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Summary — The mutagenicity of trenbolone, a synthetic androgen, was studied in a number of genotoxicity tests using *in vitro* and *in vivo* systems for gene mutations, chromosomal mutations and primary DNA damage demonstration. Only 2 tests were found to be positive or dubious: the *in vitro* micronucleus test in SHE cells (however, this test was negative in 2 other cell lines: C3H and CHO cells) and the Ames test for 1 of the 5 studies which found a positive result in TA 100 strain without metabolic activation. Repetition of this study with pure trenbolone showed no genotoxic activity; trenbolone was therefore considered to be devoid of genotoxic activity.

genotoxic / mutagen / trenbolone / Ames test

Résumé — Trenbolone: application du test d'Ames. Résultats récents. *La mutagénicité de la trenbolone, un androgène de synthèse, a été étudiée en utilisant de nombreux tests de génotoxicité* in vitro *et* in vivo *permettant de démontrer des mutations géniques, des mutations chromosomiques et des altérations primaires de l'ADN. Seuls 2 de ces tests se sont avérés positifs ou douteux : le test du micronucleus* in vitro *sur cellules SHE (mais ce test était négatif avec 2 autres lignées cellulaires : cellules C3H et cellules CHO) et le test d'Ames pour l'un des 5 auteurs qui trouve un résultat positif sur la souche TA 100 sans activation métabolique. Toutefois la répétition de l'essai en utilisant de la trenbolone pure n'a démontré aucune activité génotoxique. La trenbolone est donc dépourvue d'activité génotoxique.*

génotoxique / mutagène / trenbolone / test d'Ames

INTRODUCTION

Trenbolone, a synthetic androgen, is administered to farm animals as a growth promoter. The mutagenic activity of trenbolone has been widely studied and the majority of studies have concluded that trenbolone lacks mutagenic activity. With the Ames test, in 1 of the 5 studies a slightly positive result was found in TA 100 strain. We therefore discuss mutagenicity results in an attempt to reach a conclusion regarding trenbolone genotoxic risk.

SUMMARY OF MUTAGENIC STUDIES PERFORMED ON TRENBOLONE

The Ames test is just one small part of the complete series of mutagenicity assays performed on trenbolone; thus in order to determine the status of trenbolone, it is certainly of use to provide quick overview

of mutagenicity studies. Covalent binding tests will be excluded, as these will be treated in detail elsewhere.

Point mutation test

As regards point mutation assays, with the exclusion of the Ames test which we shall consider later, Richold (1988) implemented 3 series of tests on cell culture (table I).

The first was performed on V79 cells; the second on CHO cells. In both cases, mutation at the HPRT locus was used as marker, the doses studied were calculated with the maximum doses which could be tested considering cytotoxicity. As reference, the third assay was carried out on mouse lymphoma cells L5178Y. Mutation at the thymidine kinase locus was used, and the doses studied ranged between 10–40 µg/ml. These 3 assays on mammalian cells proved to be negative both in the presence and absence of metabolic activation with only a slight increase but without a dose–effect relationship being noted in the mouse lymphoma cells and which could be considered as negative.

Table I. Gene mutation tests in cell cultures (Richold, 1988).

Type of assay	*Dose range*	*Results*
V79/HPRT with S9	6.25 – 100	Negative
V79/HPRT without S9	6.25 – 100	Negative
CHO/HPRT with S9	25 – 180	Negative
CHO/HPRT without S9	25 – 180	Negative
L 5178 Y/TK with S9	10 – 40	Negative
L 5178 Y/TK without S9	10 – 40	Negative

Chromosomal mutation tests

In vitro

As regards chromosomal mutation assays *in vitro,* tests were carried out by detection of chromosomal aberrations in lymphocytes and CHO cells using metaphase analysis and micronucleus on CHO by Richold (1988) and with micronucleus on SHE cells and C3H cells by Schiffman *et al* (1988).

Both chromosomal aberration assays using metaphase analysis were negative, while the micronucleus assay was positive in SHE cells but negative in CHO and C3H cells. This tends to show species-specificity. However, the human cells proved to be non-sensitive to this type of effect, since Richold's study (1988) on lymphocytes demonstrated the absence of clastogenic effect of trenbolone (table II).

In vivo

Three chromosomal mutation assays were carried out *in vivo*: a mouse micronucleus assay using bone-marrow in rat; the other 2 detect chromosomal damage in rat bone marrow (metaphase analysis) and germinal cell tissue (dominant lethal assay). All these *in vivo* studies were clearly negative (table III).

Primary DNA damage tests

Among the assays for primary DNA damage, that of unscheduled DNA synthesis on Syrian hamster embryo cells without metabolic activation and on HeLa cells with and without metabolic activation, the SOS chromotest on *E coli,* the Rec-assay in *B subtilis,* and the *in vitro* sister chromat-

Table II. *In vitro* chromosomal mutation tests.

Type of assay	*Dose range*	*Results*	*References*
Chromosomal aberrations			
Human lymphocytes with S9	6 – 60 µg/ml	Negative	1
Human lymphocytes without S9	6 – 60 µg/ml	Negative	1
CHO cells with S9	1 – 10 µg/ml	Negative	1
CHO cells without S9	25 – 180 µg/ml	Negative	1
Micronucleus			
SHE cells without S9	$10^{-6} - 10^{-4}$	Positive	2
C3H 10 T 1/2 without S9	$5 \times 10^{-6} - 10^{-4}$	Negative	2
CHO cells with S9	1 – 10 µg/ml	Negative	1
Cho cells without S9	6 – 60 µg/ml	Negative	1

(1) Richold (1988); (2) Schiffmann *et al* (1988).

id exchange assay on V79 cells also proved to be negative (table IV).

It is interesting to note that chromosomal aberrations observed in SHE cells are not correlated with primary DNA damage in the same cell strain.

If we recapitulate on all the data concerning genotoxicity assays, after a complete study of the mutagenic activity of trenbolone, it would seem that only 1 assay is ambiguous but considered to be negative, and another positive in Syrian hamster cells, but without primary DNA damage and negative in human or Chinese hamster cells. All the other tests are negative.

Table III. *In vivo* chromosomal mutation tests (Richold, 1988).

Type of assay	*Dose range (mg/kg)*	*Results*
Bone marrow chromosomal aberrations		
Rat	100 – 200	Negative
Mouse micronucleus	100	Negative
Germ cell chromosomal aberrations		
Rat	100 – 200	Negative

THE AMES TEST

Five studies have been performed using the Ames test (table V). The first, carried out by Ingerowski *et al* (1981), utilised doses ranging from 1 000 – 3 000 µg/plate, but only the results of the 1 000 µg/plate were exploitable, the other doses being toxic. The study was performed by incorporation into a solid medium, with and without S9 from rat liver as metabolic activation, on 5 strains of *Salmonella typhimurium*. Substance purity was not specified. No significant variation in the number of revertants was noted.

Table IV. Primary DNA damage tests.

Type of assay	*Dose range*	*Results*	*References*
SOS chromotest *(E coli)*	3.3 – 100 µM	Negative	1
Rec-assay *(B subtilis)*		Negative	1
Unscheduled DNA synthesis/HeLa cells	1 – 256 µg/ml	Negative	2
Unscheduled DNA synthesis/HeLa cells	2.5 – 15 µg/ml	Negative	3
Unscheduled DNA synthesis/SHE cells	2.5 – 15 µg/ml	Negative	3
Sister chromatid exchanges/V79 cells	3.3 – 100 µM	Negative	1

(1) Scheutwinkel *et al* (1986); (2) Richold (1988); (3) Schiffmann *et al* (1985).

Table V. Summary of AMES test studies.

S typhimurium *strains (a)*	*Metabolic activation*		*Method*	*Purity*	*Dose range (µg/plate)*	*Results*	*References (c)*
	Without	*With*					
5, 7, 8, 9, 0	+	+ (rat)	Plate incorporation	U (b)	1 000 – 3 000	Negative	1
9, 0	+	+ (rat and hamster)	Preincubation	U	0.06 – 2	Negative	2
5,7, 8, 9, 0	+	+ (rat)	Plate incorporation	> 99%	15 – 1 500	Negative	3
9, 0, 2	+	+ (rat : 5 – 30%)	Preincubation	> 97%	12 – 1 000	Weakly positive in TA 100 without S9	4
0	+	+ (rat : 5 – 30%)	Preincubation	> 97%	5 – 50	Weakly positive in TA 100 without S9	4
9, 0, 2	+	+ (rat : 10%)	Plate incorporation	> 99%	5 – 500	Negative	5
0	+	+ (rat : 10%)	Preincubation	> 99%	30 – 120	Negative	5

(a) 5 = TA 1535, 7 = TA 1537, 8 = TA 1538, 9 = TA 98,0 = TA 100, 2 = TA 102. (b) U = Unknown. (c) References : (1) Ingerowski *et al* (1981); (2) Schiffmann *et al* (1985); (3) Richold (1988); (4) Lutz *et al* (1988); (5) Marzin (1989).

The second study was performed by Schiffman *et al* (1985), using only 2 strains, TA 98 and TA 100. The technique used was 20- or 120-min preincubation in liquid medium, with or without rat or hamster liver S9. The doses studied ranged between 0.06–2 µg/plate. These doses are relatively low. Substance purity was not specified. No mutagenic effect was detected.

In the third study, carried out by Richold (1988), 5 strains from Ames were studied with or without S9 rat liver metabolic activation induced by Arochlor. The technique used was that of incorporation in solid medium. Substance purity was > 99%. No mutagenic activity was detected for doses ranging from 15–1 500 µg/plate.

The fourth study was carried out by Lutz *et al* (1988) and comprised 3 assays : the first assay way carried out on 3 *Salmonella* strains: TA 100, TA 98 and TA 102, with and without S9 rat liver metabolic activation at 0–5 and 30% in S9 mix, using the preincubation test, in liquid medium for 20 min. This first assay was considered as being slightly positive for the TA 100 strain, in the absence of metabolic activation.

On examining the detailed results, it can be noted that doses ≥ 111 µg/plate were evidently toxic and that the 37 µg/plate dose increased the spontaneous number of mutants by only 24, while the standard deviation was 20. We can consider that this assay is negative; however, the authors continued their investigations.

Despite this modest result, in a second assay the authors decided to study a narrower dose range using the same methodology and in the second assay showed that the 40 µg/plate dose, with the maximal effect, increased the number of mutants by 46, for a standard deviation which was also in the region of 20. They were also able to calculate an equation: No of revertants = 160 + 0.78 x µg trenbolone per plate. We can see that the slope is not very significant.

The third assay, by Lutz *et al* (1988) was performed with 2 doses: 0–30 and 60 µg/plate. By using 13–15 replicates, they demonstrated a weak increase in mutants of the same amplitude, that is to say 43 induced revertants per plate, or 0.71 mutant/µg of trenbolone. The authors concluded that trenbolone possessed mutagenic activity as regards the Ames test. Fortunately judgment is not always so severe, otherwise no products could be marketed and if we classified a compound as positive when we observed an increase of 40 mutants on TA 100, everything would be mutagenic, and continuing to perform the Ames test would have no meaning.

It is difficult to accept the conclusion regarding the mutagenic activity of trenbolone with this low increase in the number of mutants; if this were the case, the term mutagenic would be devoid of meaning. Most authors consider that doubling of the number of mutants must be observed before it can be concluded that mutagenic activity is present. If the increase is more modest, as in this instance, we can draw the conclusion of an ambiguous or doubtful result and try to determine the reasons for such a weak increase.

What about the purity of the substance used? Although in the original study of Lutz *et al* (1988) the radiolabeled substance used was only > 97% pure, following an answer which appeared 1 year later in the same review, the authors said that the product used to carry out the Ames test was not that described in the section *Materials and Methods* (Lutz and Slater, 1989). But no precision about purity was given. However, such results have caused us to consider the possibility of an effect resulting from impurities.

In conclusion, as regards these 4 studies, 3 are clearly negative : 2 correctly carried out by incorporation in solid medium at high doses; one carried out with preincubation in liquid medium but at low doses. One is ambiguous, being correctly carried out at the maximum doses which could be studied but with a substance which may have contained impurities. Two hypotheses may thus be considered; either that the equivocal effect is due to impurities, or that trenbolone is very slightly mutagenic but with activity which can only be detected after preincubation without metabolic activation.

We have repeated the 2 types of study in solid medium and with preincubation, using an extremely pure substance of over 99% purity (Marzin, 1989).

The first assay in solid medium was carried out on 3 strains (TA 98, TA 100 and TA 102), with doses ranging between 5–500 µg/plate. This assay was undeniably negative.

In the assay with preincubation in liquid medium, we confined the study to TA 100, which had presented an equivocal response in the Lutz *et al* (1988) study, and used the same solvent (ethanol), with a high number of plates (12 per dose), and the same doses of 0, 30, 60 but also 90 and 120 µg/plate. Under these conditions, no mutagenic activity was detected. Although these results have come under discussion in an answer by Lutz and Schlatter (1989), published in the *Archives of Toxicology*, we consider that the response is undeniably negative.

Under these conditions, we can conclude that pure trenbolone presents no genotoxic activity as regards *Salmonella typhimurium* TA 100 strain, whatever the methodology used.

It is apparent from these studies, particularly those on the Ames assay, that we may conclude, reiterating the statement made by Margaret Richold, that "trenbolone is devoid of genotoxic activity and is not an initiator of cancer". This means that if trenbolone presents a carcinogenic risk, it would act *via* an epigenetic mechanism. Finally, to temporarily close this debate, we shall cite the conclusion made by Lutz and Schlatter (1989): "we consider the risk from trenbolone residues in meat not to be a relevant genotoxicity problem".

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