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GENOTOXICITY AND CARCINOGENICITY

Induction of micronuclei in V79 cells by the anabolic doping steroids tetrahydrogestrinone and trenbolone

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Abstract The synthetic steroid tetrahydrogestrinone is a new "designer drug" and was recently detected to be illegally used in sports. It is chemically closely related to trenbolone that is known as an animal growth promoter. The potencies of trenbolone, tetrahydrogestrinone and testosterone to induce micronuclei in V79 cells in vitro were determined. CREST analysis was employed to differentiate between aneugenic or clastogenic mechanisms. Cytotoxicity and an influence on the cell cycle were assessed in parallel. Incubations with testosterone, at concentrations between 3 and 300 μM, failed to induce micronuclei. By contrast, tetrahydrogestrinone and trenbolone increased the rate of micronuclei significantly, up to a doubling of the micronuclei rate of untreated controls. Tetrahydrogestrinone and trenbolone displayed a bell-shaped dose-response curve, with maximal effects observed at 3 and 30 μM, respectively. The micronuclei induced by tetrahydrogestrinone and trenbolone were predominantly kinetochor (CREST) positive, pointing to an aneugenic mode of action. This may be related to the specific structure of both molecules with a system of activated double bonds. As the genotoxic effect of tetrahydrogestrinone at a chromosomal level appears at a low concentration range, it cannot be ruled out that tetrahydrogestrinone presents a genotoxic hazard on a chromosomal level under conditions of its current misuse in sports.

Keywords Trenbolone · Tetrahydrogestrinone · Testosterone · Anabolic steroids · Doping · Sport · Genotoxicity · Micronucleus assay

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Introduction

Anabolic steroids are used as growth promoters in animal husbandry (Anonymous 1987; WHO 1988), and their current misuse by athletes for doping raises political concern (Deftos 2006). Aside from the hormonal activity of steroids, there is a possibility of genotoxic effects (Joosten et al. 2004). The synthetic androgenic/anabolic steroid tetrahydrogestrinone (THG) is a new "designer drug" that was recently detected to be illegally used in sports (Catlin et al. 2004; Friedel et al. 2006; Thevis et al. 2005). Its chemical structure is closely related to that of trenbolone (TB) that has been used as an animal growth promoter for almost 20 years (Anonymous 1987; WHO 1988) and also misused by bodybuilders (Daniels et al. 2006). The structural formulae of these compounds, compared to the physiological androgen testosterone (T), are depicted in Fig. 1.

As an animal growth promoter, the genotoxicity of TB has been evaluated using different endpoints in vitro and in vivo. The database gives an inconsistent picture (Marzin 1991; Metzler and Pfeiffer 2001): although TB appears to bind to DNA to a very limited extent (Barraud et al. 1984; Lutz et al. 1988), it appears negative in most bacterial and mammalian cell mutagenicity and DNA repair assays. Equivocal or contradictory results were obtained for morphological cell transformation (Schiffmann et al. 1985; Tsutsui et al. 1995), and in assays reflecting genotoxicity at

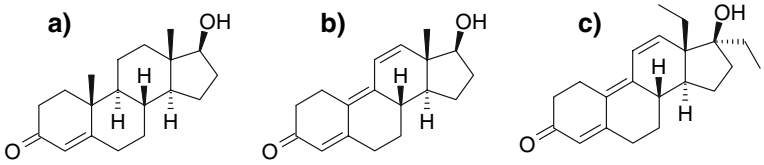

Fig. 1 Chemical structure of **a** testosterone (T), **b** trenbolone (TB) and **c** tetrahydrogestrinone (THG)

a chromosomal level, namely the mouse lymphoma assay, the chromosomal aberration test, and micronucleus (MN) induction in vitro in different cell types (Richold 1988). However, a strong irreversible binding of TB and/or its metabolites to tissue proteins has been reported, most likely connected with its activated system of conjugated double bonds (Ryan and Hoffmann 1978). This, in principle, raises concern of an interaction with proteins involved in karyokinesis (Bonacker et al. 2004a).

The genotoxicity of the structurally similar "designer drug" THG (Death et al. 2004) has not been investigated at all. In order to investigate the chromosomal genotoxicity of TB and THG we determined their potencies of micronuclei induction in V79 cells and compared these with that of the natural androgen testosterone (T). A CREST analysis was employed to differentiate between aneugenic or clastogenic mechanisms. Cytotoxicity and influence on the cell cycle were assessed in parallel.

Materials and methods

Chemicals and biochemicals

T was purchased from Sigma-Aldrich (Taufkirchen, Germany). TB was obtained through Organon, Department of Toxicology and Drug Disposition (Oss, The Netherlands). THG was originally synthesized by Thevis et al. (2005) and also characterized for hormonal activity as described by Friedel et al. (2006).

Vincristine (VCR), methylmethane sulfonate (MMS), acridine orange, propidium iodide (PI), 4′,6′-diamidino-2-phenyl-indole (DAPI), HBSS (Hanks' balanced salt solution), spermine tetrahydrochloride, Na_2EDTA, mercaptoethanol, and RNase were purchased from Sigma-Aldrich (Taufkirchen, Germany); KCl and Triton-X-100 from Fluka (Buchs, Switzerland).

CellTiter Blue™ was from Promega (Mannheim, Germany). The primary antibody for the CREST analysis, so-called "positive control serum", was obtained from DPC Biermann (Bad Nauheim, Germany); the secondary antibody, FITC conjugated anti-Human IgG, was from Sigma. Dimethylsulfoxide (DMSO), methanol, acetone, acetic acid, TRIS, NaCl, and Tween 20 were products of Merck (Darmstadt, Germany). Cell culture medium (RPMI 1640), trypsin-EDTA, and PBS (phosphate buffered saline 10×) were obtained from Invitrogen (Karlsruhe, Germany) and fetal calf serum (FCS) from Biochrom KG (Berlin, Germany). "Acridine orange working solution" was prepared as follows: 5 ml acridine orange stock solution (1 mg/ml dist. water) were mixed with 7 ml 0.3 M KH_2PO_4, 7 ml 0.3 M Na_2HPO_4 and 80 ml dist. water.

Cytotoxicity assay

Cytotoxicity of the test compounds was determined in V79 hamster lung fibroblast cells (DSMZ, Braunschweig, Germany) by means of the CellTiter Blue™/Alamar Blue assay (Nociari et al. 1998; O'Brien et al. 2000) to indicate the range of concentrations (close to and below cytotoxicity) suitable to examine the chemicals in the micronucleus assay.

The CellTiter Blue™ assay, a fluorometric method, uses the indicator dye resazurin to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Non-viable cells rapidly lose their metabolic capacity, do not reduce the indicator dye, and do not generate a fluorescence signal.

Briefly, 5×10^3 V79 cells per well were plated in black 96-well tissue-culture plates with clear bottom (Costar from VWR International, Bruchsal, Germany), allowed to attach for 24 h and treated with the test substances, added as DMSO solutions, in the culture medium for 18 h. Final concentration of DMSO was 0.1%. Cells were washed twice with warm HBSS and subsequently incubated with 20% CellTiter Blue™ (Promega) in HBSS for 3 h (Goegan et al. 1995). The fluorescence intensity was read at 540 and 595 nm using a SpectraFluor Plus microplate reader (Tecan, Crailsheim, Germany).

The IC_{20} and IC_{50} values were taken from the concentration response curves.

Micronucleus (MN) assay

The MN assay was performed in vitro according to Matsuoka et al. (1992) with slight modifications described elsewhere (Bonacker et al. 2004a; Dorn et al. 2007). Initially, 2×10^5 V79 cells were seeded into 25 cm^2 flasks (Greiner) and cultured for 48 h at 37°C. The medium was changed, and the cells were exposed to graded concentrations of the test substances, negative and positive control items. Positive controls were methylmethane sulfonate (227 µM) and vincristine (10 nM); negative controls were

cultures treated with medium alone and 0.1% DMSO, respectively. Concentrations of the test compounds examined in the MN assay were generally below the limit of cytotoxicity.

Cells were harvested by disaggregation with trypsin/EDTA (0.25% trypsin in PBS with 0.02% EDTA), suspended in complete medium with 20% FCS and centrifuged at 200*g* for 8 min. The cells were then subjected to hypotonic conditions with 0.4% KCl, and to fixation with methanol/acetic acid (4:1) (4 cycles and centrifugation in between). Cells were mounted onto slides, air-dried at ~55°C and stained with a solution of Acridine Orange (50 µg/ml) and immediately examined using a Leitz DM LB fluorescence microscope (Leica, Wetzlar, Germany) at 400-fold magnification and a filter setting providing blue excitation at 440–490 nm and emission of 520 nm.

MN were scored according to the criteria of Countryman and Heddle (1976) and Fenech (1993): structures surrounded by a nuclear membrane, having an area of less than one third of that of the main nucleus, being located within the cytoplasm of the intact cell and not linked to the main nucleus via nucleoplasmic bridges. Multinucleated cells and cells with more than six MN were not scored to avoid a mix-up with apoptotic cells.

Per culture, 2 × 1,000 cells with well-preserved cytoplasm were examined and analyzed for micronuclei. The number of MN scored was recorded and micronucleus rate calculated as number of MN per 1,000 cells for each condition/concentration. Each compound was assayed at least in two independent experiments.

CREST analysis

A distinction between aneugens and clastogens was achieved by the CREST analysis according to Renzi et al. (1996) and Russo et al. (1992), with some modifications (Bonacker et al. 2004b). Vincristine (VCR, 10 nM) and methylmethane sulfonate (MMS, 227 µM) served as aneugenic and clastogenic positive controls, respectively. The test conditions were those used in the standard MN assay, except that the cells were seeded directly onto sterile slides (2.5×10^4 cells/slide) and treated in Quadriperm® dishes (Viva Science, Hannover, Germany). After incubation with the compounds for 18 h (at 37°C under 5% CO_2) the cells were rinsed 2 min with 1× PBS, treated with 0.075 M KCl for swelling (15 min, 37°C), fixed with ice-cold (−20°C) methanol (30 min) and acetone (10 min), and permeabilized with PBS plus 0.1% Tween 20.

Then cells were incubated with 50 µl of the primary antibody (CREST serum 1:50 in PBS/0.2% Tween 20) for 24 h (37°C, 5% CO_2), washed with PBS/0.1% Tween 20 to eliminate the excess of primary antibody and again incubated with the second antibody (FITC-conjugated goat anti-human antibody, 1:100 in PBS/0.5% Tween 20) for 1 h. The cells were rehydrated in Soerensen buffer, counterstained with 100 µl DAPI/PI staining solution (1 µg/ml DAPI + 0.1 mg/ml propidium iodide (PI), mixed 3:1 v/v), mounted with antifade and analysed by fluorescence microscopy.

The induction of MN was evaluated by scoring a total of 1,000 cells per slide with a Leitz DM LB fluorescence microscope (Leica, Wetzlar, Germany) at a 400-fold or 1,000-fold magnification. MN were located by fluorescence light (UV excitation, 340–380 nm, emission 425 nm), checked with a PI filter (excitation 515–560 nm, emission 590 nm), and finally classified using the FITC filter (blue excitation, 450–490 nm, emission 525 nm).

MN were classified as "CREST-positive" when bright spots were clearly observed, "CREST-negative" when no spots were observed, or "unclear" when either opaque spots or bright background were observed. A positive CREST reaction reveals that the MN consists of one or more complete chromosomes and indicates primarily aneugenic effects (Miller and Adler 1990; Schuler et al. 1997).

Cell cycle analysis

The distribution of untreated and substance-treated V79 cells in the different cell cycle phases was determined using propidium iodide DNA staining and analysis of DNA content by flow cytometry (Dolezel et al. 1989; Krishan 1975). The background of this method is the DNA content of cells in G_0/G_1 phase being 2n, its increase during S phase up to 4n, which resembles cells in G_2/M phases.

Briefly, 8×10^4 cells were seeded in 6-well-dishes (Costar from VWR International, Bruchsal, Germany) and cultured for about 48 h before treatment. The medium was changed, and the cells were exposed to the test substances as DMSO solutions or to control conditions (medium only or 0.1% DMSO) for 18 h.

Cells were harvested by disaggregation with trypsin/EDTA (0.25% trypsin in PBS with 0.02% EDTA), suspended in medium with 20% FCS and centrifuged at 200*g* for 8 min. They were re-suspended in PBS, and cell density was adjusted at 1×10^6. After being centrifuged again, the cells were re-suspended in 500 µl lysis buffer (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, 15 mM mercaptoethanol, pH 7.5). 5 µl RNase (10 mg/ml) for RNA digestion and 5 µl PI (1.5 mM) for DNA staining were added, and the samples were incubated for 1 h at 4°C in the dark. Cells were finally analyzed by flow cytometry (FACSCalibur™, Becton Dickinson, Heidelberg, Germany).

Statistical analysis

The results obtained for the compound-treated samples were compared with the concurrent negative solvent control using the Student's two-tailed *t* test. Probability values of $P \leq 0.05$ were accepted as being significant.

Results

Cytotoxicity

Cytotoxicity of the test compounds has been determined by means of the CellTiter Blue™ assay; the results are depicted in Fig. 2. As apparent from the concentration-response curves, THG was the most and T the least cytotoxic of the three steroids. The IC_{20} values were 40, 75 and 155 μM for THG, TB and T, respectively. More pronounced cytotoxicity (IC_{50} values) was reached at 90, 230 and 285 μM for THG, TB and T, respectively.

Expression of genotoxicity in the micronucleus assay may be compromised by interfering cytotoxic or cytostatic effects (cell cycle arrest). Therefore, based on the cytotoxicity results a permissible range of concentrations was derived for the MN test, i.e., concentrations that do not result in an overt inhibition of cell division.

Micronucleus (MN) assay

Treatment with the physiological androgen T, at concentrations between 3 and 300 μM, failed to induce MN in V79 cells. By contrast, THG and TB increased the rate of MN significantly, compared to controls (Table 1). Interestingly, both THG and TB displayed a bell-shaped dose-response curve, with maximal effects observed at 3 and 30 μM, respectively (Fig. 3). These maximal effects of both compounds were close to a doubling of the respective background MN rates.

To differentiate between aneugenic and clastogenic modes of action, CREST analysis was performed for the two synthetic steroids that were tested positive in the MN

Table 1 Micronucleus (MN) assay results of T, TB, THG, and controls ($n = 4$ each)

Compound	Concentration (μM)	MN/1,000 cells (SD)	MN increase relative to control (SD)
T	3	14.50 (±0.71)	1.00 (±0.05)
	10	14.50 (±0.58)	1.00 (±0.04)
	30	14.50 (±1.91)	1.00 (±0.13)
	100	15.00 (±1.41)	1.03 (±0.10)
	300	15.00 (±0.00)	1.03 (±0.00)
Medium		14.50 (±1.00)	
0.1%DMSO		15.50 (±1.00)	1 (±0.07)
TB	3	9.00 (±1.63)	1.00 (±0.18)
	10	10.00 (±2.16)	1.11 (±0.24)
	30	19.25 (±3.30)	2.14 (±0.37)
	60	13.00 (±1.41)	1.63 (±0.18)
	100	15.25 (±2.99)	1.69 (±0.33)
Medium		9.75 (±1.26)	
0.1%DMSO		8.67 (±1.21)	1.00 (±0.16)
THG	1	10.50(±1.73)	1.00 (±0.16)
	3	21.00 (±2.94)	2.00 (±0.28)
	10	15.75 (±4.42)	1.50 (±0.42)
	30	14.00 (±3.65)	1.33 (±0.35)
Medium		9.75 (±1.26)	
0.1%DMSO		10.50 (±1.29)	1.00 (±0.12)

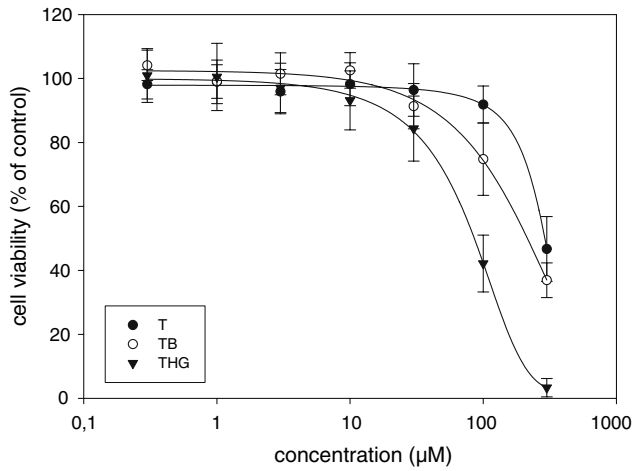

Fig. 2 Cytotoxicity of testosterone (T), trenbolone (TB) and tetrahydrogestrinone (THG); mean ± SD

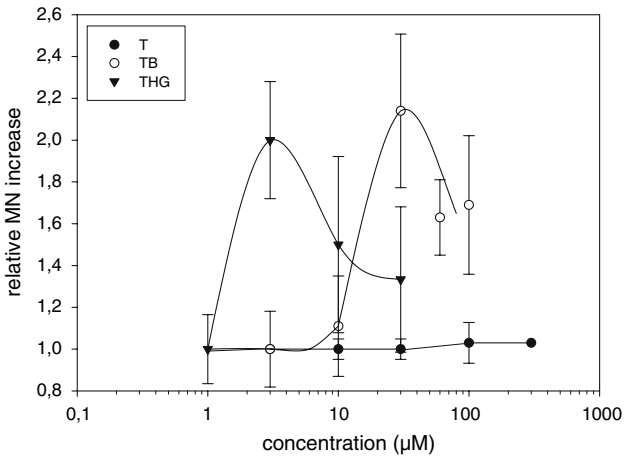

Fig. 3 Micronucleus (MN) induction of testosterone (T), trenbolone (TB) and tetrahydrogestrinone (THG) (values relative to solvent controls); mean ± SD

Table 2 CREST analysis: number and characteristics of MN induced by TB, THG and controls ($n = 4$ each)

Compound	Concentration	MN/1,000 cells (SD)		
		CREST-positive	CREST-negative	unclear
TB	30 μM	6.0 (±1.7)	2.7 (±0.6)	4.7 (±0.6)
THG	3 μM	4.0 (±0.0)	6.5 (±0.7)	3.0 (±0.0)
Medium		0.0 (±0.0)	3.0 (±1.4)	1.5 (±0.7)
0.1% DMSO		0.5 (±0.7)	4.5 (±0.7)	1.5 (±0.7)
MMS	227 μM	1.3 (±1.5)	13.3 (±3.8)	5.3 (±3.1)
VCR	10 nM	23.8 (±4.6)	7.0 (±1.4)	12.8 (±3.2)

assay. Numbers and characteristics of MN induced by the steroids and by positive and negative controls are shown in Table 2. After introducing the background corrections for steroids and positive controls (Fig. 4) the compounds were classified regarding their mode of action. The reference compounds methylmethane sulfonate, a typical clastogen, induces mainly CREST-negative MN, the reference aneugen vincristine mainly CREST-positive MN. MN induced by TB and THG were predominantly kinetochor (CREST)-positive, pointing to an aneugenic mode of action in both cases (Fig. 4).

Cell cycle analysis

Effects of steroids on the cell cycle were studied in V79 cells at a range of concentrations. Results are summarized in Table 3. No changes in cell cycle distribution were detected in V79 cells treated with THG up to 30 μM; at 60 μM the sub-G_0/G_1 cell population was increased, thus pointing to apoptosis. Testosterone treatment for 18 h at 100 and 300 μM led to a clear increase in the fraction of cells in G_2/M; lower concentrations did not show an effect. TB concentrations of 30 μM and more (100 μM) led to a marked increase in the fraction of cells in G_2/M, indicative of a dose-dependent cell cycle arrest.

Discussion

The present study on the dose-dependent induction of MN by THG, TB and T was carried out in V79 cells, a cell line that has been widely used to assess the genotoxic potential of chemicals, including hormonal steroids (Dorn et al. 2007).

Both TB (Anomymous 1987) and THG (Death et al. 2004; Friedel et al. 2006; Labrie et al. 2006) present themselves as potent androgenic and anabolic steroids. According to the present data, they reach the level of a doubling of the MN background rate in V79 cells within a relatively narrow concentration window (Fig. 3). The resulting "bell-shaped" dose-response is likely to be caused by cytotoxicity at higher concentrations (Fig. 2). The narrow window also explains the heretofore-inconsistent data on chromosomal genotoxicity of trenbolone (Metzler and Pfeiffer 2001), because in routine studies larger spaces between experimental concentrations are chosen.

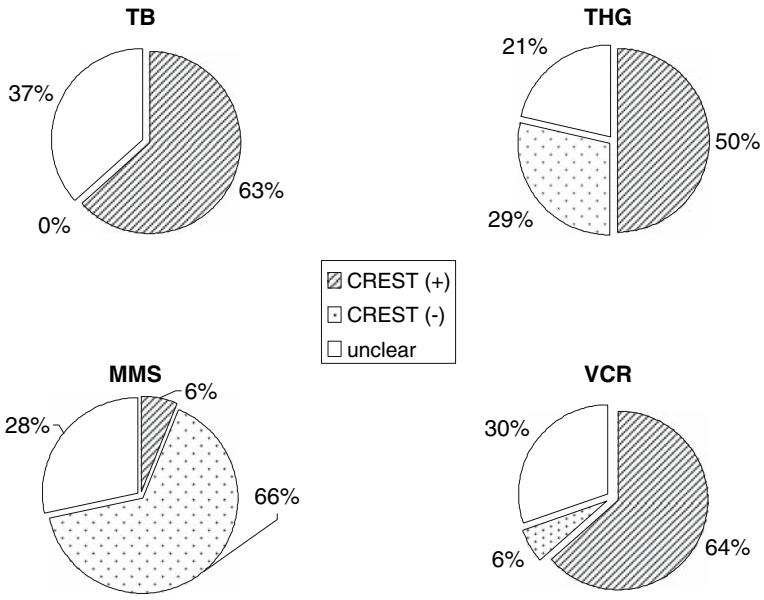

Fig. 4 CREST analysis of MN induced by trenbolone (TB) and tetrahydrogestrinone (THG). Controls: methylmethane sulfonate (MMS, 25 μg/ml); vincristine (VCR, 10 nM). Percentual distribution of *CREST-positive*, *CREST-negative* and *unclear* MN, corrected for backgound

Table 3 Cell cycle analysis: results for T, TB and THG (minimum 3 independent tests; mean ± SD)

Compound (μM)		G_0/G_1	Cell cycle phases (%)		
			S	G_2/M	Sub G_0
T	0	58.88 (±5.28)	20.74 (±3.53)	20.38 (±3.91)	
	10	57.15 (±3.03)	22.49 (±3.43)	20.35 (±1.59)	
	30	56.07 (±3.84)	21.87 (±1.76)	22.07 (±4.27)	
	100	50.50 (±6.18)	20.63 (±5.68)	28.87 (±10.59)	
	300	47.23 (±6.29)*	18.59 (±3.45)	34.18 (±2.94)*	
TB	0	61.37 (±4.16)	19.88 (±4.04)	18.74 (±0.97)	
	3	58.87 (±1.92)	21.48 (±2.87)	19.65 (±0.99)	
	10	53.10 (±7.98)	22.68 (±3.41)	24.22 (±9.46)	
	30	36.29 (±7.22)*	18.58 (±4.76)	45.13 (±11.79)*	
	100	39.57 (±9.59)*	21.45 (±1.56)	38.98 (±10.70)*	
THG	0	61.37 (±4.16)	19.88 (±4.04)	18.74 (±0.97)	
	3	62.28 (±0.99)	20.06 (±0.61)	17.66 (±1.10)	
	10	60.18 (±5.00)	21.45 (±3.21)	18.37 (±1.84)	
	30	61.97 (±2.77)	19.29 (±1.72)	18.74 (±1.32)	
	60	35.93 (±5.39)*	17.50 (±0.69)	21.13 (±4.06)	25.44 (±4.57)

* Statistically significant, $P \leq 0.05$

By contrast, the physiological hormone T did not induce MN, even at high concentrations of up to 0.3 mM (Fig. 3). This shows that the MN induction by TB and THG is independent of the hormonal effect. It is known that mutational effects of TB and T on the DNA level are almost negligible (Marzin 1991; Metzler and Pfeiffer 2001), which is paralleled by very low covalent DNA binding of both compounds in rat liver in vivo (Barraud et al. 1984; Lutz et al. 1988).

In essence, the chromosomal genotoxicity of both TB and THG, although weak, appears clear-cut and predominantly based on an aneugenic effect, according to the present data (Fig. 4). A long-known molecular feature of TB is its covalent binding to protein structures (Ryan and Hoffmann 1978; Hoffmann et al. 1984). In vitro, this is small in magnitude, but definite. This contrasts to conditions in vivo where covalent protein binding of TB is quite pronounced (Ryan and Hoffmann 1978). Chemical reduction of a pepsin/trypsin hydrolysate of TB-adducted protein with Raney nickel again liberated TB, rendering it very likely that protein sulfhydryl (SH) groups of cysteine had reacted with the activated double bond system of TB (Ryan and Hoffmann 1978). For karyokinesis, free sulfhydryl groups play a predominant role in protein interactions. An outstanding example is the aggregation (and de-aggregation) of tubulin. Almost 20 cysteine residues are accessible with disulfide reagents in the tubulin dimer, but only 4 in taxol-stabilized microtubules, and a loss of free SH-groups of tubulin is also closely associated with a loss in polymerisation competence (Britto et al. 2005).

Taking these elements together and considering the very close structural similarity between TB and THG (Fig. 1), it appears at least plausible that the observed aneugenic effects of TB and THG may be related to interactions with partial processes involved in chromosomal segregation, based on a covalent attachment of TB/THG to protein sulfhydryl groups.

There is a marked difference in the concentrations of THG and TB needed to reach the maximal effects of MN induction (THG: 3 μM, TB: 30 μM). This may reasonably be explained by different lipophilicities. The introduction of the 17α-ethyl substituent that is characteristic of THG into the TB molecule (Fig. 1) leads to a (calculated) log P (octanol–water partitioning) of 3.40 for THG, versus 1.82 for TB. Within lipid microenvironments, this causes a much higher enrichment of THG from an aqueous phase, compared to TB.

Both THG and TB are misused in doping of athletes and body-builders (Catlin et al. 2004; Death et al. 2004; Daniels et al. 2006; Thevis et al. 2005), and TB is also misused in equine sports (Guan et al. 2005). As far as doping is concerned, it must be considered that high doses may be administered. In the study reported here, THG reaches an overt chromosomal genotoxicity (doubling of the MN rate in vitro) at a concentration as low as 3 μM.

After the discovery of norbolethone (Catlin et al. 2002), THG was the second hormonally active steroid identified from use in sports doping that was never developed as a human or veterinary drug (Catlin et al. 2004). As a result, its toxicological profile has not been studied. According to the present data, it cannot be ruled out that THG presents a genotoxic hazard on a chromosomal level under conditions of its current misuse in sports.

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