BIOalternatives

Study report: **AD090102-2**
Proposal: AD090102-3

Effect of compound FOLIXIR on testosterone metabolism in reconstructed human epidermis

5α reductase activity

STUDY PROMOTER

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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.

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1. INTRODUCTION

Male pattern hair loss is a potentially reversible condition in which dihydrotestosterone (DHT) is an important etiologic factor[1]. DHT, the efficient steroid, is produced from testosterone by 5α reductase and the metabolism of testosterone is involved in hair loss since inhibitors of 5α reductase have been demonstrated to reduce alopecia.

Effects of compound **FOLIXIR** on 5α-reductase activity were thus researched in a reconstructed human epidermis (RHE) model according to a method developed and published[2] by **BIO***alternatives* which has been shown to be a useful model for the evaluation of the inhibitors of testosterone metabolism.

ABBREVIATIONS

AU	Arbitrary unit
DHT	Dihydrotestosterone
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide
OD	Optical density
RHE	Reconstructed human epidermis
RT	Room temperature
Sd	Standard deviation
sem	Standard error of the mean
SFM	Serum free medium
TLC	Thin layer chromatography

REFERENCEs

1. Olsen EA et al. (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
2. Bernard FX *et al.* (2000). Expression of type 1 5α-reductase and metabolism of testosterone in reconstructed human epidermis (SkinEthic): a new model for screening skin-targeted androgen modulators. International Journal of Cosmetic Science, **22** 397-407

2. MATERIALS AND METHODS

2.1. Biological model

- Cellular model: 9 **BIO***alternatives* reconstructed human epidermis (RHE), 10 days old batch n° 01015-130

- Culture conditions: 37°C, 5% CO_2

- Assay medium: **BIO***alternatives* maintenance medium

2.2. Test compound

Test compound	Aspect/Storage	Application	Test concentration
FOLIXIR AD090102-2	▪ Liquid ▪ Storage at RT	Topical application at 5 mg/cm²	Pure

2.3. Cytotoxicity preliminary assay

- Application: 4 RHE of 0.5 cm², in keratinocyte-SFM medium
- RHE/compound contact: 24 hours + 5 hours
- evaluation parameters: MTT reduction assay

2.4. Culture and treatment

The RHE were placed in assay medium and the test compound or the reference (finasteride at 10^{-5} M) were topically applied or not (control) and the epidermis were incubated for 24 hours. After 24 hours of treatment, the RHE were topically re-treated and incubated for 5 hours. After incubation, the test compound and reference were removed from the top of the RHE and 100 µl of the [4-^{14}C]-testosterone solution were loaded on the *stratum corneum* of each RHE (1.27 µCi/ml).

After a 24-hour incubation period, the media underneath the RHE were collected for sterols analysis. The RHE viability, at the end of the experiment, was assessed by an MTT reduction assay.
Three RHE were used for each experimental condition.

2.5. Evaluation of RHE viability by MTT assay

MTT salt: 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide was reduced by mitochondrial succinate dehydrogenase in living cells. This reaction producing formazan crystals, soluble in acidic propanol, is proportional to succinate dehydrogenase activity (mitochondrial enzyme).
Consequently, the mitochondrial succinate dehydrogenase-dependent transformation of MTT in formazan crystal is proportional to the amount of living cells in the culture and/or cellular metabolic activity.

After incubation, the medium was discarded and formazan crystals were dissolved in acidic propanol. The optical density (OD) of the extracts at 540 nm, proportional to the number of living cells, was recorded with a ThermoMax microplate reader (SoftMax, Molecular Devices).

2.6. Extractions and analysis

The steroid molecules from the 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.

The plates were autoradiographed using a phosphorImager and specific software (Packard instrument) and testosterone metabolites were quantified using Multigauge version 3.0 software (Fujifilm).

2.7. Data management

Raw data were analyzed with Microsoft Excel® software.

Formulas used in this report:

Standard error of the mean: $sem = Sd/\sqrt{n}$

The standard error of the mean (sem) is a measure of how far the sample mean is likely to be from the true population mean. The sem is calculated as the sd divided by the square root of sample size.

Percentage of viability: $Viability\ (\%) = (OD_{sample} / OD_{control}) \times 100$

3. RESULTS

3.1. Cytotoxicity preliminary assay

Table 1

The results of the MTT reduction assay and the observation of RHE confirmed, in accordance with the study promoter, the concentrations to be tested (see paragraph 2.2).

3.2. Testosterone metabolism

Tables 2 & 3, Figure 2

After 24 hours of culture, the testosterone metabolism rate was very high and dihydrotestosterone (DHT) was clearly identified in the steroid profile. DHT was the major metabolite in the control epidermis. After 24 hours, about 76% of the applied testosterone was converted into DHT. Other important metabolites which were also detected were 4-androstene-3,17-dione, 5α-androstane-3α, 17β-dione and androstane-3β, 5α, 17β-diol.

3.2.1. Effect of finasteride

Tables 2 & 3, Figures 2 & 3

Finasteride, tested at 10^{-5} M, strongly inhibited the transformation of testosterone into DHT (75% inhibition compared to the control, Table 2). Furthermore, as expected, finasteride induced a strong accumulation of 4-androstene-3,17-dione and also decreased the amount of 5α-androstane-3α, 17β-dione and androstane-3β, 5α, 17β-diol (Table 3). These results were expected and validated the assay (Figure 1).

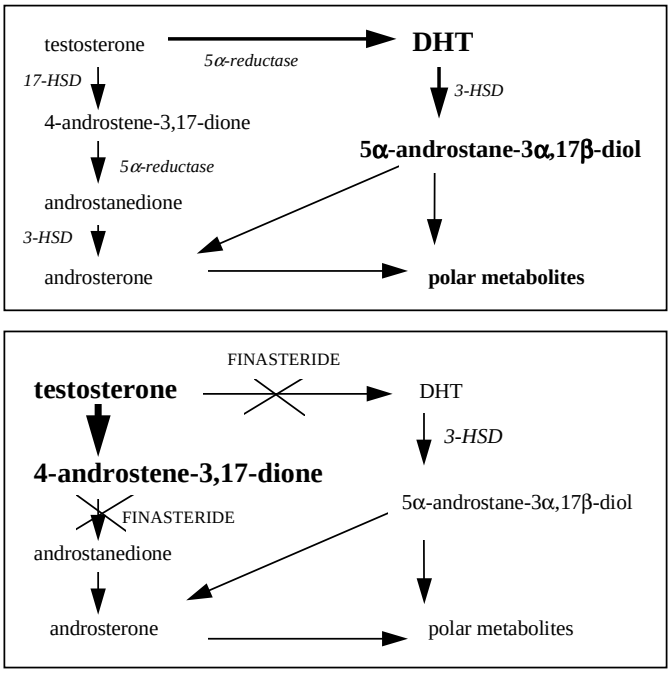

Figure 1: Schematic simplified pathway for testosterone metabolism. Effects of finasteride from Bernard F-X *et al.*, Int. J. Cosmetic Science, **22** 397-407 (2000).

3.2.2. Effect of compound FOLIXIR

Tables 2 & 3, Figures 2 & 3

Test compound **FOLIXIR**, tested at 5 mg/cm², highly inhibited the transformation of testosterone into DHT (57% inhibition compared to control) without affecting cellular viability. Furthermore, like the reference finasteride, the treatment with compound **FOLIXIR** induced an important decrease of 5α-androstane-3α, 17β-dione and androstane-3β, 5α 17β-diol and a consequent accumulation of 4-androstene-3,17-dione.

4. CONCLUSION

Under these experimental conditions, test compound **FOLIXIR** showed a strong inhibitory effect on 5α reductase activity since it significantly decreased DHT formation and thus this compound showed "Finasteride-like" properties. Moreover compound **FOLIXIR** presented no cytotoxicity.

5. TABLES AND FIGURES

Table 1: Effects of compound **FOLIXIR** on RHE viability - Preliminary cytotoxicity assay.

Test compounds	Application	Absorbance at 540 nm (OD)	Mean absorbance at 540 nm (OD)	sd (OD)	% of viability	Viability (%)	sem (%)
Control	-	0.994	1.14	0.136	87	**100**	6
		1.055			93		
		1.249			110		
		1.262			111		
FOLIXIR	Topically applied at 5 mg/cm²	0.797	0.800	0.004	70	**63**	4
		0.803			70		
		0.624	0.638	0.019	55		
		0.651			57		

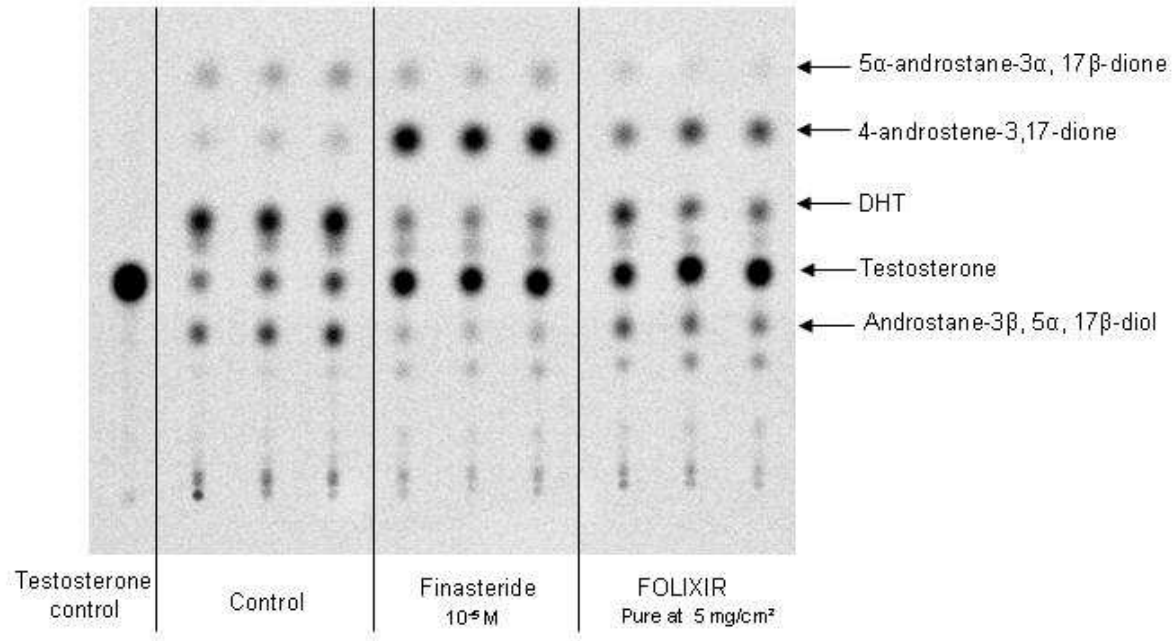

Figure 2: Effects of compound **FOLIXIR** on testosterone metabolism in reconstructed human epidermis. Chromatography and autoradiography of testosterone and other metabolites.

Table 2: Effects of compound **FOLIXIR** on DHT production.

			Total		Testosterone			DHT					DHT/testo	% viability in final assay
Treatment	**Concentration**	**# lane**	**Relative Intensity (AU)**	**Mean**	**Relative Intensity (AU)**	**% total**	**Mean (%)**	**Relative Intensity (AU)**	**% total**	**Mean (%)**	**% Control**	**sem (%)**	**ratio**	
Control	-	1	3027	**4223**	1109	37	**36**	2841	94	76	**100**	13	**2.12**	**100**
		2	3929		1576	40		2910	74					
		3	5713		1773	31		3445	60					
Finasteride	10^{-5} M	4	7222	**7021**	3866	54	**55**	1389	19	19	**25**	0	**0.35**	**-**
		5	6222		3535	57		1204	19					
		6	7618		4053	53		1402	18					
FOLIXIR	Pure at 5 mg/cm²	10	5102	**5711**	2974	58	**69**	2363	46	33	**43**	9	**0.48**	**95**
		11	6653		4510	68		1573	24					
		12	5379		4352	81		1540	29					

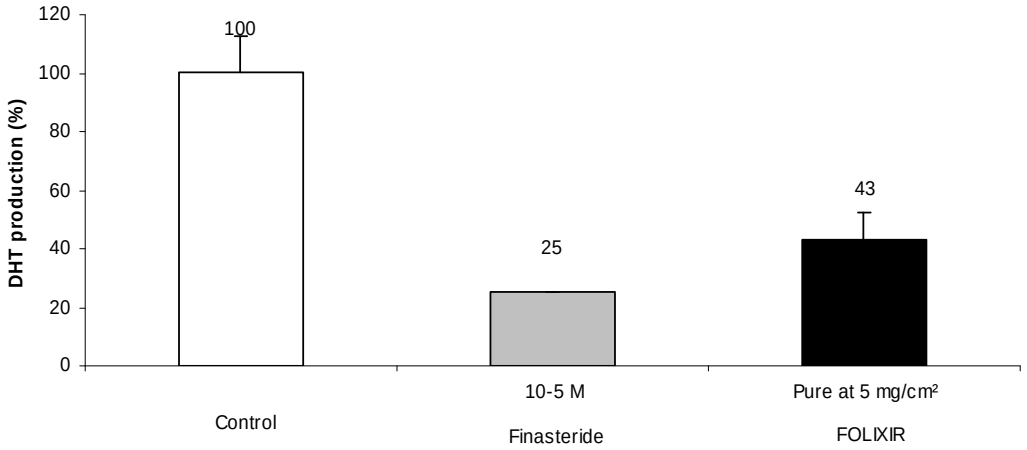

Figure 3: Effects of compound **FOLIXIR** on DHT production in reconstructed human epidermis.

Table 3: Effect of compound **FOLIXIR** on the production of other testosterone metabolites.

			Total		4-androstene-3,17-dione						% viability in final assay
Treatment	**Concentration**	# lane	Relative Intensity (AU)	Mean	Relative Intensity (AU)	% total	Mean (%)	**% Control**	**% Control**	**sem** (%)	
Control	-	1	3027	**4223**	467	15.4	**12.4**	124	**100**	15	**100**
		2	3929		508	12.9		104			
		3	5713		510	8.9		72			
Finasteride	10^{-5} M	4	7222	**7021**	3822	52.9	**52.7**	426	**424**	13	**-**
		5	6222		3455	55.5		447			
		6	7618		3788	49.7		400			
FOLIXIR	Pure at 5 mg/cm²	10	5102	**5711**	1320	25.9	**29.7**	208	**239**	20	**95**
		11	6653		1920	28.9		232			
		12	5379		1853	34.5		277			

			Total		5α-androstane-3α, 17β-dione						% viability in final assay
Treatment	**Concentration**	# lane	Relative Intensity (AU)	Mean	Relative Intensity (AU)	% total	Mean (%)	**% Control**	**% Control**	**sem** (%)	
Control	-	1	3027	**4223**	787	26.0	**22.7**	115	**100**	10	**100**
		2	3929		916	23.3		103			
		3	5713		1065	18.6		82			
Finasteride	10^{-5} M	4	7222	**7021**	802	11.1	**10.3**	49	**46**	2	**-**
		5	6222		639	10.3		45			
		6	7618		736	9.7		43			
FOLIXIR	Pure at 5 mg/cm²	10	5102	**5711**	570	11.2	**8.1**	49	**36**	7	**95**
		11	6653		389	5.9		26			
		12	5379		393	7.3		32			

			Total		androstane-3β, 5α, 17β-diol						% viability in final assay
Treatment	**Concentration**	# lane	Relative Intensity (AU)	Mean	Relative Intensity (AU)	% total	Mean (%)	**% Control**	**% Control**	**sem** (%)	
Control	-	1	3027	**4223**	1298	42.9	**38.5**	111	**100**	8	**100**
		2	3929		1578	40.2		104			
		3	5713		1858	32.5		84			
Finasteride	10^{-5} M	4	7222	**7021**	625	8.7	**7.9**	22	**21**	1	**-**
		5	6222		492	7.9		21			
		6	7618		546	7.2		19			
FOLIXIR	Pure at 5 mg/cm²	10	5102	**5711**	1316	25.8	**20.4**	67	**53**	7	**95**
		11	6653		1142	17.2		45			
		12	5379		983	18.3		47			