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Research Article

# β-Sitosterol as a Competitive Inhibitor of the Human Type 2 5α- Reductase

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## Abstract

β-sitosterol (SIT) improves symptoms and urinary flow parameters in treating benign prostatic hyperplasia. Normal and abnormal prostate growth is driven by the androgen dihydrotestosterone (DHT) formed from testosterone (T) by the catalytic action of the steroid 5α-reductase type 1/2 (SRD5A1/2). The effect of SIT as an inhibitor of the SRD5A activity in the human prostate has scarcely been studied.

This study aimed to determine the effect of SIT as an inhibitor of the activity of SRD5A2. So, the concentration of SIT was assessed, which blocks 50% of its activity ($IC_{50}$). The $IC_{50}$ value was 130 nM for 5RD5A2.

Additionally, the kinetic parameters of 5RD5A2 activity were calculated from the plots of T concentration versus DHT formed in the presence or absence of SIT. Kinetic parameters indicated a DHT formation rate of 27.54 ± 2.72 ng/mg protein/h both in the absence and the existence of SIT. At the same time, Km values were 8.03 ± 0.67 μM in the absence of the inhibitor, 20.8 ± 5.6 μM in the presence of SIT, 0.7 μM and 46.94 ± 8.8 μM in the presence of SIT, 1.4 μM.

The pharmacological effect of SIT was compared with that of finasteride on the hamster prostate. Results indicated that T-treatment increased the prostate weight of castrated hamsters compared to the vehicle-treated neutered control. The SIT plus T treatment decreased this gland's weight similarly to the T plus finasteride.

***Keywords:*** *β-Sitosterol; Type 2 5α-Reductase; Human Prostate; Finasteride; Competitive Inhibitor*

## Introduction

The Stigmast-5-en-3β-ol (IUPAC name), also known as β-sitosterol (SIT) or 5-cholestene-24β-ethyl-3β-ol is a bioactive phytosterol biosynthesized from squalene in plants. Its structure is like that of cholesterol, with an ethyl group in C-24 (1, Figure 1), and its function is to regulate the permeability and fluidity of plant cell membranes [1]. Moreover, animals can ingest this compound from their diet [2].

β-sitosterol has a long history of pharmaceutical use, given that it has not shown significant side effects [1]. It has also been reported to inhibit the growth and metastasis of the human prostate cancer cell line PC-3 [3]. It has been shown to significantly improve the symptoms and parameters of urinary flow in treating benign prostatic hyperplasia [4,5].

Its activity as an inhibitor of the steroid 5α-reductase type 1/2 (SRD5A 1/2) has also been demonstrated [6]. The SRD5A 1/2 catalyzes the reduction of $\Delta^4$, 3- ketosteroids such as testosterone (T, 2 Figure 1), progesterone (P, 3 Figure 1), and 4-androstenedione, (4-dione, 4, Figure 1) [7,8]. Besides, it is an enzyme that depends on NADPH to reach action [9].

Currently, three SRD5A isotypes are recognized, encoded by different genes: the SRD5A1, SRD5A2, and SRD5A3, with other biochemical properties and tissue localization [10]. The amino acid sequence of these three isotypes has been estimated from complementary DNA sequencing studies [8,11]. Also, their response to the finasteride (FIN, 5 Figure 1) inhibitor is distinct, which at the time, evidenced the presence of two different enzymes with the same catalytic function. SRD5A1 and SRD5A2 have their maximum activity at pH = 6 - 8 and 4.5 - 6.0, respectively. These two isoenzymes also show different kinetic parameters. In optimal activity conditions, SRD5A2 indicates a higher $V_{max}/K_m$ value and greater activity than SRD5A1 [8,12]. T is converted to the more potent reduced natural androgen dihydrotestosterone (DHT, 6 Figure 1), figure 2, due to SRD5A 1/2 (NADP: 4-ene-3-oxosteroid 5α-oxidoreductase EC 1.3.99.5) in the androgen-dependent tissues. The concentration of DHT in androgen-dependent tissues such as the prostate is independent of this metabolite level in the blood [10,13]. DHT has 2 to 5 times more affinity for the androgen (AR) receptor than T [14]. DHT is also essential for fetal sexual differentiation and growth of the prostate gland, male external genitalia, and pubertal growth of facial and body hair [14].

The normal prostate shows a major activity of SRD5A2 than SRD5A1 to produce DHT from T [8,11,12]. Furthermore, SRD5A2 is present at relatively high levels in the genital skin, epididymis, seminal vesicles, and liver [8,11,12]. The non-genital and genital skin, the liver, certain brain regions, epididymis, seminal vesicles, the testis, the adrenal gland, and the kidney express the SRD5A1 [8,11,12].

The prostate cannot produce DHT from the weak androgen dehydroepiandrosterone (DHEA, 7 Figure 1) and other adrenal circulating precursors [14]. Aggressive, hormone-resistant prostate cancer can produce T and DHT intracrine. Higher T concentrations in this tumor are generated than DHT because of the down-regulated expression of SRD5A2 and upregulated expression of FASN, CYP17A1, HSD3B1, HSD17B3, CYP19A1, and UBT2B17 [15]. In normal and hypogonadal men, DHT can induce baldness independently of over a wide range of serum T or DHT concentrations [16]. Furthermore, benign prostatic hypertrophy (BPH) is an androgen-dependent disease in which SRD5A2 plays an important role [17].

Herbal products used as SRD5A inhibitors to treat androgen-dependent diseases such as BPH, prostate cancer, polycystic ovarian syndrome, androgenic alopecia, and acne are increasingly popular due to perceived safety [18]. This work reports the activity of SIT as a competitive inhibitor of SRD5A2 and its ability to reduce prostate weight in castrated hamsters treated with T. The idea is to contribute to the knowledge of the action mechanism of SIT on the formation of DHT.

## Experimental Study

### Chemicals and radioactive materials

The T (1,2,6,7-$^3$H), with a specific activity of 95 Ci/mmol, was supplied by Perkin Elmer Life and Analytical Science (Boston, MA). Radioinert T, 5α-dihydrotestosterone (DHT), and 5-cholesten-24β-ethyl-3β-ol (SIT) were purchased from Steraloids (Wilton, NH, USA). The SIT was recrystallized until the melting point reached 136 - 137°C [Sen A., *et al*. 2012].

Sigma Chemical Co. (St. Louis, Missouri) provided NADPH. Finasteride (N-(1,1- di-methoxymethyl)-3-oxo-4-aza-5α-androst-1-eno-17β-carboxamide), an aza-steroid sold in Mexico as Proscar® (a treatment for benign prostate hypertrophy) or as Propecia® (a treatment for

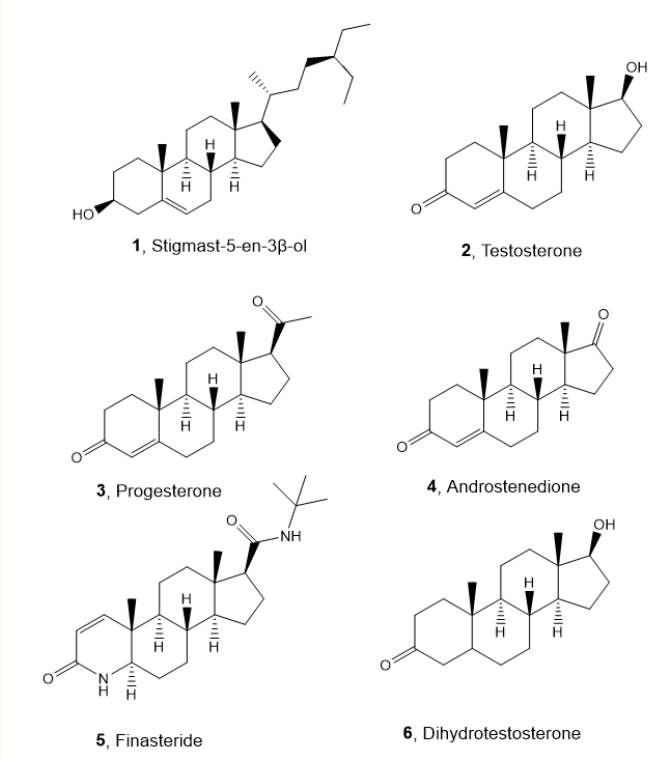


***Figure 1:*** *Structures of different steroids.*

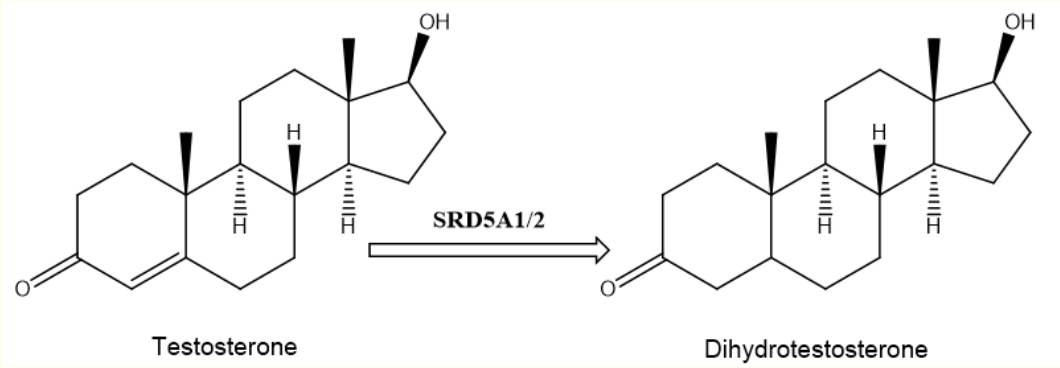


***Figure 2:*** *Testosterone converts to dihydrotestosterone in the androgen-dependent tissues because of the catalytic function of the 5α-reductase enzyme.*

male pattern hair loss) (Merck, Sharp, and Dohme), was used as a positive control in these experiments. The FIN was obtained from Proscar® by extraction. The tablets were crushed and extracted with chloroform, the solvent removed *in vacuo*, and the residue filtered by silica gel column chromatography. The melting point of the isolated FIN (252 - 254°C) was identical to that reported in the literature [19].

### Human tissue

The prostate of a 53-year-old man who died of kidney failure was removed 4 hours after death at the Pathology Department of the General Hospital of Mexico City. The Ethics Committee of the Hospital approved this Protocol. The prostate was soaked in a 150 M NaCl solution and frozen at −78°C. The prostate was thawed and homogenized in buffer A (20 mM sodium phosphate, pH 6.5, containing 0.32 M sucrose, 0.1 mM of DL-dithiothreitol (DTT), and one mM phenylmethyl-sulfonyl fluoride (PMSF); Sigma- Aldrich (CDMX, Mexico). The homogenate was centrifuged at 1500g for 60 minutes [20], and the supernatant was discarded. The residue was dissolved with buffer A solution in a 1:1 weight/volume proportion, and an aliquot was assayed protein content by the Bradford method. The suspension was maintained at −78°C until further use as a source of 5RD5A2 enzyme activity.

### Animal treatments and prostate recollection

The Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM, Xochimilco) approved the protocols for animal use in these experiments following the regulation contained in NOM-062-ZOO 1999.

Thirty adult male golden hamsters (2.5 months of age; 150 - 200g) were used for the *in vivo* experiments. Hamsters were gonadectomized under isoflurane anesthesia 30 days before the tests started. The hamsters were kept in animal-keeping conditions in a controlled temperature (22°C) room with a 12h alternation of light and dark periods, with food and water *ad libitum*.

Six sham-operated hamsters were also maintained in the same conditions as untreated controls. The gonadectomized hamsters (n = 30) were separated into five groups of six animals per cage and treated with daily subcutaneous injections of 1 mg/kg of T or T plus 2 mg/Kg of FIN [21] or with T plus 3.2 mg/kg of SIT [6]. The treatments were applied for six consecutive days, and later these hamsters were sacrificed with $CO_2$. The prostates of the SHAM and the experimental hamsters were removed and weighed.

### Biological activity of SIT

The biological activity of SIT was determined using the method previously reported for different compounds [9,21-23].

### *In vitro* assays

The quantifications of the experiments were performed under specific optimum conditions and were not affected by losses during extraction and analysis.

### Determination of the 5α-reductase activity

Briefly, we measured the activity of 5RD5A2, following radiolabeled T to DHT conversion. We prepared one set of tubes containing 1 mM dithiothreitol (DTT) mixed in 40 mM sodium phosphate buffer at pH 6.5 [23] and two mM NADPH in a final volume of 1 mL.

The reaction, performed in quadruplicate, started when the enzyme fraction was added to this mixture. 50 µg/mL of the human prostate membrane fraction was added to the assay. After incubation at 37°C, we stopped the reaction by adding dichloromethane (1 mL) and mixing. In addition, we similarly prepared a control for each assay without the enzyme fraction. This assay was repeated on three independent occasions.

The samples were extracted (5×) with dichloromethane, and the solvent collected in one tube was dried. The extract was redissolved in 50 µL of chloroform/methanol = 1:1 and applied to a thin layer chromatography plate, HPTLC Keiselgel 60 F254 (Merck, CDMX) using

the auto spotter (Analtech, Newark, Delaware). DHT and T-standards were used on each side of the plate in two lanes. The HPTLCs were eluted thrice in a mixture of chloroform/acetone = 9:1 [24,25] and air-dried. Radioactivity was scanned using an AR-2000 Bioscanner (Bioscan, Washington DC). The T standard was identified by fluorescence (UV lamp; λ = 254 nm, UVP, Upland, CA), and the DHT standard was visualized using a phosphomolybdic acid reagent.

The radioactive zones that showed identical chromatographic behavior (Rf value) to the T or DHT standards were identified. Finally, the yield of tritiated T transformed into DHT was calculated from the plot displayed by the Bioscanner, considering the total radioactivity in the lane. The SRD5A2 action was calculated, considering the radioactivity recovery in control and specific activity of [$^3$H] T.

The retention factor (Rf) of standard T was 0.56. The radioactive zone corresponding to this Rf value displayed 70% of the total radioactivity on the plate. At the same time, the Rf for the DHT standard was 0.67, and the radioactive zone corresponding to this value was 20 - 27% of the total radioactivity on the plate. This result was similar in the presence or absence of dimethyl sulfoxide in the assay.

The results of the conversion of T to DHT represented 100% of the activity of the SRD5A2. These values were used to evaluate the inhibition of the action of this enzyme caused by SIT. The unmodified [$^3$H]T (Rf 0.56) was identified from the control plate (without tissue) because the radioactive spike exhibited a chromatographic behavior similar to that of the T standard.

### Determination of the concentration of SIT inhibiting the activity of isoenzyme 5RD5A2 by 50%

T to DHT conversion was performed under the conditions described in section determination of the 5α-reductase activity for 5RD5A2. The reaction was conducted in the presence of different concentrations of SIT ($1 \times 10^{-10}$ to $10^{-4}$ M dissolved in 50 µL of dimethylsulfoxide). The reaction started when adding 50 µg/ mL of the prostate membrane fraction. The tests were prepared in quadruplicate on three different occasions.

The procedures for extracting, purifying, and identifying DHT were the same as in section determination of the 5α-reductase activity. T conversion to DHT in the presence or absence of SIT was calculated as described in section determination of the 5α-reductase activity. The $IC_{50}$ values for SRD5A were determined from plots of –Log M [SIT] vs. the amount of DHT converted. Data were graphed using SigmaPlot 12 software inhibition curves (Systat Software, INC., San Jose, CA).

### Determination of Ki values

The inhibition constant of SIT on the activity of SRD5A2 was determined at two fixed different T-concentrations [26] and in the presence of different concentrations of SIT ($1 \times 10^{-10}$ to $10^{-4}$ M dissolved in 50 µL of dimethylsulfoxide) as we detailed in determination of the concentration of SIT inhibiting the activity of isoenzyme 5RD5A2 by 50% section. Figure 4 displays the results, and the Ki value was selected from this figure's plot.

### Determination of kinetic parameters of the activity of 5RD5A2 in the absence or presence of SIT

Kinetic parameters of 5RD5A2 were determined by incubating different concentrations of labeled T, unlabeled T, and in the presence or absence of SIT, as shown in table 1. The reaction started when 50 µg/mL of the prostate membrane protein was added to the assay, as detailed in section determination of the 5α-reductase activity for 5RD5A2. Assays were performed in quadruplicate on three different occasions. The reaction conditions and procedures for extracting, purifying, and identifying formed DHT were identical, as described in section determination of the 5α-reductase activity. T conversion in the presence or absence of SIT was calculated as described in section determination of the 5α-reductase activity.

| Steroid | Concentration (µM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Labeled T | 2X10-3 | 2X10-3 | 2X10-3 | 2X10-3 | 2X10-3 | 2X10-3 | 2X10-3 | 2X10-3 |
| Unlabeled T | 0.5 | 0.7 | 1.25 | 1.69 | 3.8 | 6.30 | 14 | 27 |
| SIT | - | - | - | - | - | - | - | - |
| SIT | 0.7 | | 0.7 | | | 0.7 | 0.7 | 0.7 |
| SIT | 1.4 | | 1.4 | | | 1.4 | 1.4 | 1.4 |

***Table 1:*** *Different steroid concentrations were added to the assays to determine the kinetic parameters of the SRD5A2 in the presence or absence of SIT.*

## Results and Discussion

### β-sitosterol as an inhibitor of the activity of the SRD5A2 isoenzymes

Increasing concentrations of SIT decreased the action of SRD5A2 obtained from the human prostate (Figure 3). The $IC_{50}$ value for SIT was calculated from the graph shown in figure 3, and the result was 130 nM.

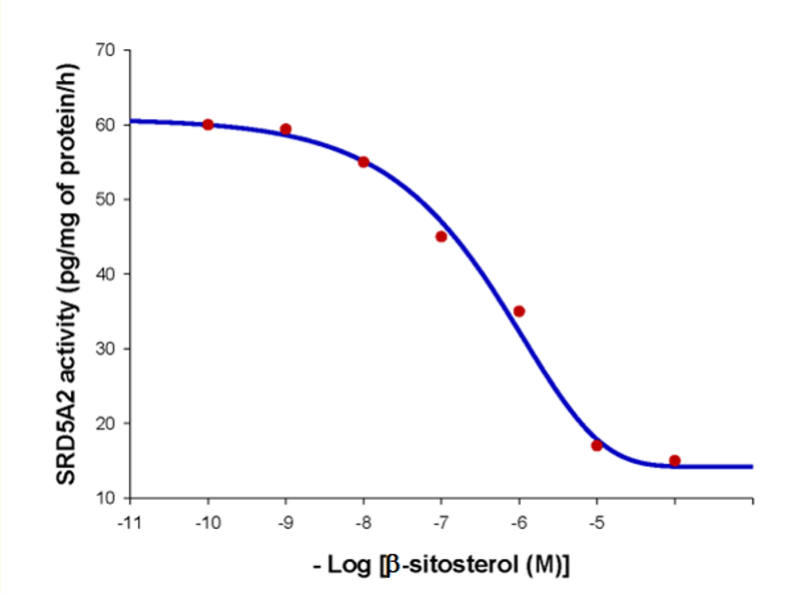


***Figure 3:*** *Graphic determination of the IC50 values of SIT to inhibit SRD5A2 activity The SIT-increasing concentrations decreased the action of SRD5A2 obtained from the human prostate to produce DHT. Assays were performed in quadruplicate on three separate occasions, as detailed in the methodology.*

The Ki value for SRD5A2 was 4.7 µM of SIT (Figure 4). The SIT displayed an affinity for SRD5A2 obtained from the human prostate.

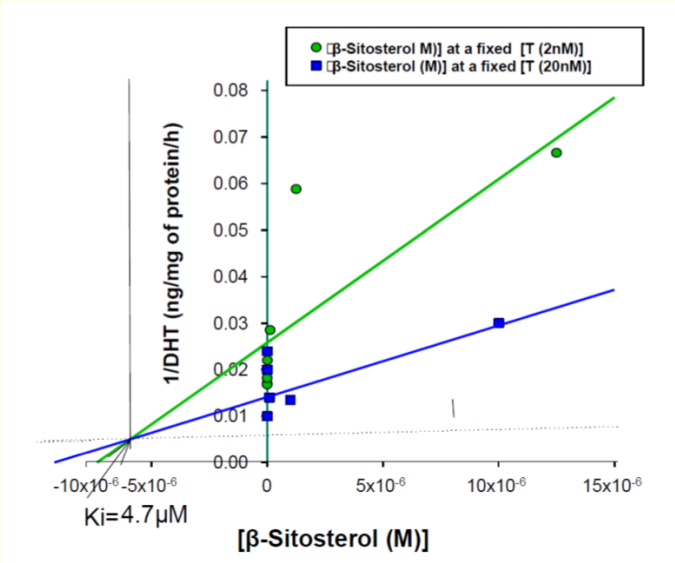


***Figure 4:*** *Ki-determination at two different testosterone (T)-concentrations. Plots were fit using the sigma plot processor.*

Figure 5 shows that SIT is a competitive inhibitor [27] of SRD5A2 activity present in the human prostate. The graphical analysis indicated a DHT formation of 27.54 ± 2.72 ng/mg protein/h both in the absence and the presence of SIT. At the same time, Km values were 8.03 ± 0.67 μM in the absence of the inhibitor, 20.8 ± 5.6 μM in the presence of SIT, 0.7 μM and 46.94 ± 8.8 μM in the presence of an SIT concentration of 1.4 μM.

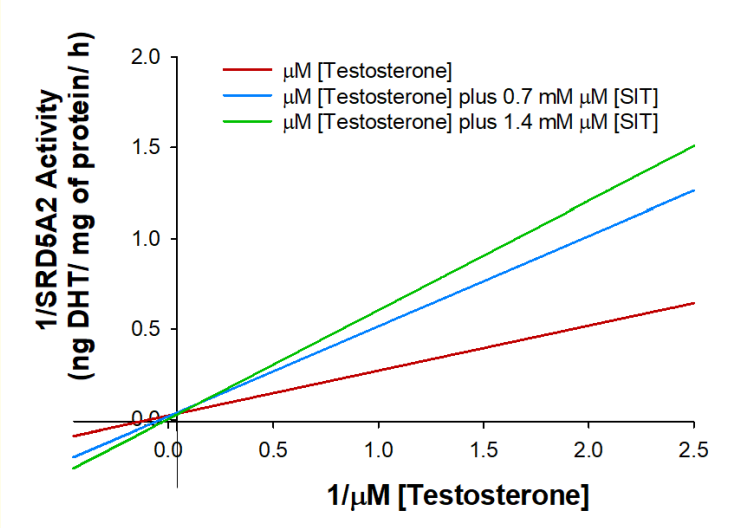


***Figure 5:*** *Competitive inhibition of SIT (at two different concentrations) on SRD5A2 activity obtained from human prostate. Data were obtained in triplicate, as detailed in the methodology.*

## Pharmacological experiments

Figure 6 shows that after the gonadectomy, the weight of the prostate decreased significantly compared to the weight of this gland in the SHAM group. The vehicle treatment of the castrated hamsters produced no change in the prostate heaviness. However, the treatment with 200 µg of T for six days significantly increased the weight of this gland compared with the castrated vehicle-treated. The T plus FIN mixture significantly decreased prostate weight compared to the T treatment. Similarly, the T plus SIT treatment significantly reduced it compared with the T treatment.

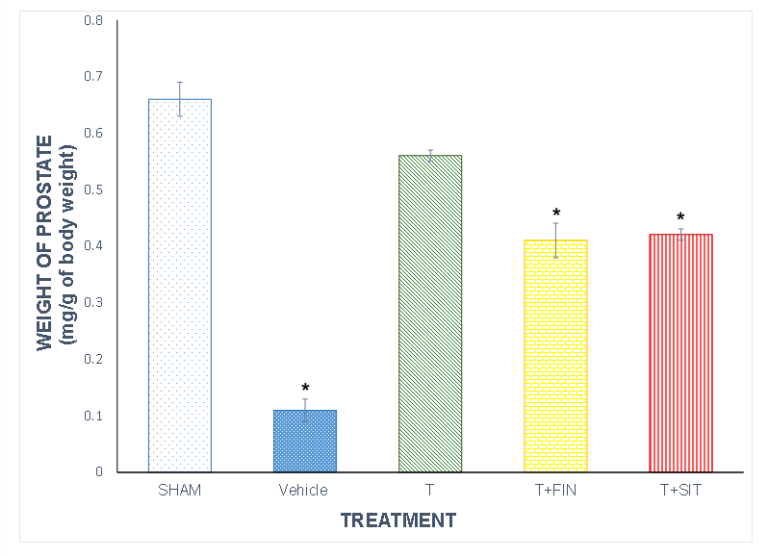


***Figure 6:*** *Effect of different treatments on the prostate weight of castrated and SHAM hamsters treated with daily injections for six days. The results are expressed as mg of prostate tissue/g body weight ± standard deviations of n = 6 hamsters of each group. *P < 0.005 vs. gonadectomized treated with T (testosterone). Dunnett's test compared the obtained means. FIN (finasteride), SIT (β-sitosterol).*

FIN is a competitive inhibitor of prostatic SRD5A2 activity, considered irreversible [28], with an $IC_{50}$ value of 8.5 nM [25]. It has a much higher affinity for SRD5A2 than SRD5A1. Its average bioavailability is 63%, from 5 mg tablets, with a maximum plasma concentration of 37 ng/mL, reaching 1 - 2 hours after ingestion of the dose [33].

SIT demonstrated that it is absorbed less efficiently than cholesterol, 4% SIT, and 27% cholesterol; its absorption is slightly higher in women than men [29,30]. Furthermore, the adrenal glands, liver, ovaries, testis, and intestinal epithelia show greater uptake of SIT. The SIT is eliminated very quickly from the body, mainly in the feces and bile, but even after four days of dosing, SIT traces can be found in these materials [29,30]. SIT disponibility is 9% after oral administration, and the half-life is about three hours with a half-distribution life of 129 hours [30].

It is necessary to consider that SIT could cleave at C-17 and form dehydroepiandrosterone in the liver of hamsters. This reaction is feasible because of the presence of liver oxygenases [31]. Dehydroepiandrosterone shows a high affinity for SRD5A2 ($IC_{50}$ = 25 nM) [32] despite being a weak androgen.

## Conclusion

The overall data indicated that SIT decreases the prostate weight similarly to FIN because both display acceptable tissue disponibility and half-life. Furthermore, FIN and SIT are competitive inhibitors of the SRD5A2 activity, and both show a high affinity for SRD5A2. However, the $IC_{50}$ for FIN (8.5 nM) is lower than that of SIT (130 nM). Pharmacokinetic data indicate that treatment with SIT is quite safe. In addition, the pharmacological experiments did not show any toxic effect of this phytosterol.

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This research received no external funding.

## Conflicts of Interest

The authors declare no conflict of interest.

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