD need chronic glucocorticoid (cGC) therapy as soon as they are diagnosed with the disease, both to correct the deficiency in cortisol and to reduce androgen secretion by adrenal cortex [2].

An organ system that has the potential to be profoundly affected by cGC therapy is the skeleton, and GC-induced osteoporosis (GIO) is the most common form of secondary osteoporosis [3]. GIO results in an early, transient increase in bone resorption accompanied by a decrease in bone formation, which is maintained for the duration of GC therapy. Although many patients remain asymptomatic, fractures occur in 30–50% of GCs-treated patients [4].

Recently, several studies have helped to clarify the mechanisms responsible for GIO, highlighting the molecular events occurring in skeletal cells.

Three principal cell types are involved in bone modeling and remodeling: osteoblasts (OBs), osteoclasts (OCs), and osteocytes, each with distinct and varying functions. The actions of these cells are modulated and coordinated by autocrine, paracrine, and endocrine regulators, such as cytokines, growth factors, and hormones. In bone cells, at the molecular level, GCs regulate various functions including osteoblastogenesis, osteoclastogenesis, and the apoptosis of osteoblasts and osteocytes [5].

In this paper, we focus on the physiology and biosynthesis of endogenous steroid hormones as well as on the effects of GCs on bone cells, highlighting the pathogenetic mechanism of GIO in children with 21-OHD.

2. Physiology and Biosynthesis of Steroid Hormones

Steroid hormones serve many essential roles in mammalian physiology, ranging from promoting development to regulation of metabolism. Two of the major steroidogenic tissues in mammals include the adrenal glands and gonads [6].

Based on its functional actions, steroid hormones are classified into five principal classes: estrogens (estradiol, estrone, and estriol), progestins (progesterone), androgens (testosterone, A4, and dihydrotestosterone), glucocorticoids (cortisol, corticosterone), and mineralcorticoids (aldosterone, deoxycorticosterone) [7].

The main adrenal steroids that enter the circulation are aldosterone, which is important in salt homeostasis and acid excretion; cortisol, which is involved in a range of homeostatic processes including carbohydrate, protein, and fat metabolism and regulation of immune processes; dehydroepiandrosterone (DHEA) and androstenedione, the primary source of circulating androgens in women [8].

Cortisol and adrenal androgen production are regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The production of corticotropin releasing hormone (CRH) by the hypothalamus stimulates adrenocorticotropic hormone (ACTH) release by the anterior pituitary gland which in turn stimulates the synthesis of cortisol by the adrenal cortex.

All steroid hormones are derived from cholesterol through a complex series of chemical modifications [9]. Figure 1 shows the biosynthesis of steroid hormones in adrenal glands and gonads.

The rate-limiting step in steroid biosynthesis is importation of cholesterol from cellular stores to the matrix side of the mitochondria inner membrane. The first enzymatic step in steroid synthesis is the conversion of cholesterol, a C27 compound, to the C21 steroid pregnenolone [10]. This is catalyzed by the mitochondrial cytochrome P450 enzyme CYP11A. Pregnenolone is the common precursor for all other steroids and, as such, may undergo metabolism by several other enzymes. To synthesize mineralocorticoids, 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum and mitochondria converts pregnenolone to progesterone. This is 21-hydroxylated in the endoplasmic reticulum by CYP21A2 to produce deoxycorticosterone (DOC). Aldosterone is produced by the 11 β-hydroxylation of DOC to corticosterone, followed by 18-hydroxylation and 18-oxidation of corticosterone by CYP11B2 enzyme. To produce cortisol, the major glucocorticoid in man, CYP17 converts pregnenolone to 17α-hydroxypregnenolone [11]. 3β-HSD utilizes 17α-hydroxypregnenolone as a substrate, producing 17α-hydroxyprogesterone. The latter is 21-hydroxylated by CYP21A2 to form 11-deoxycortisol, which is converted to cortisol by CYP11B1 in mitochondria. The 17,20-lyase activity of CYP17 converts 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA, a C19 steroid, and sex hormone precursor). DHEA is further converted by 3β-HSD to androstenedione. In the gonads, this is reduced by 17β-hydroxysteroid dehydrogenase to testosterone. In pubertal ovaries, aromatase (CYP19) can convert androstenedione and testosterone to estrone and estradiol, respectively. Testosterone may be further metabolized to dihydrotestosterone by steroid 5α-reductase in androgen target tissues [9].

3. Abnormal Steroids in 21-Hydroxylase Deficiency

Inefficient cortisol synthesis in 21-OHD patients signals the anterior pituitary to increase ACTH release, with subsequent overstimulation and hyperplasia of the adrenals.

Rather than cortisol and aldosterone, the adrenals produce excess of sex hormone precursors that are further metabolized to active androgens (testosterone and dihydrotestosterone) and to a lesser extent estrogens (estrone and estradiol) [12].

The most definitive hormonal diagnostic test for 21-OHD is an ACTH-stimulation test, which measures the serum concentrations of 17α-hydroxyprogesterone, the main substrate for 21-hidroxylase, at 0 and 60 min after ACTH administration [13].

Three forms of 21-OHD can be distinguished by means of clinical, hormonal, and molecular-genetic criteria: the classical salt wasting (SW), classical simple virilizing (SV), and nonclassical forms (NC). In SW-CAH, affected children present with salt loss during the neonatal period, and females foetuses will develop virilizing malformations of external genitalia. Patients with SV-CAH do not develop life-threatening salt loss, but female newborns present virilized genitalia, and boys may develop precocious pseudopuberty during early childhood. NC-CAH is characterized by various degrees of late-onset symptoms. The most common symptoms are premature pubarche in children, acne, hirsutism, and menstrual irregularities in young women [14].

Children with 21-OHD need chronic cGC therapy as soon as they are diagnosed with the disease in order to reduce excessive ACTH and consequent increase androgen production, by substituting for deficient cortisol and when necessary mineralocorticoid synthesis [15].

During childhood, the main aims of the medical treatment of CAH are to prevent salt loss and virilization, to achieve normal stature and to undergo normal puberty [16].

Undertreatment exposes the patient to the risk of adrenal crisis and allows increased adrenal androgen production, with consequent advancement of bone age and loss of growth potential. Overtreatment, however, results in growth retardation, truncal obesity, and osteopaenia, through the effects of steroids on growth hormone secretion and bone metabolism [15].

Hydrocortisone (HC) is considered the drug of first choice in CAH during infancy and childhood [17].

4. Molecular Genetics of 21-OHD

The gene encoding 21-hydroxylase, *CYP21A2*, is located in the HLA region III on the short arm of chromosome 6 (6p21.3) closely linked to a nonfunctional pseudogene *CYP21A1P* [1]. Both genes consist of 10 exons sharing a high degree of homology with a nucleotide identity of 98% on exon and of 96% on intron level [1]. The high homology of

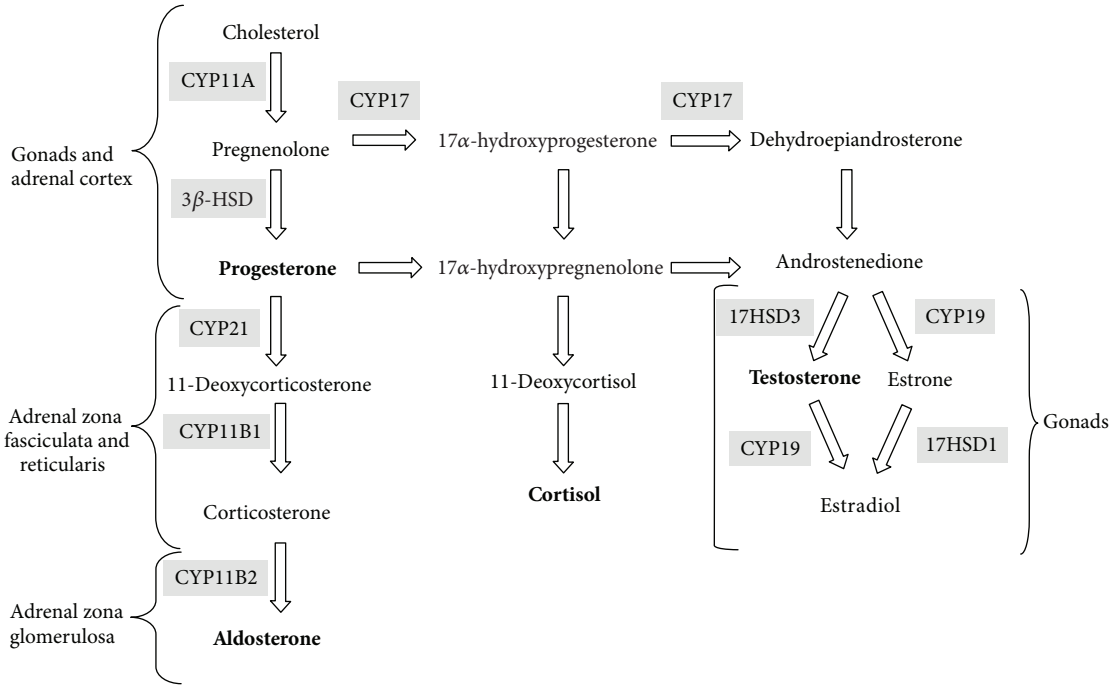

Figure 1: Biosynthesis of steroid hormones in adrenal glands and gonads. Enzymes are highlighted. Final steroid hormone product is in bold.

these regions causes misalignment during meiosis, resulting in intergenic recombinations that are responsible for 95% of the mutations associated with 21-OHD; the remaining 5% of mutations appear to be the result of spontaneous mutations rather than gene conversion events [18].

Approximately 95% of all inactivating mutations of *CYP21A2* comprise deletions/large gene conversions of the entire gene and/or a few point mutations [12].

NC and classical forms of 21-OHD are associated with distinct genotypes, characterized by varying levels of enzyme activity. The genotype for the classical form of 21-OHD is predicted to be a severe mutation on both alleles at the 21-hydroxylase locus, with markedly reduced enzymatic activity generally associated with SW. Patients with NC form of 21-OHD are predicted to have mild mutations on both alleles, or one severe and one mild mutation of *CYP21A2* (compound heterozygotes) [13]. A good genotype-phenotype correlation has been shown in 98% of 21-OHD patients; however, rare cases of nonconcordance have important implications in prenatal diagnosis of 21-OHD and genetic counseling [13].

The Endocrine Society Clinical Practice Guidelines from 2010 recommends genotyping for purposes of genetic counseling and for confirmation of the diagnosis especially in NC-CAH when the ACTH-stimulation test is equivocal [17].

5. Molecular Effects of GCs on Bone Cells

5.1. Osteoblasts. The reduction in OB number and function has a central role in the pathogenesis of GIO, leading to a suppression of bone formation characteristic of GCs excess. The mechanism includes inhibition of replication and differentiation and enhanced apoptosis of OBs [19, 20].

GCs decrease the replication of osteoblastic lineage cells, reducing the pool of cells that may differentiate into mature OBs [5].

In the presence of GCs, bone marrow stromal cells differentiation is redirected towards adipocyte lineage. Mechanisms involved include the induction of peroxisome proliferator-activated receptor $\gamma 2$ (PPARγ) and the regulation of nuclear factors of the CAAT enhancer-binding protein family (C/EBPs), adipocyte P2, aP2; the differentiation-dependent adipocyte protein is a downstream target gene of PPARγ and C/EBPα [21] abundantly expressed in the cytoplasm and nuclear region of adipocytes [22]. PPARγ and C/EBPα might also indirectly reduce OBs proliferation, decreasing IGF-I transcription [19].

An additional effect of GCs is represented by inhibition of Wnt-β-catenin signaling [19], a key pathway for promoting osteoblastogenesis. GCs suppress the canonical Wnt-β-catenin signalling pathway in OBs, enhancing the expression of Dickkopf-1 (DKK1), an extracellular Wnt inhibitor which prevents Wnt binding to its receptor complex, and destabilizing β-catenin via activation of glycogen synthase kinase 3-b [23, 24]. Moreover, GCs inhibit OB differentiation through the repression of bone morphogenetic protein 2 (BMP2), which has a key role in bone formation [25, 26].

GCs impair the function of the differentiated mature cells, inhibiting OB-driven synthesis of type I collagen (by transcriptional and posttranscriptional mechanisms) [27], the major component of the bone extracellular matrix, with

a consequent decrease in bone matrix available for mineralization [19].

Moreover, GCs modify osteocalcin gene expression via the GC-responsive elements, which have been identified in the osteocalcin promoter [28, 29].

The proapoptotic effects of GCs on OBs are explicated by modulating the expression of proapoptotic and antiapoptotic genes, such as *BCL2, BIRC5,* and *BCL2L11* [30, 31]. O'Brien et al. demonstrated the requirement of GC signaling in late-stage differentiation of OBs for apoptosis *in vivo* [20]. Dexamethasone (Dex) induction of the protein Bim, a proapoptotic Bcl-2 family member, enhances the activities of its downstream effectors, caspases -3, -7, and -8, and has been suggested as a key regulator of glucocorticoid receptor-dependent OB apoptosis [32].

5.2. Osteocytes. The loss of osteocytes might be particularly important in terms of bone structure because these mechanosensors are essential in the repair of bone microdamage. Loss of osteocytes might disrupt the osteocyte–canalicular network, resulting in a failure to detect signals that normally stimulate the replacement of damaged bone. GCs affect the function of osteocytes, by modifying the elastic modulus surrounding osteocytic lacunae. As a result, the normal maintenance of bone through this mechanism is impaired, and the biomechanical properties of bone are compromised [33]. Another direct effect of GCs on osteocytes is the induction of apoptosis through activation of caspase 3 [34].

5.3. Osteoclasts. The initial bone loss occurring in patients exposed to GCs might be secondary to increased bone resorption by OCs [3].

OCs are members of the monocyte-macrophage family, derived from the fusion of marrow-derived mononuclear phagocyte, the OC precursors (OCPs), which circulate in peripheral blood (PB) [35]. These cells differentiate under the influence of two cytokines, namely macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor k-B ligand (RANKL). RANKL expressed on OBs and stromal cells as a membrane-bound protein and cleaved into a soluble molecule (sRANKL) by metalloproteinase [36] promotes differentiation and fusion of OCPs and activates mature OCs to reabsorb bone by binding to its specific receptor RANK. Osteoprotegerin (OPG), a soluble decoy receptor secreted by OBs and bone marrow stromal cells, competes with RANK in binding to RANKL, preventing its osteoclastogenic effect [36].

GCs increase the expression of RANKL and decrease the expression of OPG in stromal cells and OBs [37]. GCs also enhance the expression of M-CSF, which in the presence of RANKL induces osteoclastogenesis [37]. Moreover, GCs have been demonstrated to upregulate receptor subunits for osteoclastogenic cytokines of the glycoprotein 130 family [38]. In a work by Takuma et al. [39] are explained the effects of GCs on OC formation. In particular, this study demonstrated that Dex downregulates endogenous interferon-β production, an autocrine cytokine that normally inhibits OCs differentiation, allowing osteoclast progenitors to be freed from its differentiation-depressing effect and to proceed toward the phenotype of mature OCs.

6. Glucocorticoid Receptor-Mediated Effect of GCs

The GC-induced effects described above appear to be dependent on the duration and concentration of GC treatment and possibly on the differentiation stage of bone cells [4, 40], while data on the exact role of glucocorticoid receptor (GR) in mediating GCs actions are limited.

GR is a ligand-regulated transcription factor, a member of the nuclear-receptor (NR) superfamily that controls gene expression linked to several processes like inflammation, stress responses, glucose homeostasis, lipid metabolism, proliferation, and apoptosis development [41]. In the absence of ligand, GR is associated to the hsp90 chaperone heterocomplex and primarily localizes in the cytoplasm, while the GR-ligand complex is mainly nuclear. In the nucleus, the activated GR regulates gene expression through two modes of action [42, 43]. A direct mechanism involves GR homodimer binding to positive or negative glucocorticoid response elements (GREs) located in the promoter region of target genes, leading to transcription activation or repression, respectively. The activated GR may also function through an indirect mechanism by interacting as a monomer with other transcriptional factors, such as NF-kB or AP-1 [44], without direct binding to DNA. Both GR modes of action would be independent, and it has been postulated that GC beneficial effects (immunosuppressant and anti-inflammatory effects) are associated to the indirect-transrepression mechanism, while the side effects are associated to the direct transactivation one.

Therefore, extensive efforts are aimed at developing selective GR agonists (SEGRAs) as novel therapies with an improved risk/benefit ratio. The concept of SEGRAs is based on the fact that they largely mediate their effects via transrepression by GR monomers and not through transactivation by GR dimers. Moreover, SEGRAs will serve as a tool to further investigate the molecular basis of GC side effects.

Compound A (CpdA), a plant-derived phenyl aziridine precursor, is a well-investigated agent that mediates its effects by binding the GR [45]. In a recent work, Thiele et al. [46] assessed the effects of CpdA on bone metabolism in a mouse model of GIO. In particular, they examine the effects on the skeleton of CpdA and prednisolone (PRED) using quantitative computed tomography, bone histomorphometry, serum markers of bone turnover, and gene expression analysis. Mice treated with PRED showed a reduction of the total and trabecular bone density in the femur and in the spine, increase of osteoclast number, serum CTX-1 and the skeletal RANKL/OPG ratio, reduced skeletal expression of osteoblast markers, and increased serum levels of DKK-1. None of these effects were observed with CpdA, and consistent with the *in vivo* data, CpdA did not increase the RANKL/OPG ratio in MLO-Y4 cells and failed to transactivate DKK-1 expression in bone tissue, BMSCs, and osteocytes. This study underlines

the bone-sparing potential of CpdA and confirms that GC enhanced DKK1 and RANKL expression significantly, in accordance with previous studies.

7. Pathogenetic Mechanism of GIO in Children with 21-OHD

Previous reports on 21-OHD patients showed increased [47], decreased [48–57], or normal bone mineral density (BMD) [58–62].

These contradictory results may be explained by heterogeneous populations and methods, as the reports differ with respect to age selections and GC regimens [15]. cGC therapy is known to generate bone loss in many ways: a direct suppression of osteoblastic activity [63] and an inhibition of digestive calcium absorption with secondary hyperparathyroidism and increased bone resorption by osteoclasts [64]. Two studies have evaluated fractures in CAH patients [56, 65]. The study by Falhammar et al. [56] included 61 women with 21-OHD and 61 age-matched women as controls. Results indicated a higher frequency of fractures in women with CAH. When only osteoporotic fractures (vertebrae, wrist, and hip) were considered, the difference almost reached significance ($P = 0.058$). This is of importance for CAH patients, even if this finding has to be confirmed in larger studies, which should evaluate differences in lifestyle between patients and controls, as the trauma leading to fractures was not ascertained. The second study [65] reported vertebral compression fractures in a young adult male with 21-OHD, the onset of which likely corresponds to excessive GC dosing during adolescence.

Biochemical markers of bone turnover have been partially evaluated in patients with CAH [50, 52, 55, 56, 58], and the literature data are inconclusive. Bone turnover was found to be lower in patients with CAH than in controls, and osteocalcin levels correlated positively with growth velocity and negatively with BMD [50, 58]. Another study showed higher bone-specific alkaline phosphatase (ALP) and serum β-C-telopeptide of type I collagen (CTX) concentrations in young CAH patients compared with control subjects [55]. In the report of Falhammar et al. [56], the bone resorption marker CTX was found to be reduced in the older group of patients both compared with controls and younger patients. This was not in accordance with the findings of Sciannamblo et al. [55] and Zimmermann et al. [57] that observed elevated CTX concentrations in young individuals, some who are still growing. The authors concluded that the CAH patients treated for many years had predominantly low bone formation but also unexplained low bone resorption [56].

Faienza et al. [66] demonstrated a high osteoclastogenic potential of peripheral blood mononuclear cells (PBMCs) in children with 21-OHD on long-term GC treatment. In particular, spontaneous osteoclastogenesis, without adding MCSF and RANKL, and significantly higher osteoclasts resorption activity occurred in 21-OHD patients. Conversely, MCSF and RANKL were essential to trigger and sustain osteoclastogenesis in controls. This spontaneous osteoclastogenesis seems to be supported by both the presence of circulating OCPs and factors released by T cells. In particular, Faienza et al. identified a significant percentage of CD11b-CD51/CD61- and CD51/61-RANK-positive cells, which are OCPs strongly committed. Moreover, evidences supporting a T cell regulation of osteoclastogenesis came from 21-OHD patients' T-cell-depleted PBMC cultures, in which the addition of exogenous M-CSF and RANKL was necessary for OC formation. In fact, T-cells from 21-OHD patients expressed high levels of RANKL and low levels of OPG with respect to controls. Furthermore, 21-OHD patients had higher soluble RANKL and lower OPG serum levels compared with controls. Moreover, we, very recently, demonstrated high DKK1 levels in sera and circulating monocytes, T lymphocytes, and neutrophils from 21-OHD patients [67]. The serum from patients containing elevated levels of DKK1 can directly inhibit osteoblast differentiation *in vitro* as well as affect the expression of RANKL in osteoblasts [66]. We also found a correlation between both DKK1 and RANKL or CTX serum levels in patients. Thus, chronic GC treatment in 21-OHD patients may contribute both to the alteration of bone resorption and formation [66, 67].

8. Conclusions

Despite the conflicting results in the literature about the bone status on GC-treated patients with 21-OHD, many reports consider these subjects to be at risk for osteoporosis and fractures. Furthermore, it should be a useful monitoring bone status in treated 21-OHD children, checking BMD and bone turnover markers, in order to avoid GIO in adulthood.

Other studys should be performed to analyze the expression of regulators of bone resorption and bone formation in 21-OHD patients.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Three New Steroidal Glycosides from the Roots of Cynanchum stauntonii

Jin-Qian Yu, Zhi-Hui Zhang, An-Jun Deng, and Hai-Lin Qin

State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

Correspondence should be addressed to An-Jun Deng; denganjun@imm.ac.cn

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Three new steroidal glycosides, named as stauntosides L, M, and N (**1–3**), along with one known C_{21} steroidal glycoside, anhydrohirundigenin monothevetoside (**4**), were isolated from the 95% ethanol extract of the roots of *Cynanchum stauntonii*. The structures of these new compounds were elucidated on the basis of extensive spectroscopic analyses, mainly 1D and 2D NMR, HRESI-MS, and chemical methods.

1. Introduction

Cynanchum stauntonii (Decne.) Schltr. ex Levl. is a perennial medicinal herb from the family of Asclepiadaceae, which is widely distributed in south-central region of China. The dried-up roots of *C. stauntonii*, along with that of another species of the same genus, *C. glaucescens* (Decne.) Hand.-Mazz., has been used as antitussives and expectorants to treat diseases in the history of China [1]. Both of which are given the name of "Bai-qian" in traditional Chinese medicine (TCM) [2]. The main chemical constituents isolated from *Cynanchum* species are steroids, especially the steroidal saponins with aglycones assignable to either the normal four-ring C_{21} steroid skeleton or the aberrant 13,14 : 14,15-disecopregnane-type skeleton or the equally abnormal 14,15-secopregnane-type skeleton, respectively [3, 4]. It is known that C_{21} steroids and their glycosides are of considerable bioactivities, such as hypolipidemic and antitumor activities. However, chemical investigation into the title plant is very rare up to now with, to the best of our knowledge, only three papers have reported several steroids, including four ones by our group eight years ago [1]. The ongoing investigations in our group intend to enrich the information about the chemical constituents and their bioactivities of this plant which has led to the isolation and elucidation of some known and new steroidal glycosides [5]. In this paper, we describe three new steroidal glycosides (**1–3**) and one known analogue, anhydrohirundigenin monothevetoside (**4**) (Figure 1), from the roots of *C. stauntonii*. The isolated new steroidal glycosides contained steroid aglycones with either the 13,14 : 14,15-disecopregnane-type skeleton or the 14,15-secopregnane-type skeleton and were given the trivial names stauntosides L-N, respectively.

2. Materials and Methods

2.1. General Methods. Optical rotations were measured on a Perkin-Elmer 241 digital polarimeter at 20°C. IR spectra were recorded on a Nicolet 5700 spectrometer. 1D and 2D NMR spectra were taken on a Varian INOVA-500 spectrometer or a Varian NMR System-600 NMR spectrometer with tetramethylsilane as internal standard. ESIMS and HRESIMS were obtained using an Agilent 1100 series LC/MSD Trap SL mass spectrometer. Preparative HPLC was performed on a Shimadzu LC-6AD system equipped with a SPD-10A detector, and a reversed-phase C18 column (YMC-Pack ODS-A U 20×250 mm, 10 μm) was employed. Column chromatography (CC) was undertaken over silica gel (200–300 mesh). TLC was carried out with glass plate precoated silica gel G. Spots were visualized under UV light and by spraying with 10% H_2SO_4 in 95% EtOH followed by heating. GC was

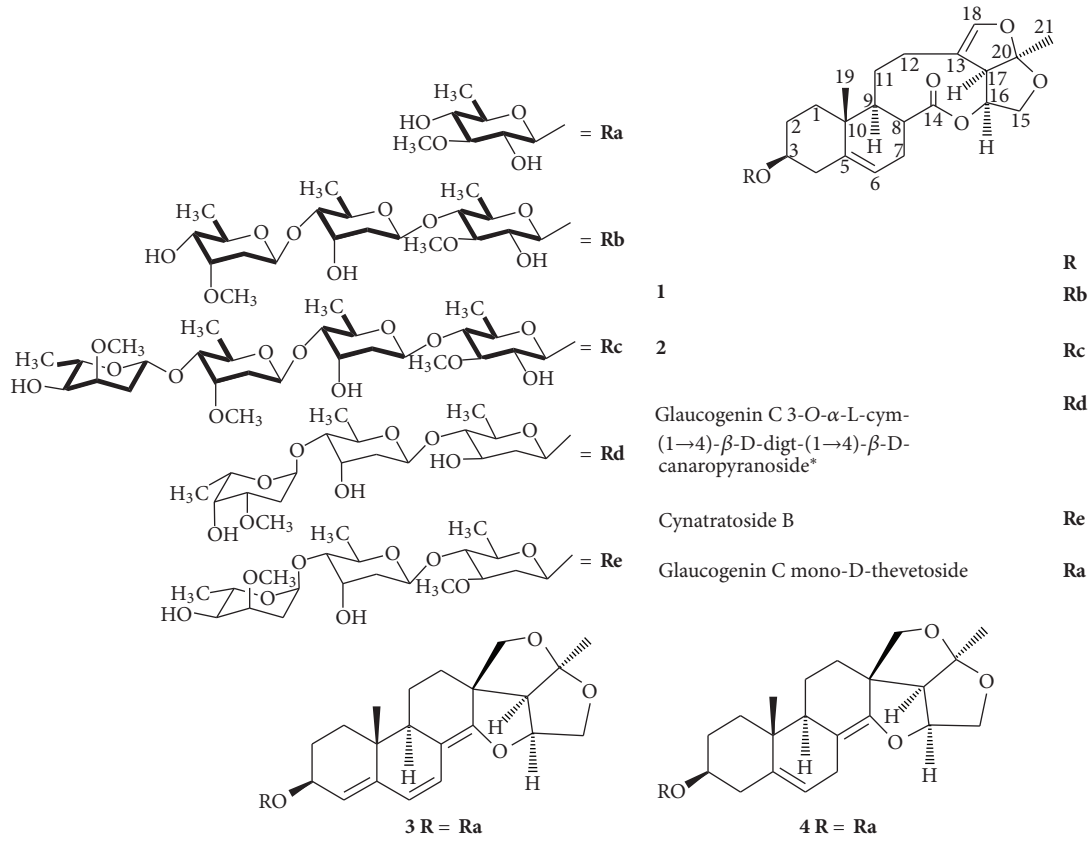

FIGURE 1: The structures of compounds **1–4** and the reference compounds, and the key ^{1}H, ^{1}H-COSY correlations in oligosaccharide moieties. (—: ^{1}H, ^{1}H-COSY). *cym: cymaropyranosyl; digt: digitoxopyranosyl.

conducted on an Agilent 7890A instrument. Reference compounds, glaucogenin C 3-*O*-*α*-L-cymaropyranosyl-(1 → 4)-*β*-D-digitoxopyransyl-(1 → 4)-*β*-D-canaropyranoside, cynatratoside B, and glaucogenin C mono-D-thevetoside which were used to identify the monosaccharides obtained in the acid hydrolysis, including their absolute configuration, were isolates from the title plant in our previous work [5]. Acetonitrile used in preparative HPLC procedure was in HPLC grade, and other solvents were of analytical grade.

2.2. Plant Material. The roots of *C. stauntonii* were collected from Tongbai County, Henan Province of central China, in August 2011. It was identified by Associate Professor Lin Ma (a savant in plant systematics from Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College). A voucher specimen (ID-S-2426) was deposited in the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China.

2.3. Extraction and Isolation. The dried-up and pulverized roots (30 Kg) of *C. stauntonii* were extracted three times under reflux conditions with 95% EtOH. The combined ethanolic solution was concentrated in vacuo to yield a dark-brown residue (ca. 5000 g). The residue was suspended in 80% aqueous ethanol (ca. 10000 mL) and then extracted with petroleum ether and EtOAc successively in separatory funnel, each for several times until the upper solvent being very transparent. The combined EtOAc solution was washed three times with 5% aqueous solution of $NaHCO_3$ (3 × 1000 mL) and then H_2O (2 × 1000 mL), respectively, to pH 7. After the removal of the organic solvent, 190 g of brown residue was obtained. This resulting residue was fractionated by CC over silica gel eluted with gradient solvents of $CHCl_3$–MeOH (100 : 0-1 : 1) to yield 13 fractions (designated as fractions 1 to 13) according to their TLC profiles. Fraction 3 (68 g) was further separated by CC over silica gel using a stepwise gradient solvents of petroleum ether/EtOAc (25 : 1 → 1 : 1) as eluents to yield seven further subfractions (F3-1-F3-7, also according to the detection of TLC). Fraction F3-5 (7.0 g) was applied to Flash C18 column chromatography eluted with CH_3OH/H_2O (40% → 100%) to give six subfractions (F3-5-1-F3-5-6). Fraction F3-5-4 (1.1 g) was applied to preparative HPLC system (mobile phase: CH_3OH/H_2O (70 : 30, v/v); flow rate: 5 mL min^{-1}; UV detection at 210 nm) resulting in the isolation of compound **1** (36 mg). Fraction F3-6 (3.5 g) was applied to Flash C18 column chromatography

eluted with CH_3OH/H_2O (40% $\rightarrow$ 100%) to give six subfractions (F3-6-1-F3-6-6). Compound **2** (60 mg) was obtained by recrystallization from F3-6-4. Fraction F3-7 (3.0 g) was applied to Flash C18 column chromatography eluted with CH_3OH/H_2O (40% $\rightarrow$ 100%) to give seven subfractions (F3-7-1-F3-7-7). Fraction F3-7-2 (0.2 g) was applied to preparative HPLC system (mobile phase: CH_3CN/H_2O (35 : 65, v/v); flow rate: 5 mL min^{-1}; UV detection at 210 nm and 280 nm) resulting in the isolation of compound **3** (12 mg) and compound **4** (22 mg).

The known compound anhydrohirundigenin monothevetoside (**4**) [1] was identified by comparison of their spectroscopic data (^{1}H and ^{13}C NMR, MS) with the literature values.

*2.3.1. Stauntoside L (***1**). White amorphous powder (CH_3OH–$CHCl_3$), $[\alpha]_D^{20}$ + 17.7 (c = 1.14, CH_3OH, 20°C). IR(KBr) ν_{max}: 3479, 2933, 1735, 1652, 1452, 1381, 1309, 1162, 1072, 1003, 871, and 606 cm^{-1}. ESI-MS (positive mode) m/z: 817.5 $[M+Na]^+$. HRESI-MS (positive mode) m/z: 817.4002 $[M+Na]^+$, calcd for $C_{41}H_{62}O_{15}Na$, 817.3981. ^{1}H NMR (500 MHz, C_5D_5N) for aglycone: δ 0.77 (3H, s, H-19), 1.53 (3H, s, H-21), 3.54 (1H, d, J = 9.0 Hz, H-17), 3.80 (1H, m, H-3), 3.93 (1H, m, H_β-15), 4.23 (1H, t, J = 7.7 Hz, H_α-15), 5.32 (1H, d, J = 5.0 Hz, H-6), 5.43 (1H, m, H-16), 6.47 (1H, s, H-18). ^{1}H NMR (500 MHz, C_5D_5N) data of the sugar moiety: see Table 1. ^{13}C NMR (125 MHz, C_5D_5N): see Table 2.

*2.3.2. Stauntoside M (***2**). White amorphous powder (CH_3OH–$CHCl_3$), $[\alpha]_D^{20}$ + 0.90 (c = 1.00, CH_3OH, 20°C). IR(KBr) ν_{max}: 3482, 2933, 1733, 1652, 1452, 1382, 1308, 1164, 1077, 1006, 872, and 610 cm^{-1}. ESI-MS (positive mode) m/z: 961.6 $[M+Na]^+$. HRESI-MS (positive mode) m/z: 961.4767 $[M+Na]^+$, calcd for $C_{48}H_{74}O_{18}Na$, 961.4776. ^{1}H NMR (500 MHz, C_5D_5N) for aglycone: δ 0.77 (3H, s, H-19), 1.53 (3H, s, H-21), 3.54 (1H, d, J = 8.0 Hz, H-17), 3.82 (1H, m, H-3), 3.94 (1H, m, H_β-15), 4.23 (1H, m, H_α-15), 5.32 (1H, d, J = 5.0 Hz, H-6), 5.43 (1H, dd, J = 8.0, 17.0 Hz, H-16), 6.47 (1H, s, H-18). ^{1}H NMR (500 MHz, C_5D_5N) data of the sugar moiety: see Table 1. ^{13}C NMR (125 MHz, C_5D_5N): see Table 2.

*2.3.3. Stauntoside N (***3**). White amorphous powder (CH_3OH-$CHCl_3$), $[\alpha]_D^{20}$ + 200.7 (c = 1.01, CH_3OH, 20°C). IR(KBr) ν_{max}: 3487, 2937, 1682, 1452, 1381, 1326, 1256, 1187, 1061, 1030, 867, 833, 686, and 492 cm^{-1}. ESI-MS (positive mode) m/z: 525.2 $[M+Na]^+$. HRESI-MS (positive mode) m/z: 525.2465 $[M+Na]^+$, calcd for $C_{28}H_{38}O_8Na$, 525.2459. ^{1}H NMR (600 MHz, C_5D_5N) for aglycone: δ 0.80 (3H, s, H-19), 1.57 (3H, s, H-21), 2.22 (1H, dd, J = 11.4, 5.7 Hz, H-9), 2.78 (1H, d, J = 8.4 Hz, H-17), 3.82 (1H, dd, J = 10.9, 4.5 Hz, H_β-15), 4.03 (1H, d, J = 8.4 Hz, H-18$_a$), 4.07 (1H, d, J = 8.4 Hz, H-18$_b$), 4.28 (1H, br d, J = 10.9 Hz, H_α-15), 4.61 (1H, m, H-3), 4.81 (1H, m, H-16), 5.81 (1H, br s, H-4), 5.90 (1H, d, J = 9.6 Hz, H-6), 6.64 (1H, d, J = 9.6 Hz, H-7). ^{1}H NMR (600 MHz, C_5D_5N) data of the sugar moiety: see Table 1. ^{13}C NMR (150 MHz, C_5D_5N): see Table 2.

Table 1: The ^{1}H NMR chemical shifts of the sugar moieties of compounds **1–3** in C_5D_5N (δ in ppm, J values in Hz).

H	**1**[a]	**2**[a]	**3**[b]
	β-D-the	β-D-the	β-D-the
1′	4.81 d(7.5)	4.82 d(8.0)	4.90 d(7.8)
2′	3.93	3.94	3.99
3′	3.68	3.71	3.67
4′	3.72	3.70	3.67
5′	3.66	4.21	3.77
6′	1.45 d(6.5)	1.46 d(6.0)	1.62 d(6.0)
3′-OCH_3	3.94 s	3.94 s	3.92 s
	β-D-digt	β-D-digt	
1″	5.51 dd(9.5,1.5)	5.52 d(10.0)	
2″	1.68, 2.35	2.01, 2.43	
3″	3.71	4.64	
4″	3.49	3.48 dd(9.5,2.5)	
5″	4.30	4.31	
6″	1.42 d(6.5)	1.42 d(6.0)*	
3″-OCH_3			
	β-D-cym	β-D-cym	
1‴	5.11 dd(10.0,1.5)	5.13 d(9.5)	
2‴	2.00, 2.42	1.67, 2.40	
3‴	4.64	3.92	
4‴	3.47	3.39 dd(9.5,2.5)	
5‴	4.11	3.67	
6‴	1.46 d(6.0)	1.30 d(6.5)	
3‴-OCH_3	3.44 s	3.52 s	
		α-L-cym	
1⁗		5.19 d(3.5)	
2⁗		2.07, 2.38	
3⁗		3.85	
4⁗		4.06	
5⁗		4.31	
6⁗		1.56 d(6.5)*	
3⁗-OCH_3		3.31 s	

*Not differentiated.
[a]500 MHz; [b]600 MHz.
the: thevetopyranosyl; digit: digitoxopyranosyl; cym: cymaropyranosyl.

2.4. Acid Hydrolysis of Reference Compounds and Compounds **1–3**. Each solution of 6 mg of reference compounds, glaucogenin C 3-*O*-α-L-cymaropyranosyl-(1 $\rightarrow$ 4)-β-D-digitoxopyranosyl-(1 $\rightarrow$ 4)-β-D-canaropyranoside, cynatratoside B, and glaucogenin C mono-D-thevetoside, and the new compounds **1–3**, was refluxed within 10% HCl (3 mL) at 75°C for 2.5 h. After cooling, the reaction mixture was extracted thoroughly with $CHCl_3$, the $CHCl_3$ layer was washed with water, and then the water fraction was combined with the original aqueous layer. The aqueous layer was evaporated under vacuum, then rediluted with water and reevaporated in vacuo repeatedly to eliminate the surplus HCl and furnish a final neutral residue. The residue was analyzed by TLC with silica gel G as adsorbents, 10% $H_3PO_4 \cdot 12MoO_3$

Table 2: The ^{13}C and DEPT NMR chemical shifts of compounds **1**–**3** in C_5D_5N.

(a)

C	Aglycon moiety		
	1[a]	**2**[a]	**3**[b]
1	36.5 t	36.5 t	33.6 t
2	30.0 t	30.0 t	27.8 t
3	78.2 d	78.2 d	75.4 d
4	39.1 t	39.0 t	125.2 d
5	140.6 s	140.6 s	144.5 s
6	120.4 d	120.4 d	125.7 d
7	30.0 t	30.0 t	122.6 d
8	40.7 d	40.7 d	108.2 s
9	53.2 d	53.2 d	44.2 d
10	38.7 s	38.7 s	35.6 s
11	23.9 t	23.9 t	20.5 t
12	28.4 t	28.4 t	30.8 t
13	118.5 s	118.5 s	54.9 s
14	175.5 s	175.5 s	155.3 s
15	67.8 t	67.8 t	72.1 t
16	75.5 d	75.5 d	86.2 d
17	56.2 d	56.2 d	62.1 d
18	143.8 d	143.8 d	77.5 t
19	17.8 q	17.8 q	17.7 q
20	114.4 s	114.4 s	118.5 s
21	24.8 q	24.8 q	22.7 q

(b)

C	Sugar moiety		
	1[a]	**2**[a]	**3**[b]
	β-D-the	β-D-the	β-D-the
$1'$	102.3 d	102.3 d	103.5 d
$2'$	74.6 d	74.6 d	75.1 d
$3'$	85.8 d	85.8 d	88.2 d
$4'$	82.9 d	82.9 d	76.0 d
$5'$	71.6 d	71.6 d	72.8 d
$6'$	18.7 q	18.7 q	18.7 q
$3'$-OCH_3	60.5 q	60.5 q	61.0 q
	β-D-digt	β-D-digt	
$1''$	99.0 d	99.0 d	
$2''$	39.0 t	39.1 t	
$3''$	67.7 d	67.7 d	
$4''$	83.2 d	83.2 d	
$5''$	68.8 d	68.8 d	
$6''$	18.5 q	18.5 q*	
	β-D-cym	β-D-cym	
$1'''$	99.8 d	99.6 d	
$2'''$	35.6 t	34.9 t	
$3'''$	78.8 d	77.4 d	
$4'''$	74.1 d	82.3 d	
$5'''$	71.0 d	69.3 d	
$6'''$	18.9 q	18.6 q	
$3'''$-OCH_3	58.0 q	57.3 q	

(b) Continued.

C	Sugar moiety		
	1[a]	**2**[a]	**3**[b]
		α-L-cym	
$1''''$		101.2 d	
$2''''$		30.9 t	
$3''''$		75.8 d	
$4''''$		67.5 d	
$5''''$		67.7 d	
$6''''$		17.8 q*	
$3''''$-OCH_3		55.0 q	

*not differentiated.
[a]125 MHz; [b]150 MHz.
the: thevetopyranosyl; digit: digitoxopyranosyl; cym: cymaropyranosyl.

(phosphomolybdic acid hydrate) in 95% EtOH as detection reagent for spraying, followed by heating the plate to develop the colors, and solvent A, $CHCl_3$–CH_3OH (8-1), and solvent B, EtOAc-acetone (2.5-2) as solvent systems, respectively, for development of sugars. The Rf values of D-digitoxose, D-thevetose, and L-cymarose were determined, by interactive comparison among the three reference compounds, in the order of 0.84, 0.70, and 0.34 over solvent A, and of 0.88, 0.75, and 0.40 over solvent B, respectively.

2.5. Determination of the Absolute Configurations of Monosaccharides. The absolute configurations of D-digitoxose, L-cymarose, and D-thevetose were determined as per the method published by Hara et al. [6]. The monosaccharides obtained on acid hydrolysis, as described above, were dissolved in pyridine and reacted with L-cysteine methyl ester hydrochloride at 60°C for 1 h. Equal volume of acetic anhydride was added and heating was carried out for another 1 h. Acetylated thiazolidine derivatives were injected into GC system. The absolute configurations of the sugars were determined by comparing the retention times with those of acetylated thiazolidine derivatives synthesized from the known sugars obtained through acid hydrolysis of the reference compounds. (Also, the retention times of D-digitoxose, L-cymarose, and D-thevetose were determined by interactive comparison. GC conditions in the test: column, HP-5, 30 m × 0.25 mm, 0.25 μm; detection FID; carrier gas, N_2; injection temperature, 250°C, detection temperature, 280°C, column temperature, 150°C (0 min), 10°C/min to 250°C (20 min). tR D-digitoxose 13.09 min, tR L-cymarose 13.46 min, and tR D-thevetose 16.07 min).

The D-cymarose involved in this paper was not detected by GC method because of the lack of reference sugars, but, from the results of the typical monosaccharides, it can be concluded that the absolute configurations of the monosaccharides composed of the sugar units can be really determined by comparison of their spectroscopic data with those reported in the literature. This determination is also because of the very common kind of D-cymarose in the case of the *Cynanchum* species.

3. Results and Discussion

All three new compounds were obtained as white lamellae or amorphous powder and showed up positive Liebermann-Burchard and Keller-Kiliani reactions, suggesting their glycosidic steroidal category with 2-deoxysugar units existing in their sugar moieties [7].

*3.1. Stauntoside L (***1**). The positive HRESI-MS of **1** gave a pseudomolecular ion peak at *m/z* 817.4002 $[M+Na]^+$, corresponding to the molecular formula $C_{41}H_{62}O_{15}$. The IR spectrum showed the absorption bands for hydroxy (3479 cm^{-1}), carbonylic (1735 cm^{-1}), and olefinic (1652 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** revealed the diagnostic signals of steroidal glycoside, with a 13,14 : 14,15-disecopregnane-type skeleton aglycone being exhibited by two tertiary methyls resonated at δ 0.77 (3H, s, H-19) and 1.53 (3H, s, H-21) and one methyleneoxy group resonated at δ 3.93 (1H, m, H_β-15) and 4.23 (1H, t, J = 7.7 Hz, H_α-15), and with three sugar units being shown by three anomeric proton signals at δ 4.81 (1H, d, J = 7.5 Hz, H-1′), 5.51 (1H, dd, J = 9.5, 1.5 Hz, H-1′′), and 5.11 (1H, dd, J = 10.0, 1.5 Hz, H-1′′′), which correlated to the corresponding anomeric carbon signals at δ_C 102.3 (C-1′), 99.0 (C-1′′), and 99.8 (C-1′′′), respectively, in the HSQC spectrum, and three secondary methyls at δ 1.45 (3H, d, J = 6.5 Hz, H-6′), 1.42 (3H, d, J = 6.5 Hz, H-6′′), and 1.46 (3H, d, J = 6.0 Hz, H-6′′). In addition, two characteristic olefinic proton signals at δ 5.32 (1H, d, J = 5.0 Hz, H-6) and 6.47 (1H, s, H-18) and two methoxyls at δ 3.44 (3H, s) and 3.94 (3H, s) were also determined in the ^{1}H NMR spectrum, the later olefinic signal was obviously deshielded and the two methoxyls were compatible with two methylated deoxypyranoses when examining the ^{13}C and DEPT NMR data which exhibited forty-one carbon signals, with seven methyls, nine methylenes, twenty methines, and five quaternary carbons being categorized (Table 2). With the exception of the ^{13}C and DEPT NMR signals assignable to three monosaccharides, the remaining resonances were very similar to those of glaucogenin C, a known steroidal aglycone isolated previously from *C. atratum* [8]. The main differences were observed for glycosidation shifts at C-2 (−2.3), C-3 (+7.1), and C-4 (−4.0) in aglycone moiety of **1**, so the oligosaccharide chain was determined to link with the C-3 hydroxyl of **1**, which was also confirmed, with the aid of HSQC spectrum for determining the direct carbon-proton linkages, by the long-range ^{1}H–^{13}C correlation of the signal of H-1′ with the signal of C-3 in the HMBC spectrum. After the anomeric protons were identified, the ^{1}H–^{1}H COSY experiment, coupled with the HSQC spectrum, was very effective in determining the spin systems within the sugar moieties because of the handsome differences of the chemical shifts and the relatively large coupling constants theoretically (Figure 1). One β-D-thevetopyranose, one β-D-digitoxopyranose, and one β-D-cymaropyranose in the very three sugar units were further speculated by comparing the ^{1}H and ^{13}C NMR spectroscopic data of **1** with those of stauntoside J [5], which were supported by the splitting patterns and coupling constants of the above-mentioned anomeric proton signals. These conclusions about the absolute configurations of the monosaccharides were confirmed by acid hydrolysis as described above in Acid Hydrolysis of Reference Compounds and Compounds **1–3** and Determination of the Absolute Configurations of Monosaccharides, which not only gave one D-thevetopyranose, one D-digitoxopyranose, and another kind of sugar unit, but also confirmed that the absolute configurations of the monosaccharides determined by comparison of their spectroscopic data with those reported are really consistent with reality. Also, this determination is because of the very common kind of D-cymarose in the case of the *Cynanchum* species. Because of the lack of reference substance, D-cymaropyranose units could not be determined in the GC test. The sugar sequence of **1** was demonstrated by HMBC correlations from δ_H 5.11 (H-1′′′ of β-D-cymaropyranose) to δ_C 83.2 (C-4′′ of β-D-digitoxopyranose), from δ_H 5.51 (H-1′′ of β-D-digitoxopyranose) to δ_C 82.9 (C-4′ of β-D-thevetopyranose), and from δ_H 4.81 (H-1′ of β-D-thevetopyranose) to δ_C 78.2 (C-3) (Figure 2). Thus, compound **1** was established to be glaucogenin C 3-*O*-β-D-cymaropyranosyl-(1 → 4)-β-D-digitoxopyranosyl-(1 → 4)-β-D-thevetopyranoside and was given the trivial name of stauntoside L.

*3.2. Stauntoside M (***2**). The positive HRESI-MS of **2** gave a pseudomolecular ion peak at *m/z* 961.4767 $[M+Na]^+$, corresponding to the molecular formula $C_{48}H_{74}O_{18}$. The IR spectrum showed the absorption bands for hydroxy (3482 cm^{-1}), carbonylic (1733 cm^{-1}), and olefinic (1652 cm^{-1}) groups. A detailed comparison between compounds **2** and **1** indicated that they have the consistent ^{1}H- and ^{13}C-NMR spectroscopic data from their aglycone moieties (see experimental and Table 2), which was confirmed to be glaucogenin C by detailed analysis of 2D NMR spectra (Figure 2. Complete data not shown). With the exception of the aglycone signals, the ^{1}H NMR spectrum of **2** revealed the diagnostic signals of four sugar units by four anomeric proton signals at δ 4.82 (1H, d, J = 8.0 Hz, H-1′), 5.52 (1H, br d, J = 10.0 Hz, H-1′′), 5.13 (1H, br d, J = 9.5 Hz, H-1′′′), and 5.19 (1H, d, J = 3.5 Hz, H-1′′′′), which correlated to the corresponding anomeric carbon signals at δ_C 102.3 (C-1′), 99.0 (C-1′′), 99.6 (C-1′′′), and 101.2 (C-1′′′′), respectively, in the HSQC spectrum, and four secondary methylic signals at δ 1.46 (3H, d, J = 6.0 Hz, H-6′), 1.42 (3H, d, J = 6.0 Hz, H-6′′ or H-6′′′′), 1.30 (3H, d, J = 6.5 Hz, H-6′′′), and 1.56 (3H, d, J = 6.5 Hz, H-6′′′′ or H-6′′). The ^{1}H, ^{1}H-COSY experiment, coupled with the HSQC spectrum, established the spin systems within the sugar moiety (Figure 1). By comparing the ^{1}H- and ^{13}C-NMR spectroscopic data of **2** with those of **1** and stauntoside H [5], the structures of the four sugar units were suggested, that is, one β-D-thevetopyranose, one β-D-digitoxopyranose, one β-D-cymaropyranose, and one α-L-cymaropyranose, which were further supported by the splitting patterns of the above-mentioned anomeric proton signals. Compound **2** was subjected to acid

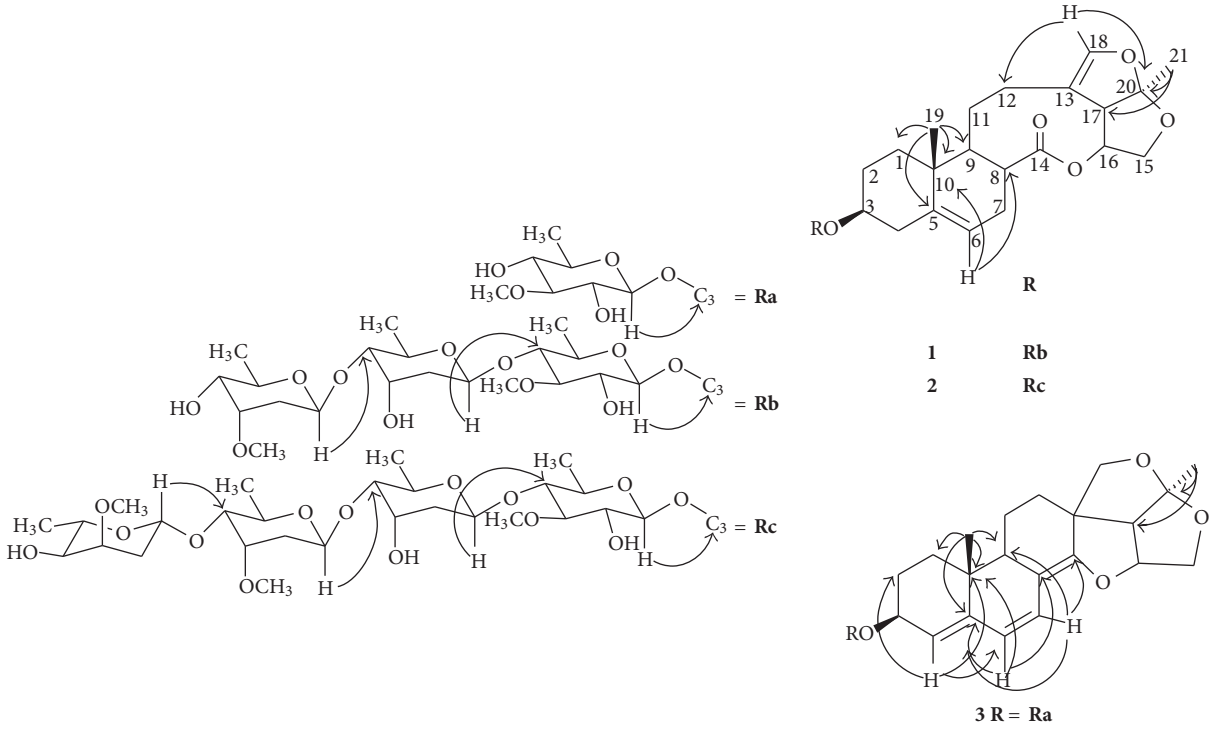

Figure 2: Principal HMBC correlations of the new compounds (**1–3**). (→ : HMBC).

hydrolysis and GC analysis as described above in Acid Hydrolysis of Reference Compounds and Compounds **1–3** and Determination of the Absolute Configurations of Monosaccharides, which gave D-thevetopyranose, D-digitoxopyranose and L-cymaropyranose, and another kind of sugar unit. Because of the lack of reference substance, D-cymaropyranose unit could not be determined in the GC test. The linkages of the four sugars were ascertained by the HMBC spectrum, which showed long-range ^{1}H–^{13}C correlations from δ_H 5.19 (H-1$''''$ of α-L-cymaropyranose) to δ_C 82.3 (C-4$'''$ of β-D-cymaropyranose), from δ_H 5.13 (H-1$'''$ of β-D-cymaropyranose) to δ_C 83.2 (C-4$''$ of β-D-digitoxopyranose), from δ_H 5.52 (H-1$''$ of β-D-digitoxopyranose) to δ_C 82.9 (C-4$'$ of β-D-thevetopyranose), and from δ_H 4.82 (H-1$'$ of β-D-thevetopyranose) to δ_C 78.2 (C-3) (Figure 2). Hence, the structure of compound **2** was elucidated to be glaucogenin C 3-*O*-α-L-cymaropyranosoyl-(1 → 4)-β-D-cymaropyranosoyl-(1 → 4)-β-D-digitoxopyranosoyl-(1 → 4)-β-D-thevetopyranoside and was given the trivial name of stauntoside M.

*3.3. Stauntoside N (***3**). Compound **3** was determined to possess the molecular formula $C_{28}H_{38}O_8$ by its pseudomolecular ion peak at m/z 525.2465 $[M + Na]^+$ in the positive HRESI-MS experiment. The IR spectrum showed the absorption bands for hydroxy (3487 cm^{-1}) and olefinic (1682 cm^{-1}) groups. The ^{1}H NMR spectroscopic data of **3** (see experimental and Table 1) revealed the diagnostic signals of steroidal glycoside, with an aglycone of 14,15-secopregnane-type skeleton being exhibited by two tertiary methyls resonated at δ 0.80 (3H, s, H-19) and 1.57 (3H, s, H-21), two methineoxy groups at δ 4.61 (1H, m, H-3) and 4.81 (1H, m, H-16), and two methyleneoxy groups at δ 3.82 (1H, dd, J = 10.9, 4.5 Hz, H_β-15) and 4.28 (1H, br d, J = 10.9 Hz, H_α-15), and at δ 4.03 (1H, d, J = 8.4 Hz, H-18$_a$) and 4.07 (1H, d, J = 8.4 Hz, H-18$_b$), and with one sugar unit being shown by one anomeric proton signal at δ 4.90 (1H, d, J = 7.8 Hz, H-1$'$), which correlated to the anomeric carbon signal at δ_C 103.5 (C-1$'$) in the HSQC spectrum, and one secondary methyl at δ 1.62 (3H, d, J = 6.0 Hz, H-6$'$). In addition, three characteristic olefinic proton signals at δ 6.64 (1H, d, J = 9.6 Hz, H-7), 5.90 (1H, d, J = 9.6 Hz, H-6), and 5.81 (1H, s, H-4) and one methoxyl at δ 3.92 (3H, s) were also determined. The ^{13}C and DEPT NMR spectra exhibited twenty-eight carbon signals, with four methyls, six methylenes, twelve methines, and six quaternary carbons being categorized (Table 2). Comparison of ^{1}H and ^{13}C NMR spectroscopic data of **3** with those of stauntoside C [5], as well as the information obtained from HSQC experiments, demonstrated that most signals of **3** were superimposable to its counterparts in stauntoside C, except for the sugar unit. On acid hydrolysis, **3** afforded thevetose. The absolute configuration of thevetose was determined to be D-type through GC analysis as described above in *2.4* and *2.5*. Coupled with the coupling constant of the anomeric proton, the sugar unit was solidly determined to be β-D-thevetopyranose. Furthermore, by comparing with anhydrohirundigenin monothevetoside [1] and glaucogenin-C β-D-thevetopyranoside [9], the HMBC experiment confirmed the connectivities in compound **3** which showed the significant long-range ^{1}H–^{13}C correlations from δ_H 4.90 (H-1$'$) to δ_C

75.4 (C-3), and from δ_H 4.61 (H-3) to δ_C 103.5(C-1′) (Figure 2). Therefore, compound **3** was elucidated as deoxyamplexicogenin A 3-*O*-β-D-thevetopyranoside and was given the trivial name of stauntoside N.

4. Conclusions

In recent years, only several papers have described phytochemical investigations of *C. stauntonii* and led to a small amount of steroidal glycosides being reported. In the present work, we reported on three new steroidal glycosides, named as stauntosides L, M, and N, from *C. stauntonii*. Here, the structure elucidation, mainly undertaken by means of spectroscopic and chemical evidence, provided unambiguous information about the aglycone skeletons and structures, the position of the glycosidic linkage, and the sequence of the monosaccharides in the sugar moiety. In addition, it should be emphasized that the main and active ingredients of *Cynanchum* species are steroidal glycosides [10]. In conclusion, this study has enriched the information about the compounds of the title plant and further established that *C. stauntonii* is a significant source of steroidal glycosides.

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Review Article

Role of Sex Steroid Hormones in Bacterial-Host Interactions

Elizabeth García-Gómez,[1,2] Bertha González-Pedrajo,[2] and Ignacio Camacho-Arroyo[1]

[1] *Facultad de Química, Departamento de Biología, Universidad Nacional Autónoma de México, Mexico, DF 04510, Mexico*
[2] *Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico, DF 04510, Mexico*

Correspondence should be addressed to Ignacio Camacho-Arroyo; camachoarroyo@gmail.com

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Sex steroid hormones play important physiological roles in reproductive and nonreproductive tissues, including immune cells. These hormones exert their functions by binding to either specific intracellular receptors that act as ligand-dependent transcription factors or membrane receptors that stimulate several signal transduction pathways. The elevated susceptibility of males to bacterial infections can be related to the usually lower immune responses presented in males as compared to females. This dimorphic sex difference is mainly due to the differential modulation of the immune system by sex steroid hormones through the control of proinflammatory and anti-inflammatory cytokines expression, as well as Toll-like receptors (TLRs) expression and antibody production. Besides, sex hormones can also affect the metabolism, growth, or virulence of pathogenic bacteria. In turn, pathogenic, microbiota, and environmental bacteria are able to metabolize and degrade steroid hormones and their related compounds. All these data suggest that sex steroid hormones play a key role in the modulation of bacterial-host interactions.

1. Introduction

Sex steroid hormones such as progesterone, estradiol, and testosterone play a number of important physiological roles including reproduction, differentiation, development, cell proliferation, apoptosis, inflammation, metabolism, homeostasis, and brain function [1]. They are mainly synthesized by gonads, the adrenal gland, and the placenta and are released into the blood stream to act both in peripheral target tissues and the central nervous system [2]. Sex steroid hormones exert their function by binding to either specific intracellular receptors that act as ligand-dependent transcription factors (classical mechanism) or membrane receptors that stimulate several signal transduction pathways (nonclassical mechanism) [1, 3–5].

Interestingly, sex steroid hormones also participate in the communication between microorganisms and mammal hosts. This type of communication is commonly referred to as "interkingdom signaling" and can be used by microbial pathogens to activate their virulence factors and control the course and outcome of infection [6]. Notably, human and animal males, in general, are more susceptible to protozoan, fungal, bacterial, and viral infections than females [7]. This susceptibility could be due to the lower immune responses presented in males than in females, since innate responses, antibody-mediated, and cellular responses are typically lower in males than in females [7–9].

Numerous studies have reported the effects of sex steroid hormones on the dimorphic sex differences in the response to microbial and viral infections. In addition to affecting host immunity, sex hormones alter gene expression and behavior that influence susceptibility and resistance to infection [7]. This paper mainly focuses on the participation of sex hormones in the interaction between pathogenic bacteria and their hosts, their involvement in the host mechanisms used to minimize and eradicate the infection, as well as in the pathways used by bacteria to evade the immune response.

2. Mechanism of Action of Sex Steroid Hormones

Many actions of estradiol, progesterone, and testosterone are mediated by the classical or genomic mechanism of action

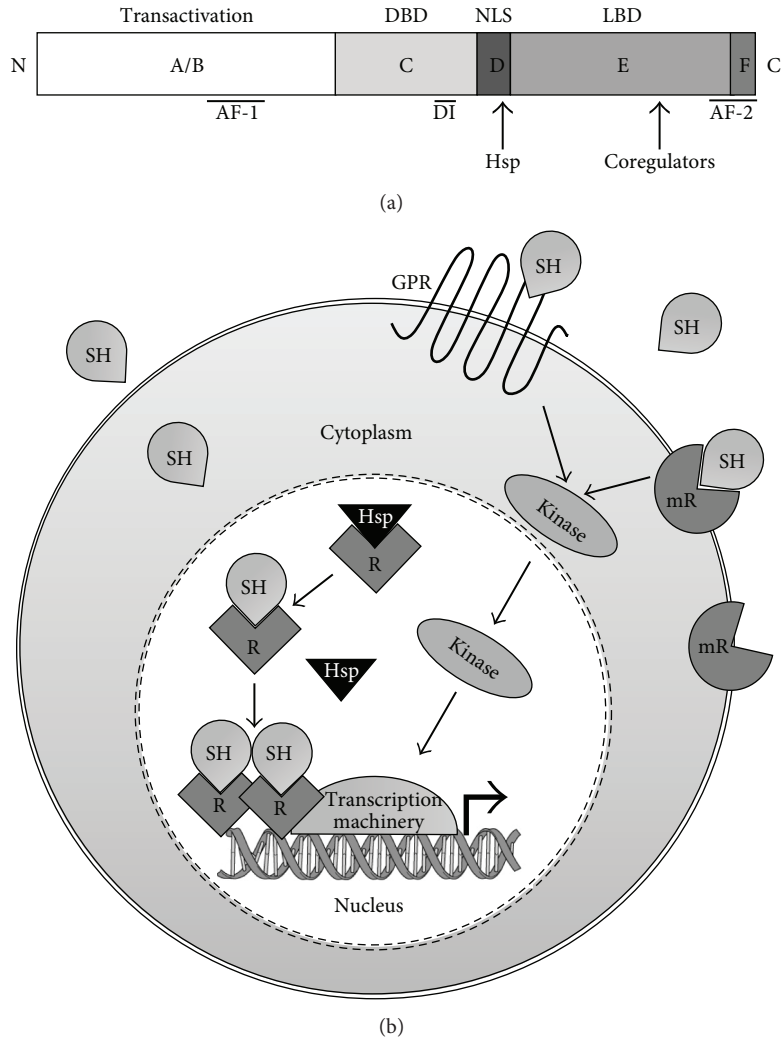

Figure 1: Mechanisms of action of sex steroid hormones. (a) Schematic representation of the main functional domains of sex steroid intracellular receptors. Transactivation domain (A/B) contains a transcriptional activation function (AF1). The C domain contains the DNA-binding domain (DBD) and a dimerization interface (DI). The hinge region (D domain) contains the nuclear localization signal (NLS) and binding sites for chaperones (Hsp). The ligand-binding domain (LBD) is contained in the E domain, which also contains part of AF-2 region and a site for coregulators association. The F domain includes part of AF-2. Domains are not represented to scale, modified from [10]. (b) Classical and nonclassical mechanisms of action of sex steroid hormones. Through the classical mechanism, sex hormones (SHs) exert their function by binding to specific intracellular receptors (R). In the absence of ligand, receptors are associated with heat-shock proteins (Hsps); when the hormone interacts with its specific intracellular receptor, it induces conformational changes that allow the dissociation of Hsp, promoting dimerization, phosphorylation, and receptor binding to hormone response elements located in the promoter region of target genes. Then, receptors act as ligand-dependent transcription factors, recruit coregulators, and associate to the basal transcription machinery. Alternatively, through a nonclassical mechanism, sex hormones bind to membrane receptors (mRs) that in many cases are coupled to G proteins, which stimulate several signal transduction pathways, for example, through kinase activation, modified from [11].

that involves specific intracellular receptors, ER, PR, and AR, respectively, which are members of the nuclear receptor superfamily of ligand-dependent transcription factors [11, 12]. Two PR isoforms have been reported in humans, which are encoded by the same gene but regulated by distinct promoters. These isoforms are PR-B of 114 kDa and an N-terminal truncated form, PR-A of 94 kDa [13]. There also exist two subtypes of ER, ER-α of 66 kDa and ER-β of 55 kDa, which are transcribed from different genes [14]. Similarly, there are two isoforms of AR encoded by a single gene, AR-A and AR-B; the latter has a molecular mass of 110 kDa, while the former has a molecular mass of 87 kDa and lacks the first 187 amino acids of the N-terminal region of AR-B [15].

Sex steroid receptors are modular proteins with distinct functional domains (Figure 1(a)). The N-terminal region contains the A/B domain that has the transcriptional activation function (AF)-1. The middle region (C domain) contains the DNA-binding domain (DBD) that is the highest conserved and the dimerization region. The C domain is followed by a hinge region (D-domain) that contains a

nuclear localization signal (NLS) and the binding sites for chaperone proteins that maintain receptors in an inactive state. The E domain contains the ligand-binding region (LBD), a second AF domain (AF-2) as well as a region for coregulators association. Finally, the F domain is located at the extreme C-terminal region and contains part of the AF-2 domain [10, 11, 16] (Figure 1(a)).

According to the classical model of steroid receptors action, in the absence of ligand, nuclear receptors are associated with the heat-shock proteins Hsp70 and Hsp90. When the hormone interacts with its specific intracellular receptor, it induces conformational changes that allow dissociation of Hsp70 and Hsp90 promoting dimerization, phosphorylation, and high affinity binding to hormone response elements (HREs) located in the promoter region of target genes. Then, receptors modulate transcription by recruiting components of the basal transcriptional machinery. Sex hormone receptors also mediate transcriptional activity by recruiting a group of coactivator and adapter proteins, which function as acetyl transferases, ligases, ATPases, methylases, cell cycle regulators, RNA helicases, and docking proteins to bridge to basal transcription factors. In addition to coactivators, several corepressors have been characterized that activate a family of histone deacetylases, which activity results in failure to recruit the basal transcription machinery and inhibition of gene expression [5, 11] (Figure 1(b)).

Besides the classical mechanism of action, sex steroids can act in the cells through the nonclassical or nongenomic mechanism of action, in most cases mediated by membrane receptors. Thus, membrane progesterone receptors (mPRs) have been identified. Progesterone induces rapid responses in target cells such as spermatozoids, neurons, myometrial cells and immune cells through interaction with its mPRs, and mediates signaling via G-protein-coupled pathways [17]. Estradiol can associate with the transmembrane G-protein-coupled estrogen receptor-1 (GPR30) activating the trimeric G-protein. GPR30 plays an important role in the cardiovascular and immunological systems [18, 19]. G-protein-coupled receptors for androgens have also been identified in several cell types, including breast and prostate tumor cells, vascular and immune cells [20] (Figure 1(b)).

The signaling pathways of the nongenomic actions of sex steroids involve ion channels, enzyme-linked receptors, cyclic AMP and cyclic GMP production, mitogen-activated protein kinases (MAPKs), tyrosine kinases, and lipid kinases cascades (Figure 1(b)) [21–24]. Thus, progesterone modifies calcium influx in spermatozoa by opening membrane Ca^{+2} channels and activating the Src/$p21^{ras}$/ERK kinase pathway. Besides, progesterone can activate MAPK pathway in different cell types [21, 25, 26]. Testosterone can depolarize Sertoli cells and cause calcium influx through inhibition of K^{+}ATP channels; this hormone can also activate MAPK cascades through activation of the kinases Ras, Raf, MEK (mitogen-activated protein kinase/ERK kinase), and ERK (extracellular-signal-regulated kinase) [27]. In the case of estradiol, it can interact with GPRs in vascular cells, which activate the Src kinase that phosphorylates the epidermal growth factor receptor (EGFR) and releases metalloproteases, which trigger the release of EGF ligand from heparin. Then, EGF binds to EGFR, activating the Ras/Raf/MEK/ERK kinase system [11].

3. Modulation of Immune Responses by Sex Steroid Hormones

Sex steroid hormones markedly regulate the activity of immune cells, including lymphocytes, macrophages, granulocytes, and mast cells. The modulation of the immune system by sex steroids has both physiological and pathological implications [8, 9].

Androgen receptors have been identified in various lymphoid tissues, including the thymus and bone marrow, as well as in the spleen and in macrophages [8]. It has been reported that testosterone reduces natural killer (NK) cell activity in mice [28] and the synthesis of proinflammatory cytokines, including the tumor necrosis factor-alpha (TNFα) through the inhibition of transcriptional factors such as the nuclear factor kappa B (NFκB) [29], whereas this hormone increases the synthesis of anti-inflammatory cytokines such as interleukin 10 (IL-10) [30]. Testosterone also decreases the expression of macrophage and monocyte Toll-like receptor 4 (TLR4), which is grouped in a family of pattern recognition receptors (PRRs) and is involved in the activation of the innate immune system in response to pathogen challenge [31].

On the other hand, estrogens can enhance cell-mediated and humoral immune responses. ERs are expressed in various lymphoid tissue cells as well as in circulating lymphocytes and macrophages [8]. Estradiol contributes to resistance against infections by enhancing NK cell cytotoxicity and stimulating the synthesis of proinflammatory cytokines such as IL-1, IL-6, and TNFα [32, 33]. Estradiol also inhibits the production of IL-4, IL-10, transforming growth factor beta (TGF-β) and interferon gamma (IFN-γ) [34, 35]. Additionally, estrogens may protect immune cells against apoptosis [36].

PRs have been identified in epithelial cells, mast cells, granulocytes, macrophages, and lymphocytes [8]. Progesterone is typically known as an immunosuppressive agent since it inhibits the activation of NFκB and increases the expression of the suppressor of cytokine signaling protein (SOCS1) in macrophages [37]. Progesterone also reduces macrophage and NK cell activity [33, 38, 39] as well as antibody production by B cells [40]. Elevated concentrations of progesterone during pregnancy inhibit the development of Th1 (helper T-cell immune type 1) responses and the production of proinflammatory cytokines such as IFN-γ, while promoting Th2 immune responses, including the synthesis of anti-inflammatory cytokines such as IL-4, IL-5, and IL-10 [41].

4. Effects of Sex Steroid Hormones on Bacterial Infections

Different studies provide evidence that males exhibit greater susceptibility to bacterial challenge than their female counterparts [42]. Experimental models of infection in castrated

animals with or without hormonal substitution have been used to study the role of sex hormones in bacterial infections [43].

An approximation to determine the effects of sex hormones over bacterial infection has been the endotoxin lipopolysaccharide (LPS) administration to experimental animals to reproduce sepsis. Sepsis is driven by the overproduction of cytokines such as TNF-α, IL-1β, and IL-6 by macrophages, which detect bacteria and endotoxins via TLRs [44]. Circulating levels of these cytokines are higher in sepsis male patients and mice than their female equivalents, while levels of IL-10 are higher in female than in male patients or male mice treated with LPS [45, 46]. There is evidence that estradiol administration increases survival by decreasing the oxidative stress along the rat gastrointestinal tract following intraperitoneal LPS challenge [47]. In line with this observation, the removal of endogenous estrogens following ovariectomy increases mortality associated with LPS challenge in rats, and this effect was reverted by estrogens treatment. Besides, androgenized females have a higher rate of mortality following LPS administration [48].

Mycobacterial infections occur more frequently in males than in females. This is the case of *Mycobacterium tuberculosis* that produces a higher number of tuberculosis cases in men in all regions of the world, phenomenon that may involve sex hormones [49]. Male mice infected with *M. marinum* are more susceptible than females to mortality and bacterial colonization of lungs and spleen. When exogenous testosterone is administered, the susceptibility of female mice to infection increases, whereas castration in males attenuates the infection, demonstrating that testosterone is responsible for the increased susceptibility to *M. marinum* infection [50].

It has been demonstrated that estradiol and progesterone alter the gastric mucosal response to early *H. pylori* infection in ovariectomized gerbils, modifying the mucosa turnover. Progesterone-treated gerbils presented less gastritis, and a synthetic progesterone derivative (17-α-hydroxyprogesterone caproate) impairs the viability of *H. pylori* [51].

Another example of predisposition to infections in males is seen during Q fever, a zoonotic infection caused by *Coxiella burnetti*, which is considered a potential biological weapon. Men show symptoms, such as flu-like syndrome, pneumonia, hepatitis, myocarditis, pericarditis, meningitis, or encephalitis, more often than women. When mice were infected with *C. burnetii*, it was observed that bacterial load and granuloma number in spleen were higher in males than in females. Ovariectomized mice showed increased bacterial load in the spleen and liver, whereas the treatment of ovariectomized mice with estradiol reduced it [52].

Sex steroid hormone effects on diseases produced by bacteria depend on the infective species and sex steroid hormone levels. In contrast with the data presented above, there are bacterial infections with major incidence in women and female animal models. For example, in mice infected with *Pseudomonas aeruginosa*, indicators such as weight loss, bacterial load, and inflammatory mediators in the lung were higher in females than in males, suggesting a possible role of estrogens in female predisposition to infection by *P. aeruginosa* [53]. In support of this hypothesis, it has been observed that the administration of estradiol to male mice with pneumonia caused by *P. aeruginosa* leads to more severe inflammation in lung tissue and an increased expression of IL-17 and IL-23 [54].

It has been reported that female propensity to typhoid infection is due to estrogens, since the treatment with estradiol increases female mice susceptibility to an intraperitoneal *Salmonella typhimurium* challenge, whereas the treatment with progesterone increases the resistance to the infection and the survival time, suggesting a differential role of ovarian sex hormones in this infection [55]. Pregnant mice infected with *S. enterica* serovar Typhimurium showed a higher bacterial load in the spleen than nonpregnant mice, which correlates with a diminished splenic recruitment of dendritic cells, neutrophils, and NK cells, a decrease in IL-12 production, and increased levels of IL-6 [56].

Another example is the susceptibility of women to *Listeria monocytogenes* infection during pregnancy when estradiol and progesterone levels are very high [42]. Also during pregnancy, gingivitis and pyogenic granuloma have been related to the increased concentrations of circulating estrogens and progesterone [57]. As it can be observed, there is a clear sexual dimorphism in the susceptibility and progress of bacterial infections in human patients and rodent models of disease that are related with sex steroid hormone actions [42, 58].

Besides its role in the modulation of the immune system, sex steroid hormones have a direct effect over bacterial metabolism, growth, and expression of virulence factors. For instance, during pregnancy, the proportion of certain bacterial species associated with plaque microbiota is altered with a significant increase in the ratio of anaerobic to facultative bacteria [59]. *Prevotella intermedius* (previously *Bacteroides melaninogenicus* subsp. *intermedius* [60]) is found among these anaerobic bacteria, and interestingly, it uptakes estradiol and progesterone, which in turn enhance bacterial growth. Additionally, these sex hormones can act as substitutes for vitamin K, an essential growth factor for *P. intermedius* [59].

It has also been demonstrated that progesterone (32–127 μM) inhibits the growth of *Neisseria gonorrhoeae* and *N. meningitidis*. This effect was either bacteriostatic or bactericidal, depending on progesterone concentration [61]. Interestingly, it has been shown that during infection of primary cervical epithelial cells, the treatment with progesterone (30 nM) increases *N. gonorrhoeae* survival and replication through subversion of the activity of the host serine-threonine kinase Akt by the gonococcal phospholipase D [62]. This opposite effect of progesterone could be due to the different doses of the hormone used in each study.

Studies using mouse, rat, and guinea pig models of genital tract *C. trachomatis* infection suggest that the hormonal status of the genital tract epithelium influences the outcome of the *Chlamydia trachomatis* infection [63]. In an *in vitro* model of infection of HeLa cells with *C. trachomatis*, estradiol preexposition of cells enhances the adherence of chlamydial elementary bodies, as well as the development of bacterial inclusions [64]. Recently, it was demonstrated that the

persistence phenotype, defined as a long-term association between *Chlamydia* and their host cell in which the bacteria remain viable but nonculturable, also occurs in response to high levels of sex hormones, in particular estradiol that regulates the expression of genes related to persistence. For example, estradiol exposure results in the upregulation of the *trpB* gene, a marker for chlamydial persistence. Progesterone administration resulted in a general upregulation of genes that encode elements of carbohydrate and amino acid metabolism pathways [63]. These observations constitute an evidence of the direct influence of sex steroid hormones over expression of factors involved in virulence of a bacterial pathogen and particularly in the development of persistence.

Recently, a strain of *P. aeruginosa* isolated from the lung of a woman with cystic fibrosis showed an increased production of alginate (an extracellular polymer involved in biofilm development) in the presence of estradiol, which correlates with the exacerbation of cystic fibrosis occurring at the end of the follicular phase when levels of estradiol are high [65].

Germination rate of spores of *Clostridium sordellii*, a bacterium that produces hemorrhagic enteritis in several animals as well as infections of the human female genital tract during postpregnancy, is increased in response to progesterone. In contrast, it acts as an inhibitor of germination of spores of *C. difficile*, which is a gut pathogen associated with diarrhea. In this case, progesterone competes with bile salt taurocholate that is recognized as a germinant, probably by binding to the same receptors that recognize taurocholate in *C. difficile* spores. This is an example of how spores of two related species differentially respond to sex steroids [66]. The effects of sex steroid hormones on bacterial infections are summarized in Figure 2.

5. Bacterial Metabolism of Sex Steroid Hormones

Bacteria are capable of metabolizing sex steroid hormones through the activity of distinct enzymes such as hydroxysteroid dehydrogenase (HSD) that regulate the balance between active and inactive steroids. Bioinformatics analyses have identified genes that encode HSDs in distinct bacterial genomes. The dominating phyla that were identified to express these enzymes were Actinobacteria, Proteobacteria, and Firmicutes. A large number of HSD-expressing bacteria constitute the normal gastrointestinal microbiota, while another group of bacteria were isolated from natural habitats like seawater, soil, and marine sediments [67].

In regard to pathogenic bacteria, *Prevotella intermedius* (previously *Bacteroides melaninogenicus* subsp. *intermedius*), a gingival infective agent, uptakes progesterone and estradiol [59], while *Streptococcus mutans and Bacillus cereus* metabolize testosterone and progesterone due to the activity of 5α-steroid reductase 3β-, 17β-, and 20α-HSDs and steroid hydroxylases produced by *B. cereus*, whereas *S. mutans* produces 5α- and 5β-steroid reductases and 3α-, 17β-, and 20α-HSDs [68]. *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* also reduce testosterone to 5α-dihydrotestosterone [69].

Treponema denticola, another gingival bacterium associated with periodontitis, metabolizes cholesterol, progesterone, and testosterone using 5α-reductase, 3β- and 17β-HSD activity [70]. However, only cholesterol induces bacterial growth, whereas high concentrations of progesterone and testosterone inhibit it. The lack of sensitivity of *T. denticola* to low concentrations of progesterone and testosterone (0.0001 μg/mL) may be due to their active removal by an ATP-binding cassette (ABC) efflux transporter [71].

It has been reported that sex steroid hormones are substrates of *E. coli* multidrug efflux (MDE) pumps that are important factors in the resistance against bile acids. Two of these MDE systems, AcrAB-TolC and EmrAB-TolC, can transport estradiol and progesterone outside the bacterial cell. Additionally, when both systems were mutated, a steroid hormone-dependent growth suppression was observed [72]. Likewise, in *N. gonorrhoeae,* it has been demonstrated the participation of an efflux pump (MtrCDE) in the transport of sex hormones, which confers bacterial resistance to progesterone [73]. Efflux-deficient gonococcal mutants were more rapidly cleared from infected intact female mice than from ovariectomized mice and were more sensitive to progesterone *in vitro* [73]. These pumps may be essential for bacterial survival under conditions where steroids are present, such as in the gastrointestinal, vaginal, and urinary tracts [72].

Pathogenic bacteria also have an influence over host sex hormone metabolism. For instance, *S. enterica* infection in a murine model reduces the levels of steroid hormones such as progesterone. The analysis of the transcript levels of genes that encode several enzymes involved in the synthesis of steroid hormones reveals that the expression of some HSDs is reduced [74].

In addition to bacterial pathogens, bacteria from human microbiota play an important role in the metabolism of sex hormones. Microbiota is critical for human health since it has been implicated in the development of immune system, energy homeostasis, and protection against pathogens. Moreover, imbalances in the intestinal microbiota have also been associated with pathological processes [75]. A known cause of intestinal microbiota alteration is the use of antibiotics that can increase the susceptibility to enteric infections [76]. In a recent metabolomics study, it has been determined that the treatment of mice with streptomycin disrupts the intestinal homeostasis, through a reduction in the number of fecal bacteria and consequently by affecting the intestinal metaboloma. 87% of all metabolites detected were diminished, including steroids, suggesting that the intestinal microbiota is involved in steroid metabolism [75].

It has been observed that fecal bacteria can perform hydrolytic, reductive, and oxidative reactions of androgens and estrogens [77]. Enzymes involved in 21-dehydroxylation or 16α-dehydroxylation of steroids such as corticosteroids and sex hormones have been identified in intestinal microbiota, and interestingly, these enzymes are not present in mammalian tissues [78]. Reversible 17β reduction of androgens carried out by human intestinal microorganisms is

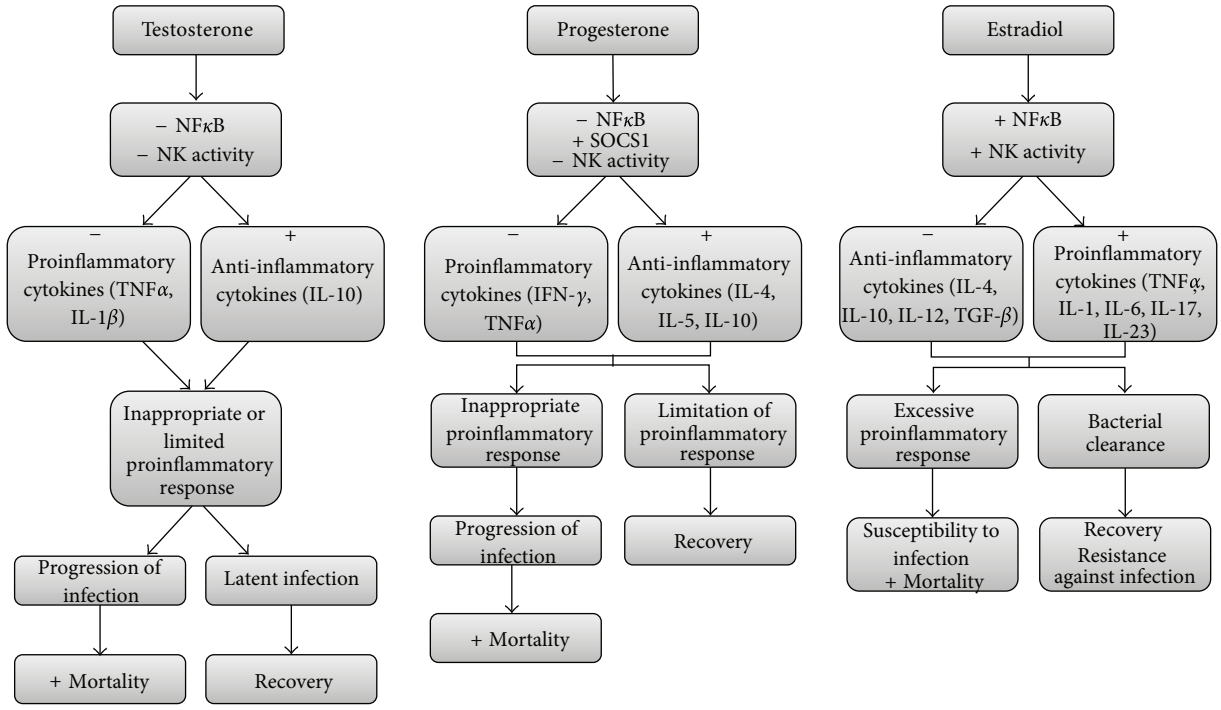

Figure 2: Effects of sex steroid hormones on bacterial infections. In general, male mammals are more susceptible to bacterial infections and its negative outcomes than their female counterparts. This is due to the suppressor effect of testosterone on the immune system, while estradiol acts as an activator of the immune system. Testosterone reduces the NK cell activity and induces the production of anti-inflammatory cytokines such as IL-10, whereas it reduces the production of proinflammatory cytokines such as TNFα through the inhibition of NFκB. This conduces to an inappropriate proinflammatory response that in turn allows the progression of the infection and its negative effects, such as an increase in mortality. In some cases, the limited proinflammatory response leads to a latent infection that can be abated and conduces to recovery. Progesterone acts as a modulator of the immune system due to its suppressing effects by reducing the NK cell activity, inducing the production of IL-4, IL-5 and IL-10 and increasing the expression of SOCS1, while inhibiting the production of IFNγ and TNFα, which avoid the development of bacterial infections, subsequent bacteremia, and sepsis. However, in high levels, for example during pregnancy, progesterone predisposes to some bacterial infections due to reduced proinflammatory responses. On the other hand, estradiol enhances the NK cell activity, and through the activation of NFκB, induces the production TNFα, IL-1, IL-6. IL-17, and IL-23, while inhibiting the production of IL-4, IL-10, IL-12, and TGF-β, and allows the bacterial clearance and recovery from infection. However, estradiol can also produce an excessive proinflammatory response and increased mortality as a consequence of susceptibility to infection and multiple organ failure. +, increase; −, reduction.

suggested to play a role in the regulation of testosterone levels and in the release of androgens in humans [78, 79].

Sex steroid metabolism is not only carried out by pathogenic or microbiota bacteria, but also by environmental bacteria, such as soil-, marine-, and sludge-associated organisms. The most studied example of steroid metabolism and steroid-dependent gene regulation in bacteria is the soil bacterium *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*) [80]. *C. testosteroni* expresses various genes that respond to steroids including receptors such as TeiR, as well as activators (TesR) and repressors (RepA and RepB) of the 3α-HSD/carbonil reductase (CR-) encoding gene, *hsdA*. These proteins participate in the adaptation of the bacteria to the environment [81, 82]. Particularly, 3α-HSD/CR is an enzyme involved in the metabolism of androgens that mediates the oxide reduction of androstenedione, 5α-dihydrotestosterone, and androsterone. Interestingly, the expression of 3α-HSD/CR is highly inducible in the presence of steroid substrates [67, 83].

A testosterone catabolic pathway that differs to that found in *C. testosteroni* has been described in *Steroidobacter denitrificans* [84], a bacterium isolated from sludge that is capable of metabolizing estradiol and testosterone [85]. This bacterium oxidizes testosterone to 1-dehydrotestosterone, which is then transformed into androsta-1,4-diene-3,17-dione that in turn undergoes a reduction reaction occurring at its A ring; probably this reaction is accomplished by an as yet unidentified 3α-HSD [84]. Some seawater bacteria can also degrade steroids, for instance the marine bacterium H5, belonging to the genus *Vibrio*, can degrade testosterone and estrogens. Additionally, two estradiol inducible genes coding a 3-ketosteroid-Δ-1-dehydrogenase and a carboxylesterase were identified [86].

Since natural and synthetic steroid hormones released into the environment are a potential health risk to humans and animals by interfering with sexual development and reproduction, among other functions, steroid-degrading bacterial species may be useful in the bioremediation of

contaminated environments, process also known as bioaugmentation [67]. The latter has been successfully applied in a variety of environments and in degradation of different pollutants such as petroleum hydrocarbon, phenol, and the herbicide atrazine [87].

Estradiol and its primary degradation product estrone have been detected in surface water, groundwater, livestock, and municipal wastes. The majority of bacteria that degrade estradiol such as *Bacillus amyloliquefaciens, B. subtilis,* and *B. cereus* have been isolated from sludge and can convert estradiol into estrone, but they cannot further degrade estrone [88]. Other estradiol-degrading bacteria isolated from activated sludge of a wastewater treatment plant that can be used in bioremediation of polluted environments correspond to genera *Aminobacter*, *Brevundimonas*, *Escherichia*, *Flavobacterium*, *Microbacterium*, *Nocardioides*, *Rhodococcus,* and *Sphingomonas*. Most of these strains cannot further degrade estrone [89].

In *Stenotrophomonas maltophilia*, a bacterium that degrades estradiol, it was determined that estrone is converted into tyrosine through the cleavage of its saturated ring, this amino acid in turn can be utilized in protein biosynthesis; however, the enzyme responsible of this conversion was not identified [90]. *Sphingomonas* strain KC8, whose genome sequence has been recently reported [91], has the capability of degrading different steroids, such as estradiol, estrone, and testosterone [92]. Although the degradation mechanism used by this bacterium has not been identified, its genome contains several genes encoding the enzymes putatively involved in estrogen degradation, such as HSD, 3-ketosteroid-Δ-1-dehydrogenase and catechol 2,3-dioxygenase [91]. Another bacterium of the *Sphingomonadaceae* family, named EDB-LI1, forms biofilms and it also degrades estrone [87].

The identification of key enzymes in biodegradation could help to discover microbial estrogen degradation pathways and suggest biomarkers to monitor estrogen degradation by a microbial community [90], which can be constituted by a mixture of distinct bacteria capable of degrading various classes of steroid hormones and their related compounds.

6. Conclusions

Sex steroid hormones play important roles in diverse functions of mammals, such as the modulation of the immune response. Testosterone, estradiol, and progesterone can differentially regulate responses against bacterial infections and alter metabolic pathways of pathogenic and microbiota bacteria. In general, testosterone acts as an immunosupressor, while estradiol acts as an activator, and progesterone acts as a modulator of the immune system. These effects are related to the sexual dimorphism found in bacterial infections, where men and male animals are in many cases more susceptible to bacterial infections than females. The stage of the menstrual or estrous cycles and pregnancy also determines the outcome of bacterial infections due to the changes in the levels of sex hormones. In some cases, administration of sex hormones may control the course of bacterial infections, functioning as a complement to antibiotic therapy. In turn, bacteria have developed mechanisms to eliminate or to exploit sex hormones in their benefit by using them as carbon and energy sources, principally through their degradation or chemical modification. Interestingly, this feature can be utilized in human benefit by using bacteria capable of degrading and eliminating steroid hormones from polluted environments. The knowledge of the specific enzymes and mechanisms involved in these processes could be helpful in the selection of appropriate bacteria to be used in bioremediation programs.

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