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Proposal n°: AD070315C-2

Study n°: AD070315B

EFFECTS OF REVIVOGEN SCALP THERAPY ON TESTOSTERONE METABOLISM IN RECONSTRUCTED HUMAN EPIDERMIS

STUDY REPORT AD070315B-2

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Date: October 18th, 2007

The investigators and the author of this report hereby certify the validity of the data presented and attest their full agreement with the conclusions presented at the end of the report.

Certified by:

Name: Franck JUCHAUX

Position: Study director

Date: October 18th, 2007

Signature

TABLE OF CONTENT

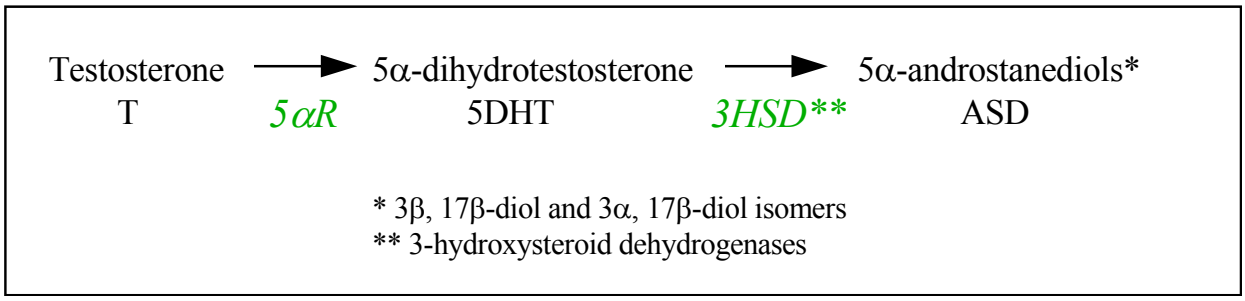
1 - INTRODUCTION

ADVANCED SKIN AND HAIR, INC. has developed the compound **Revivogen Scalp Therapy** for hair loss. Male pattern hair loss (MPHL) is a potentially reversible condition in which dihydrotestosterone is an important etiologic factor (Olsen et al, 2006). Dihydrotestosterone, the efficient steroid, is produced from testosterone by 5α reductase. Inhibition of 5α reductase activity is known to improve MPHL.

Olsen EA, Hordinsky M, Whiting D, Stough D, Hobbs S, Ellis ML, Wilson T, Rittmaster RS, 2006. The importance of dual 5alpha-reductase inhibition in the treatment of male pattern hair loss: results of a randomized placebo-controlled study of dutasteride versus finasteride. J Am Acad Dermatol., 55(6):1014-23

BIO*alternatives* performed this study in order to assess the effects of the test compound on the metabolism of testosterone in reconstructed human epidermis. This model has been shown to be useful for the evaluation of inhibitors of this metabolism *(Bernard et al. 2000; Int. J. Cosm. Sci.,22, 397-407)*.

The steroid 5α-reductase isoenzymes (5αR) transform testosterone (T) into 17β-hydroxy-5α-androstan-3-one (5-dihydrotestosterone, DHT). This reaction is crucial in the action of androgens.



2 - MATERIALS AND METHODS

2.1 Biological model

Reconstructed Human Epidermis (RHE)

- Tissues: 18 RHE (0.50 cm², 10 days), batch n° 01015-31
- Culture: at 37°C and 5 % CO_2
- Culture medium: differentiation medium

2.2 Test compound and references

Test compound	Stock-solution	Dilution	Application
Revivogen Scalp Therapy batch 07-06 (AD070315/2)	Liquid supplied by the study promoter and stored at room temperature.	-	**topical at 50 µl/RHE**

References	Stock-solution	Dilution	Application
Finasteride batch 231664	10^{-2} M in ethanol	In water	**topical at 10^{-5} M/RHE**
Avodart® batch 053721A	10^{-3} M in ethanol	In water	**topical at 10^{-6} and 10^{-5} M/RHE**

2.3 Testosterone

Testosterone: [4-^{14}C] testosterone (Amersham B76, 54 mCi/mmole, 2.35 nmole/epidermis). [4-^{14}C] testosterone stock-solution was dissolved in ethanol and diluted in sterile water (1% ethanol final).

2.4 Treatment

The RHE were topically treated (or not, control) with the test compound or the references. Three RHE were used for each experimental condition.

After **24h of treatment**, the RHE were topically re-treated and incubated for **5 hours**. After incubation, the test compound and references were removed from the top of the RHE and 100 µl of the labelled testosterone solution were loaded on the *stratum corneum* of each RHE (127 nCi/epidermis).

After a 24-hour incubation period, the media underneath the RHE were collected for sterols analysis.

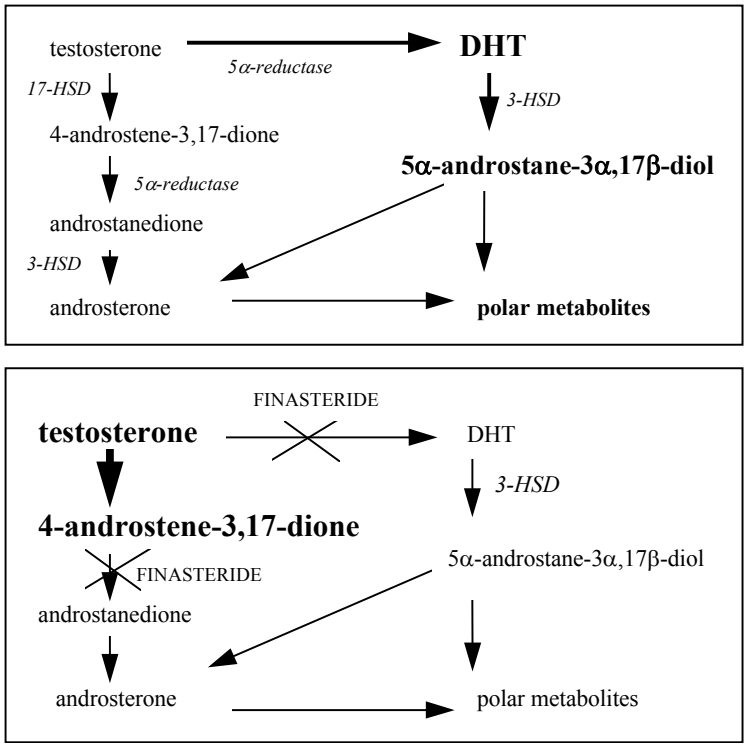
2.5 Extractions and analysis

Transepidermal diffusion assessment: the amount of testosterone that passed through the epidermal tissues was measured by liquid scintillation counting (LKB 1211 Rackbeta counter) of a fraction of culture medium.

Metabolism analysis: the steroid molecules from culture media were extracted by 2 volumes of chloroform/methanol (98:2) and dried. The various molecular species (testosterone metabolites) were separated by thin layer chromatography (**TLC**) on silica plates (RE/Silice, Whatman) in a solvent system containing dichloromethane, ethylacetate and methanol (85:15:3).
The plates were autoradiographed and testosterone metabolites were quantified using a phosphorImager and specific software (Packard instrument).

3 - RESULTS AND CONCLUSION

Testosterone metabolism



Schematic simplified pathway for testosterone metabolism. Effects of finasteride
(from Bernard F-X *et al*., Int. J. Cosmetic Science, **22** 397-407 (2000))

Tables 1 and 2

Untreated control:
After 24h of culture, the rate of testosterone metabolism was very high.
Dihydrotestosterone (DHT) was clearly identified in the steroid profile. DHT was the major metabolite in the control epidermis. After 24h, about 74% of the deposited testosterone was converted into DHT. Other important metabolites were androstane-diols (e) and 4-androstene-3,17-dione (b).

Effects of finasteride:
Finasteride at 10^{-5} M strongly inhibited the transformation of testosterone into DHT (67% inhibition compared to the control). Furthermore, as expected, finasteride decreased the amount of androstane-diols (e) and induced a strong accumulation of 4-androstene-3,17-dione (b) (Figure 1).

Effects of dutasteride:
Dutasteride at 10^{-6} M and 10^{-5} M strongly inhibited dose dependently the transformation of testosterone into DHT (respectively 80% and 86% of inhibition of the DHT production compared to the control). Furthermore, as expected, dutasteride decreased the amount of

androstane-diols (e) and induced a strong accumulation of 4-androstene-3,17-dione (b) (Figure 1).

Effects of Revivogen Scalp Therapy:

Revivogen Scalp Therapy (5 mg/cm²) strongly reduced the production of DHT (90% of inhibition of the DHT production compared to the control).

4 - TABLES AND FIGURES

Table 1: Diffusion of [^{14}C]-testosterone (and metabolites) through RHE.

Trans-epidermal diffusion (24h)

Treatment	Conc.	cpm	sd	n	% control	nmol steroid
Total testosterone	-	***326060***	**-**	*1*	**-**	*2,35*
Untreated control	-	**125307**	14977	3	**100**	0,9
Finasteride	10^{-5} M	**147747**	6335	3	**118**	1,1
Dutasteride	10^{-5} M	**150160**	10809	3	**120**	1,1
	10^{-6} M	**159213**	20653	3	**127**	1,1
Revivogen Scalp Therapy	5 mg/cm²	**30026**	1181	3	**24**	0,2

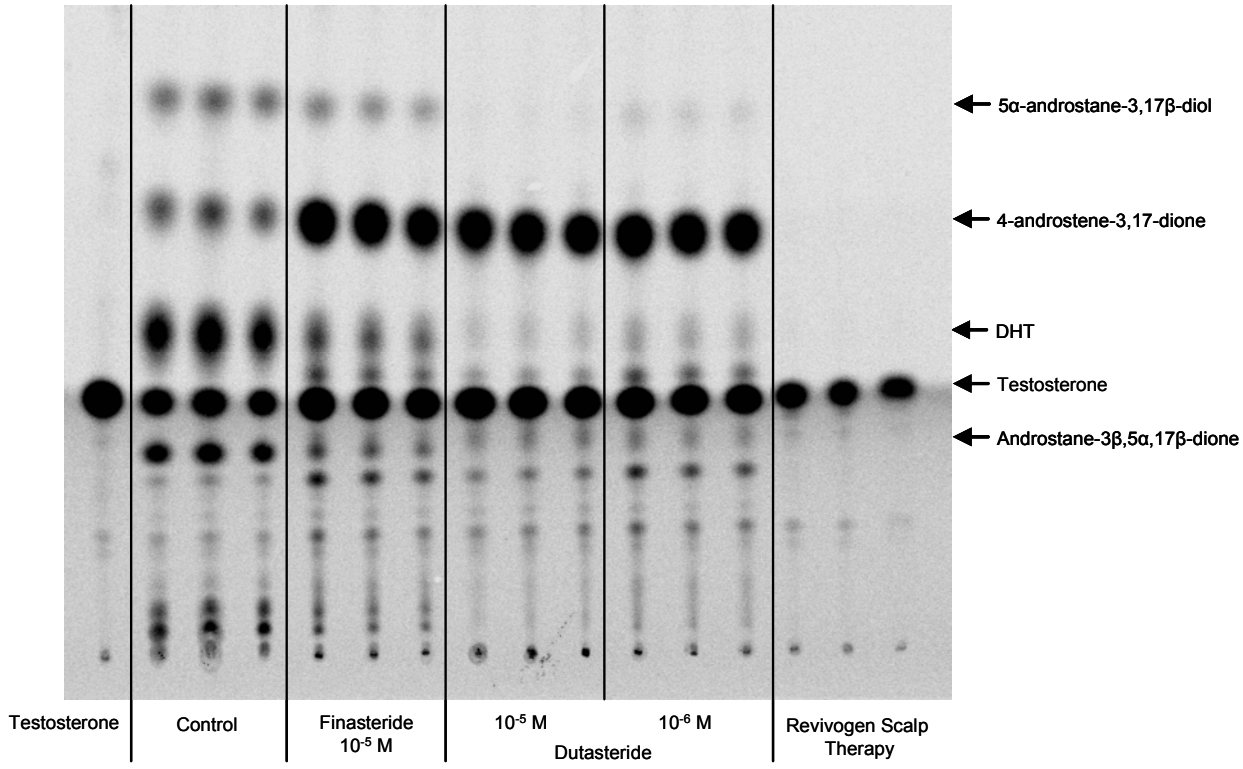


Figure 1: Thin layer chromatography and autoradiography of [^{14}C]-testosterone and metabolites after transepidermal diffusion (24h).

Table 2: Effects of **Revivogen Scalp Therapy** and the reference compounds on the production of testosterone metabolites. Instant Imager analysis of TLC in figure 2 (direct radioactivity measurement).

			Total		**Testosterone**			**DHT**			**DHT/testo**
Treatment	**Conc.**	Lane n°	AU	moyenne	AU	% total	moyenne (%)	AU	% total	moyenne (%)	ratio
Control	-	2	24474	**26091**	5821	23,8	**24,6**	4273	17,5	**17,5**	**0,71**
		3	29037		7177	24,7		5072	17,5		
		4	24763		6293	25,4		4375	17,7		
Finasteride	10^{-5}M	5	35114	**30507**	13282	37,8	**38,4**	3314	9,4	**9,1**	**0,24**
		6	30182		11691	38,7		2744	9,1		
		7	26227		10105	38,5		2325	8,9		
Dutasteride	10^{-5} M	8	24240	**25230**	11298	46,6	**46,6**	1222	5,0	**5,0**	**0,11**
		9	25088		11695	46,6		1282	5,1		
		10	26361		12264	46,5		1298	4,9		
	10^{-6} M	11	29444	**28162**	12800	43,5	**42,7**	1650	5,6	**5,8**	**0,13**
		12	28026		11772	42,0		1649	5,9		
		13	27017		11520	42,6		1567	5,8		
Revivogen Scalp Therapy	5 mg/cm²	17	13155	**12308**	6396	48,6	**46,0**	883	6,7	**7,1**	**0,15**
		18	12350		5715	46,3		877	7,1		
		19	11420		4907	43,0		858	7,5		

AU: Arbitrary Unit for radioactivity quantification

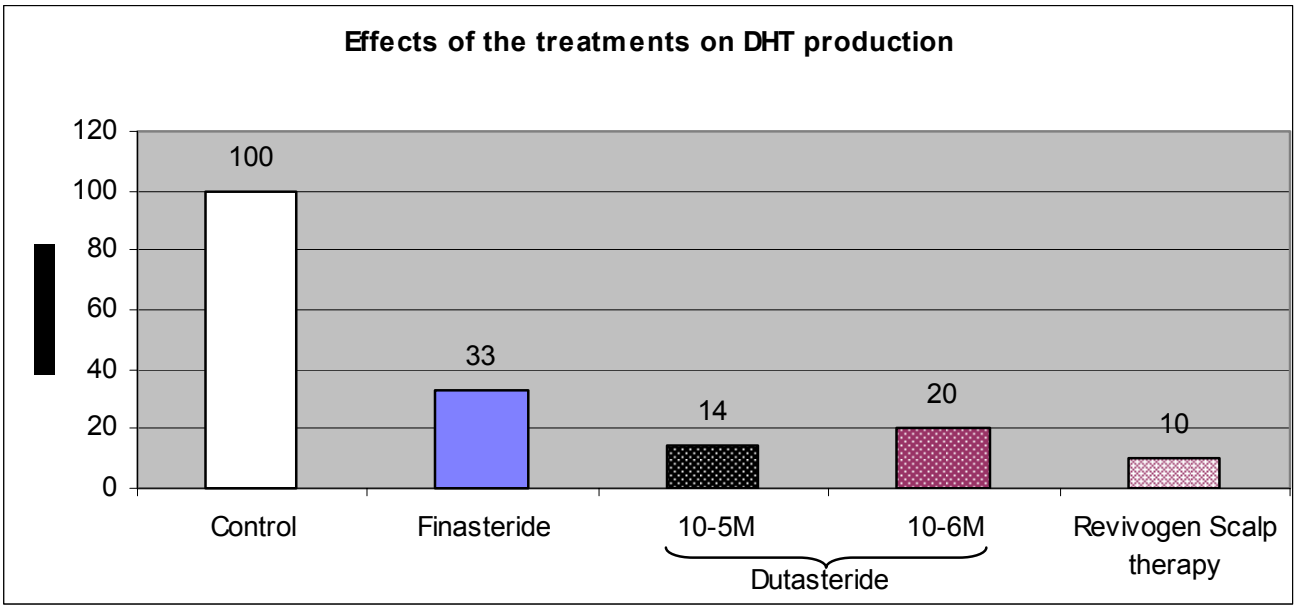


Figure 2: Effects of **Revivogen Scalp Therapy** and the reference compounds on DHT metabolism.