Assessment of chromosomal genotoxicity of steroidal hormones related to drug development

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Susanne Barbara Dorn

aus Rottweil

Oktober 2007

The present dissertation was performed according to the European Graduate College “Molecular Mechanisms in Food Toxicology”, Heinrich-Heine-Universität Düsseldorf, at the Institut für Arbeitsphysiologie an der Universität Dortmund.

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Dr. Hermann M. Bolt
1. Koreferent: Prof. Dr. Frank Wunderlich
2. Koreferent: Prof. Dr. Hartmut Greven

Tag der mündlichen Prüfung: 20.12.2007

Parts of the present thesis have been published in:

Dorn,S.B., Bolt,H.M., Thevis,M., Diel,P., and Degen,G.H. (2007) Induction of micronuclei in V79 cells by the anabolic doping steroids tetrahydrogestrinone and trenbolone. *Arch. Toxicol.* **Epub ahead of print:** [DOI 10.1007/s00204-007-0241-2].

Dorn,S.B., Degen G.H., Bolt,H.M., van der Louw,J., van Acker,F.A.A., van den Dobbelsteen,D.J., and Lommerse,J.P.M. (2007) Some molecular descriptors for non-specific chromosomal genotoxicity based on hydrophobic interactions. *Arch. Toxicol.* **Epub ahead of print:** [DOI 10.1007/s00204-007-0256-8].

Dorn,S.B., Degen,G.H., Müller,T., Bonacker,D., Joosten,H.F., van der Louw,J., van Acker,F.A.A., and Bolt,H.M. (2007) Proposed criteria for specific and non-specific chromosomal genotoxicity based on hydrophobic interactions. *Mutat. Res.* **628:** 67-75.

Table of contents

Abbreviations

8-oxoG	8-Oxoguanin
acc	Number of hydrogen bond acceptor atoms
A. dest.	aqua destillata (distilled water)
ANDRO	androstenedione
AP	apurinic/apyrimidinic
AR	androgen receptor
ARE	androgen responsive element
BCA	bicinchoninic acid
BSA	bovine serum albumin
Carboxy -H_2DCFDA	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
CAT	Chromosomal aberration test
clog D	Lipophilicity of ionizable molecules at pH=7.4
(c)log P	Lipophilicity of neutralized molecules
CREST	**C**alcinosis, **R**aynaud phenomenon, **E**sophageal dismotility, **S**clerodactyly and **T**elangiectasia (disease leading to centromere antibodies)
CTB	CellTiter Blue™
DAPI	4`6`diamidino-2-phenylindole
dipole	Molecular dipole moment
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
don	Number of hydrogen bond donor atoms
EDTA	ethylenediaminetetraacetic acid
ETHI	ethisterone
em.	emission
ex.	excitation
Equ.	Equation
FACS	fluorescence activated cell scanner
FCS	fetal calf serum (for cell culture)
Fig.	Figure
FITC	fluorescein-5-isothiocyanate
FSH	Follicle Stimulating Hormone

GFA	genetic function approximation
h	hour
HBSS	Hank's Balanced Salt Solution
IC_{20}	inhibitory concentration with cell viability reduction of 20%
IC_{50}	inhibitory concentration with cell viability reduction of 50%
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
Ig	immunoglobulin
im	intramuscular
LH	Luteinizing Hormone
log P	Lipophilicity
log S	Solubility
MAD	madol
min	minute(s)
MMS	methylmethane sulfonate
MN	micronucleus/micronuclei
MT	7α-methyltestosterone
mw	Molecular Weight
NA	19-norandrostenedione
NE	19-norethisterone
NOEC/NOEL	no-observed-effect-concentration/ -level
NR	Neutral Red
NT	19-nortestosterone
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate- buffered saline
PM	17α-propylmesterolone
polsurf	Polar surface area
PS	phosphatidylserine
QSAR	Quantitative Structure Activity Relationship
robo	Number of rotatable bonds
ROS	reactive oxygen species
rpm	rounds per minute

s	seconde(s)
SD	standard deviation
SHBG	sex hormone-binding globulin
surface	Total molecular surface
T	testosterone
Tab.	Table
TB	trenbolone
THG	tetrahydrogestrinone
Tris	tris-(hydroxymethyl)-aminomethane
V79 cells	Chinese hamster cell line (for cell culture)
v/v	volume/volume
VCR	vincristine
vol	Molecular Volume
vs.	versus
w/o	without
w/v	weight/volume
WoE	weight of evidence
Z-DEVD-R110	rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)

1 Introduction

1.1 Genotoxicity testing in drug development

In drug development the beneficial (toward) effects of a drug must be balanced against its toxic properties (untoward effects). In order to identify adverse effects of a compound, the preclinical toxicity assessment includes determination of acute and chronic toxicity, non-carcinogenic (e.g. neurotoxicity, reproduction toxicity, immunotoxicity), genotoxic and carcinogenic effects.

Genotoxicity evaluation is an integral part of the toxicological assessment of candidate compounds in preclinical drug development. As genotoxic compounds may pose a potential risk of carcinogenicity and heritable mutations, positive results in genotoxicity tests, such as clastogenicity or aneugenicity, are of key relevance in drug development. Very frequently, such positive results lead to a discontinuation of compound development (Joosten et al., 2004).

Current internationally harmonized guidelines (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S2B, 1997) recommend the conduct of *in vitro* and *in vivo* genotoxicity assays to detect the potential of candidate compounds to induce small mutations and/or chromosomal damage. In a standard genotoxicity test battery, point and frameshift mutations are usually examined by the *Salmonella typhimurium* assay *in vitro* (Ames test). On a chromosomal level, routine test procedures are the chromosomal aberration test (CAT) *in vitro* in human lymphocytes (or an *in vitro* mouse lymphoma *tk* assay). This is supplemented by an *in vivo* test for chromosomal damage using rodent hematopoietic cells (chromosomal aberrations in bone marrow cells or an analysis of micronuclei in bone marrow; ICH S2B, 1997; Müller et al., 1999).

In the development of hormonal steroids genotoxic properties of candidate compounds are taken very seriously. The research work described here is a result of a cooperation with Organon Biosciences N.V. (Oss, The Netherlands). Steroid compounds, which are presently in the preclinical drug development process at Organon, are referred to by codes (Nor-C, Nor-D, Nor-E, Nor-F, and Nor-G). A compilation of genotoxicity screening data of these drug candidates is given in Tab. 1, along with the results for the physiological androgen testosterone for comparison. This shows that positive results of chromosomal genotoxicity, assessed in the chromosomal aberration test (CAT), are an important point of discussion. Positive results in this particular test are not generally matched by positive *in vivo* data in the bone marrow micronucleus (MN) test.

Tab. 1. The physiological androgen testosterone and drug candidate steroids examined: genotoxicity profiles, according to standard genotoxicity tests

Compounds	**Ames test**	**CAT** *(in PBLs)*		**MNT** *in vivo*
		+S9	**-S9**	
Testosterone	**n.d.**	**–** (≤ 1730µM)	**–** (≤ 1130µM)	**n.d.**
Nor-C	**–**	**+** (≥ 600µM)	**+/–** (*a*: – up to 670µM; *b*: + ≥ 550µM)	**–** (≤ 2000mg/kg/d)
Nor-D	**–**	**+** (at 660µM)	**+** (≥ 430µM)	**n.d.**
Nor-E	**n.d.**	**+** (at 320µM)	**+** (at 255µM)	**n.d.**
Nor-F	**n.d.**	**–**	**+ (**≥ 400µM)	**n.d.**
Nor-G	**–**	**+ (**≥ 316µM)	**+** (at 470µM)	**–** (≤ 1000mg/kg/d)

CAT: Chromosomal aberration test
PBLs: Peripheral blood lymphocytes
MNT: Micronucleus test
a: CRO1
b: CRO2
n.d.: not detected

In toxicological screening tests it is not usual to cover a large range of concentrations; the testing strategy of the guidelines (ICH S2A, 1995) is oriented towards a limited number of tests up to cytotoxic concentrations. Usually the steroid concentrations applied in the routine chromosomal aberration test (CAT) *in vitro* are high (about 0.5mM and higher; Tab. 1), compared to those eliciting the intended pharmacological (hormonal) effect of the compounds. The rate of positive results in *in vitro* genotoxicity tests, particularly in the *in vitro* chromosomal aberration test (CAT), is known to be relatively high (Müller and Kasper, 2000; Snyder and Green, 2001). Thus, the specificity and in consequence the relevance of such positive results is debatable.

This raises the question of the toxicological relevance of effects, which are obtained at high (and even cytotoxic) concentrations only. It is connected with current discussions on threshold effects in chromosomal genotoxicity (Bolt and Degen, 2004). For years, a general assumption was that genotoxic effects cannot be linked with threshold mechanisms, while in other areas of toxicology (e.g. acute and repeated dose toxicity and reproductive toxicity) the existence of thresholds of effects was generally accepted (Madle et al., 2000). Recently, a number of "indirect" mechanisms have been described that may result in positive genotoxicity results, including inhibition of enzymes of DNA synthesis, DNA repair, or DNA-topoisomerases, and generation of reactive oxygen species (ROS; Scott et al., 1991). Such specific mechanisms are expected to exhibit no-observed-effect-

concentrations (NOECs), below which a genotoxicity would not be induced. Cytotoxicity may also contribute to indirect genotoxicity (Kirkland et al., 2007a; Kirkland et al., 2000). Additionally, processes that involve DNA degradation, like apoptosis, may lead to positive genotoxicity test results in clastogenicity assays (Meintières et al., 2001; Meintières and Marzin, 2004). Such "thresholds for genotoxicity" are a matter of current discussions (Kirkland et al., 2007b).

Approximately 30% of all marketed pharmaceuticals exhibit positive genotoxicity results when tested in the standard ICH/OECD genetic toxicology battery. Most of these molecules have not been shown epidemiologically to pose an inherent risk of cancer to patient populations at the doses and frequencies medication. This indicates the general difficulty in assigning genotoxicity-based risk estimates (Snyder, 2007), which calls for further research.

Very recently, there are movements in the interpretation and management of positive results in *in vitro* genotoxicity assays, in particular in *in vitro* mammalian cell tests, as the existing ICH guidelines on "Genotoxicity", "Carcinogenicity" and "Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals" are under revision (van Acker et al., 2007). Currently, a procedure of combining genotoxicity testing strategies has been proposed, which is shown in Fig. 1.

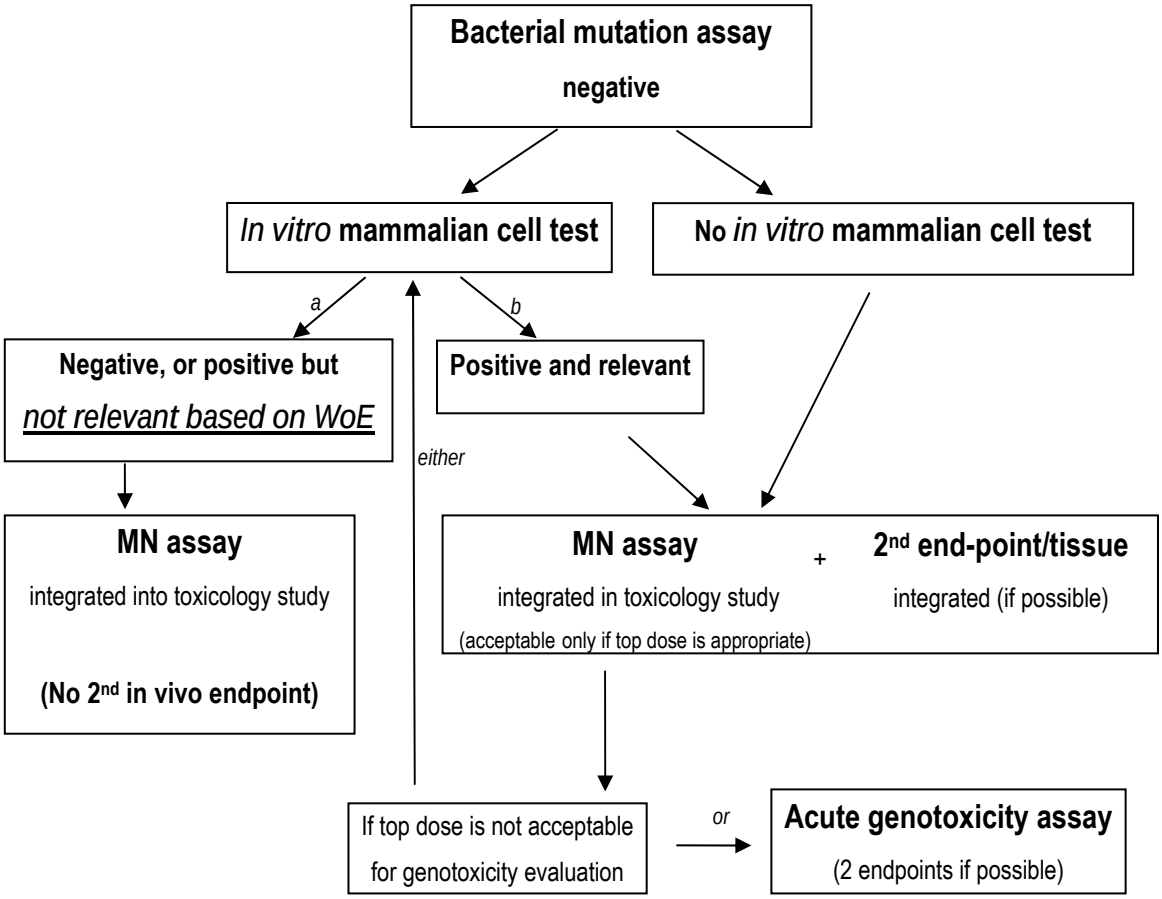


Fig. 1 Proposed revisions of guidelines for a drug genotoxicity testing strategy (according to van Acker et al., 2007); MN: micronucleus, WoE: weight of evidence.

In these guideline revisions (Fig. 1), the relevance of positive results in a single mammalian cell test *in vitro*, like the chromosomal aberration test, is assessed based on a "weight of evidence" (WoE) approach.

The chromosomal aberration test (CAT) in the standard test battery for genotoxicity testing is frequently performed in human lymphocytes with the assumption that human cells, in general, would reflect the situation in humans better than other experimental models, e.g. immortalized cell lines. However, the use of primary human cells, and of human lymphocytes in particular, has the clear disadvantage of a very wide inter-individual variability, which makes a definite interpretation sometimes difficult. Therefore, for an elucidation of mechanisms in chromosomal genotoxicity the use of a well-defined cell line may be preferable. This is the reason why V79 cells, derived from male Chinese hamster lung fibroblasts, were selected for the present investigations. This adherent cell line is widely used in toxicology research related to mutagenicity and genotoxicity testing. It is characterized by a stable diploid karyotype with 22 chromosomes and a doubling time of 12 h.

As an endpoint of chromosomal genotoxicity the formation of micronuclei (MN) was chosen for the present experiments, which plays a role of increasing importance in genotoxicity testing. The MN assay *in vitro* is easy to perform; the evaluation of MN is less subjective than that of chromosomal aberrations, and both high specificity and reproducibility of the test results have been demonstrated (von der Hude et al., 2000). An advantage is that it provides a quantitative measure (number of MN/ 1000 cells), and an additional differentiation between aneugenic and clastogenic effects can be achieved by immunostaining (CREST analysis; for methodological details, see Chapter 2.2.4.2).

1.2 Androgenic steroids of pharmacological interest

1.2.1 Androgens as drugs

Physiological androgens are 19-carbon hormonal steroids stimulating or controlling the development and maintenance of masculine characteristics in vertebrates. This includes the development activity of the accessory male sex organs. Additionally, most important is their protein-anabolic property. The primary androgenic testicular secretion product is testosterone (Fig. 2).

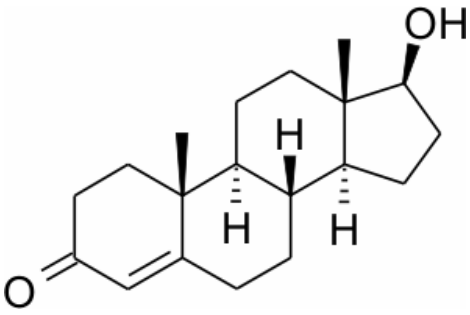


Fig. 2. Structure of the physiological androgen testosterone

In males, testosterone is produced in the testes under the endocrine regulation of Luteinizing Hormone (LH). It is also formed in the adrenal glands and in female ovaries. Testosterone and Follicle Stimulating Hormone (FSH) are essential hormones regulating the testicular sperm production.

The basic cellular responses to androgens are mediated through the androgen receptor (AR). This intracellular ligand binding protein belongs to the family of nuclear transcription factors influencing the transcription of a large number of genes through interaction with palindromic DNA sequences, the “androgen responsive elements” (ARE; Fig.3; Beato et al., 1996).

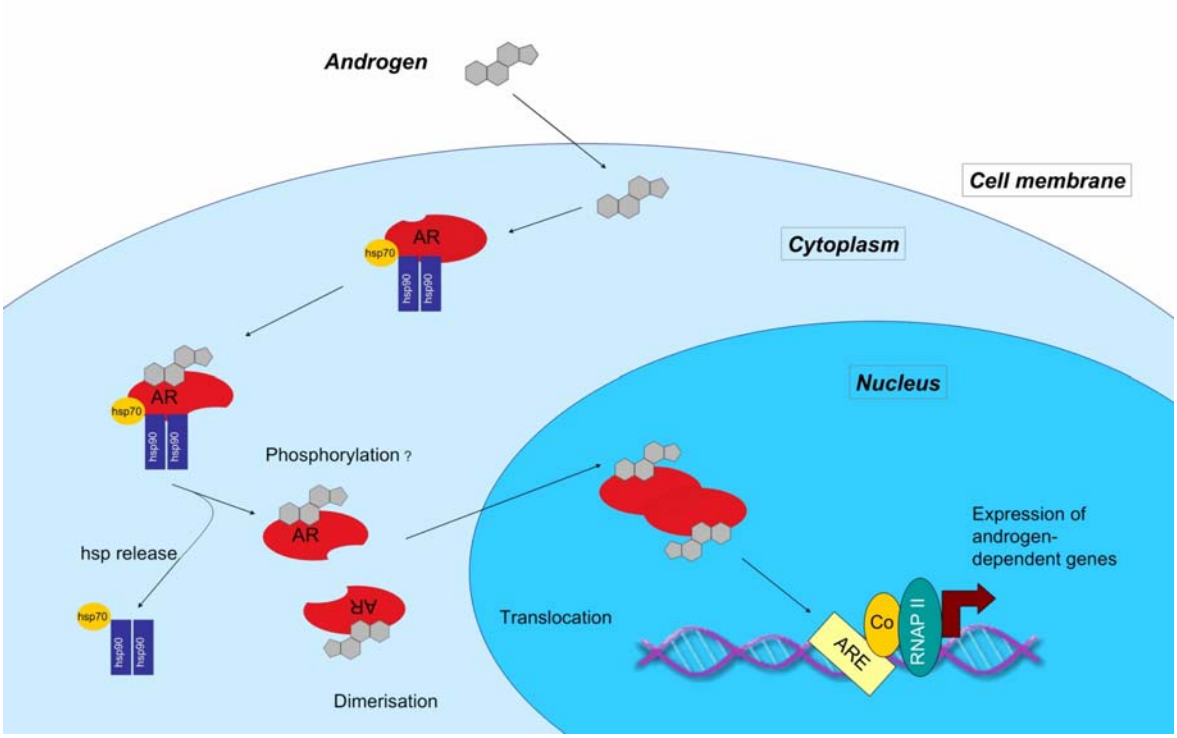


Fig. 3. Schematic overview on the cellular androgen response via androgen receptor (AR) activation. Hsp: heat shock protein; AR: androgen receptor; ARE: androgen responsive element; Co: cofactors; RNAP II: RNA polymerase II (Gobinet et al., 2002).

The daily natural testosterone production is ~7 mg (24 µmol) in men; in females it is about 10% of this (Forth et al., 1998). In the blood plasma, testosterone circulates 98% protein-bound, 54% with low affinity binding to albumin and other proteins, 44% with high affinity binding to the sex hormone-binding globulin (SHBG); only 2% of circulating testosterone is free (not bound to proteins; Södergård et al., 1982). SHBG-bound testosterone is regarded as biologically inactive (Mutschler et al., 2001).

In andropausal men ("climacterium virile"; Partial Androgen Deficiency in Aging Males (PADAM)) and during hypogonadism (due to e.g. castration after testicular tumors, eunuchism, pituitary disfunction, "pubertas tarda"), lower levels of testosterone are seen. Decreased testosterone levels give rise to a range of clinical manifestations, e.g. loss of bone mass due to increased bone resorption, loss of muscle mass, reduction of libido, and increase in serum LH. For androgen replacement therapy and also for potential application in male hormonal contraception, new, potent, and orally active androgens are developed.

Testosterone is an androgen with a relatively low oral potency, as it is rapidly metabolized (first-pass effect of metabolism in the liver).

An approach to achieve a slower metabolism and thereby a higher oral effectiveness is the introduction of side chains and substituents into the testosterone molecule. For example, esterification of the 17β-OH group of testosterone with long-chain fatty acids leads to testosterone esters, which serve as “pro-drugs” (Grootenhuis et al., 2004). Intramuscular (im) injection of testosterone esters in oily solutions results in a depot from which the compound is slowly released. Commonly used esters are testosterone propionate, undecanoate, and anantate. The androgen esters are hydrolyzed by esterases and subsequently the androgen can activate its receptors in the target organs (Bursi et al., 2001).

The effects of androgens are of pharmacological interest. In the past, “catabolic states” were considered as indications for anabolic steroids, ranging from reduced general condition, especially in geriatrics and reconvalescence, to consuming diseases, destructive bone processes and muscular dystrophy, and to radio- and cytostatic therapy of malignant tumors. Such applications are now obsolete (Forth et al., 1998; Mutschler et al., 2001), and anabolic steroids are only indicated in the therapy of aplastic anemia. The most commonly used anabolic steroid is 19-nortestosterone, a testosterone derivative demethylated at C19 (“19-nor”), and its esters. However, with this compound, a dissociation of anabolic and androgenic effects through chemical modifications is fulfilled only incompletely.

Therefore, new potent androgens, which are orally effective and metabolically stable, and in which the anabolic and androgenic properties are separated, are of continued interest for further pharmaceutical development.

1.2.2 Compounds examined in the present study

In the present study a set of different steroids was investigated with regard to their chromosomal genotoxicity. These were mainly androgens, but progestational compounds were also included. Some of the compounds showed both properties. Beside compounds with a (potential) pharmacological use, also substances were studied, which are currently misused for doping in sports (19-nortestosterone, 19-norandrostenedione, madol, tetrahydrogestrinone, and trenbolone). Fig. 4 shows the structures of the steroids studied. Further candidate steroids, which are currently in the drug development process, are coded as Nor-C, Nor-D, Nor-E, Nor-F, and Nor-G.

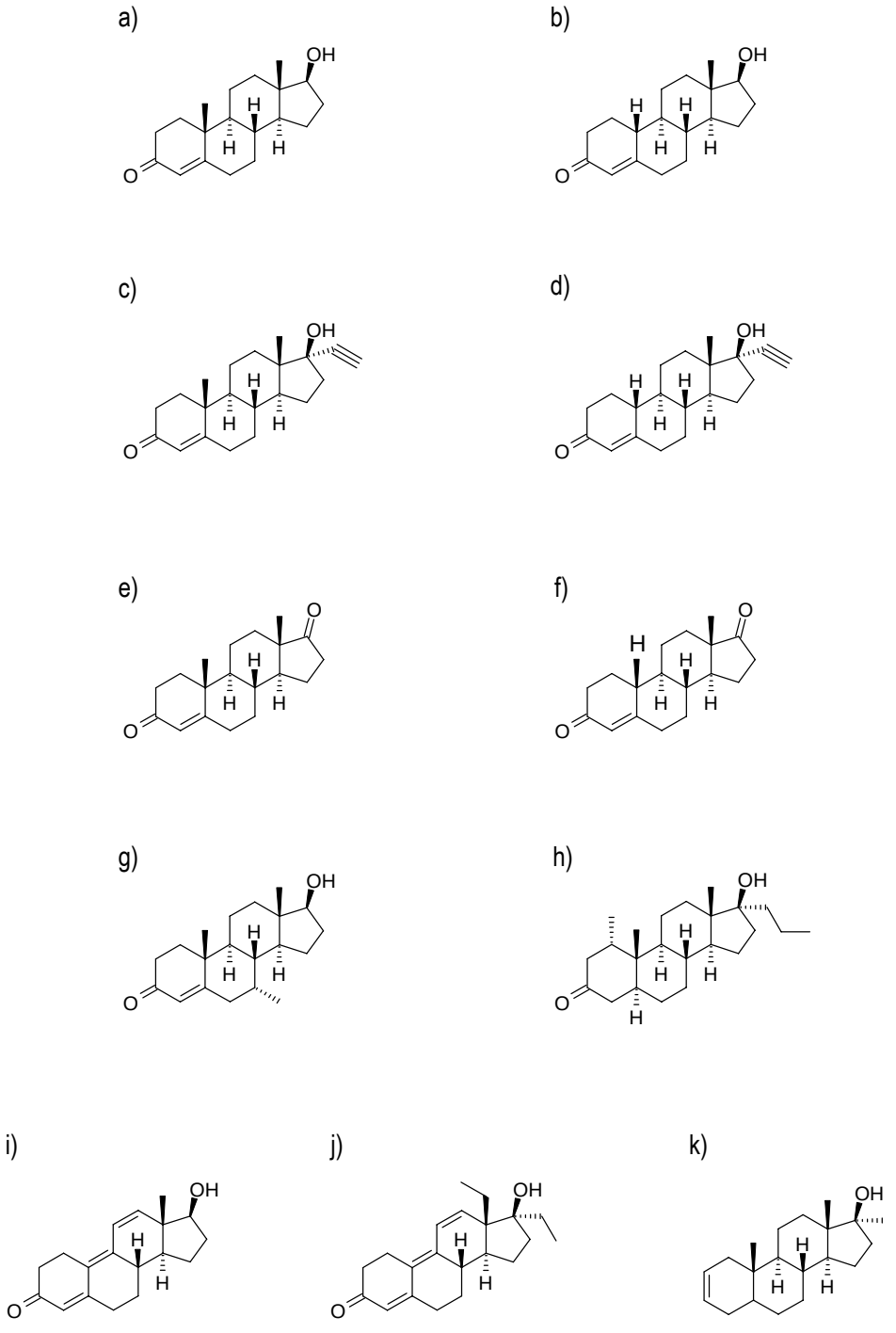


Fig. 4. Structures of the steroids studied in the present investigation: a) testosterone (T), b) 19-nortestosterone (NT), c) ethisterone (ETHI), d) 19-norethisterone (NE), e) androstenedione (ANDRO), f) 19-norandrostenedione (NA), g) 7α-methyltestosterone (MT), h) 17α-propylmesterolone, i) trenbolone (TB), j) tetrahydrogestrinone (THG), k) madol (MAD)

1.3 Relationship of molecular properties and chromosomal genotoxicity

In a series of publications *Agneta Önfelt* has addressed basic processes underlying chromosomal genotoxicity, using V79 cells as a stable experimental model (Önfelt, 1983; Önfelt, 1986; Önfelt, 1987a; Önfelt, 1987b; Önfelt and Klasterska, 1983; Schultz and Önfelt, 2000). Initially, she has studied spindle disturbances, especially the induction of c-mitosis in mammalian cells as a process leading to abnormal chromosome numbers. She also discussed compound-specific mechanisms that may lead to chromosomal genotoxicity (Önfelt, 1983; Önfelt, 1986). A concept was introduced that the lipophilic (hydrophobic) character of chemicals determines a background genotoxicity, which was declared as “non-specific”. Such non-specific interference of organic xenobiotics with relevant processes of cytokinesis and karyokinesis may occur based on the partitioning of the chemical into cellular hydrophobic compartments (Önfelt, 1987a). For a number of compounds with a “non-specific” mode of action, Önfelt (1987a) demonstrated an empirical dependence of their c-mitotic effect on lipophilicity. The latter was quantitatively described by the octanol-water partition coefficient log P, referring to the equilibrium distribution of the solute between the two liquid phases, the lipophilic n-octanol and the lipophobic water, providing a thermodynamic measure of the lipophilicity of compounds.

Some compounds showed higher genotoxicity than predicted on the basis of their lipophilicity. These could be linked to more specific modes of action, e.g. chlorophenols, colcemid, carbaryl, methyl mercury, caffeine, and some glutathione-specific agents (Önfelt, 1987a; Önfelt, 1987b; Önfelt and Klasterska, 1983).

The original concept connecting lipophilicity and genotoxicity on a chromosomal level was further corroborated by Schultz and Önfelt (2000). Aneuploidy in Chinese hamster V79 cells - studied by assessing the induction of bi- and multinucleated cells - was elicited by a variety of lipophilic organic chemicals, including aliphatic hydrocarbons and alcohols. This was opposed to effectors with a more specific known mode of action, like colcemid, cytochalasin B or diamide. Again, effectors with a known or likely specific mode of action were more active, i.e. they acted at concentrations that were consistently lower, than predicted on the basis of their lipophilicity (log P). Schultz and Önfelt (2000) generally proposed to distinguish between specific and non-specific action in the screening of potential aneugens based on lipophilicity.

Fig. 5 presents the data, as published by Schultz and Önfelt (2000). It demonstrates the relationship between lipophilicity (Log P, referring to the oil-water partition coefficient) and chromosomal genotoxicity (-Log C, with C as the concentration needed to reach the threshold of doubling of binucleated cell counts in V79 cell cultures).

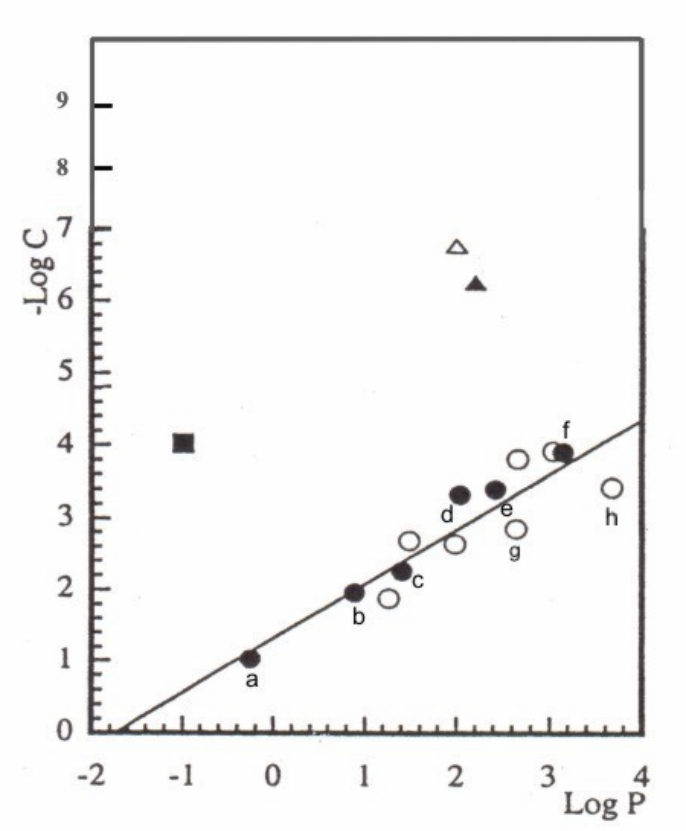


Fig. 5. Original data set as plotted by Schultz and Önfelt (2000) (modified): relationship between concentrations (C) inducing a two-fold increase in bi-nucleated V79 cells after 24 h and the octanol/water partition coefficient (P). Alcohols (closed circles): (a) ethanol; (b) butanol; (c) pentanol; (d) hexanol; (e) heptanol; (f) octanol. Aliphatics (open circles): (g) carbon tetrachloride; (h) pentachloroethane. Specific interactants: cytochalasin B (closed triangle); diamide (closed square) colcemide (open triangle; 10-fold increase of bi-nucleated cells).

So far, molecular descriptors other than log P describing the physicochemical properties of molecules have not been applied to distinguish between specific and non-specific chromosomal genotoxicity different compounds induce.

1.4 Aim of the present thesis

In view of the "weight of evidence" issue in the interpretation of genotoxicity results, as described above, the aims of the present study were defined as follows:

- It had to be investigated, whether the concept of Schultz and Önfelt (2000) and its underlying principles are applicable to hormonal steroids, which are of interest in drug development. Therefore, the genotoxicity database of Schultz and Önfelt was broadened; aneugenic as well as clastogenic compounds were included in order to differentiate between specific and non-specific chromosomal genotoxicity based on the concept of hydrophobic interactions. Datasets used for this purpose were the original dataset of Schultz and Önfelt (2000), existing sets of micronucleus assay data from the own laboratory, and new data generated on hormonal steroids in the course of the present work.
- In addition, mechanistic backgrounds of the effects of chromosomal genotoxicity should be studied. In particular, the induction of apoptosis, the formation of reactive oxygen species, the influence of cytotoxicity and lipophilicity as well as the compounds´ potency to induce a cell cycle arrest were addressed.
- As molecular descriptors other than log P had not been used so far to distinguish between specific and non-specific chromosomal genotoxicity, further procedures used in Quantitative Structure Activity Relationship (QSAR) modelling had to be applied to the experimental data sets, in order to develop a quantitative concept linking physicochemical parameters of test compounds with chromosomal genotoxicity. The introduction of additional molecular descriptors was expected to give further insights into underlying processes of genotoxicity.
- A final goal was the development of a general procedure to screen specific *versus* non-specific modes of action, with a potential of application for the safety assessment in chemical or pharmaceutical product development.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and biochemicals

2-Mercaptoethanol	Sigma-Aldrich (Taufkirchen, D)
4´,6´-Diamidino-2-phenyl-indole (DAPI)	Sigma-Aldrich (Taufkirchen, D)
5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCF-DA)	Invitrogen (Karlsruhe, D)
Acridine orange	Sigma-Aldrich (Taufkirchen, D)
Anti-Human IgG, F(ab')$_2$ fragment – FITC (goat)	Sigma-Aldrich (Taufkirchen, D)
Bovine serum albumin (BSA)	New England BioLabs (Frankfurt, D)
Camptothecin	Sigma-Aldrich (Taufkirchen, D)
Dimethyl sulfoxide (DMSO)	Merck (Darmstadt, D)
Dinatriumhydrogenphosphate (Na_2HPO_4 + 2 H_2O)	Merck (Darmstadt, D)
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich (Taufkirchen, D)
Hydrogen peroxide (H_2O_2)	Sigma-Aldrich (Taufkirchen, D)
“Positive control centromere serum“ (“CREST-serum“)	DPC Biermann (Bad Nauheim, D)
Potassium dihydrogen phosphate (KH_2PO_4)	Merck (Darmstadt, D)
Methylmethane sulfonate (MMS)	Merck (Darmstadt, D)
Neutral Red (NR)	Sigma-Aldrich (Taufkirchen, D)
Propidium iodide	Sigma-Aldrich (Taufkirchen, D)
RNase type I-A, 86 Kunitz units/mg protein	Sigma-Aldrich (Taufkirchen, D)
Spermine tetrahydrochloride	Sigma-Aldrich (Taufkirchen, D)
Tris-(hydroxymethyl)-aminomethane (Tris)	Merck (Darmstadt, D)
Triton® X-100	Fluka (Buchs, CH)
Trypan Blue (0.5%)	Serva (Heidelberg, D)
Tween® 20	Sigma-Aldrich (Taufkirchen, D)
Vincristine (VCR)	Sigma-Aldrich (Taufkirchen, D)

Other standard laboratory chemicals were purchased in *p.a.* quality from Sigma-Aldrich (Taufkirchen, D), Merck (Darmstadt, D), Roth (Karlsruhe, D), and Serva (Heidelberg, D).

2.1.2 Hormonally active steroids

7α-Methyltestosterone (MT)	Organon (Oss, NL)
17α-Propylmesterolone (PM)	Organon (Oss, The NL)
19-Norandrostenedione (NA)	Dr. M. Thevis (German Sport University, Cologne, D)
19-Norethisterone (NE)	Sigma-Aldrich (Taufkirchen, D)
19-Nortestosterone (NT)	Sigma-Aldrich (Taufkirchen, D)
Androstenedione (ANDRO)	Organon (Oss, NL)
Ethisterone (ETHI)	Organon (Oss, NL)
Madol (MAD)	Dr. M. Thevis (German Sport University, Cologne, D)
Testosterone (T)	Sigma-Aldrich (Taufkirchen, D)
Tetrahydrogestrinone (THG)	Dr. M. Thevis (German Sport University, Cologne, D)
Trenbolone (TB)	Organon (Oss, NL)

Other hormonally active steroids, being in the commercial drug development phase, were provided by Organon (Oss, NL). These are coded here as:

Nor-C
Nor-D
Nor-E
Nor-F
Nor-G

2.1.3 Kits

Annexin V-FITC Apoptosis Detection Kit I®	BD Biosciences Pharmingen (Heidelberg, D)
Apo-ONE® Homogeneous Caspase-3/7 Assay	Promega (Mannheim, D)
BCA “Uptima” Assay	Interchim (Mannheim, D)
CellTiter-Blue™ Cell Viability Assay	Promega (Mannheim, D)

2.1.4 Consumables

Cell culture flask, (25; 75; 175 cm²)	Greiner (Frickenhausen, D)
Centrifuge tubes, sterile (12; 15; 50 ml)	Greiner (Frickenhausen, D)
Coverslips (24 mm x 60 mm)	Menzel-Gläser (Braunschweig, D)
CryoTubes™	Nunc (Wiesbaden, D)
Disposable sterile filters (Ø 0.22 µm) Millex™	Millipore (Eschborn, D)
Disposable syringes (5; 10; 20 ml) Omnifix®	B|Braun (Melsungen, D)
Microscope slides (26 mm x 76 mm), frosted end	Menzel-Gläser (Braunschweig, D)
Micro test tubes (1.5 ml; 2.0 ml)	Eppendorf (Hamburg, D)
Pipette tips (10; 200; 1000 µl)	Eppendorf (Hamburg)/ Greiner (Fricken-hausen, D)
Polystyrene tube, round bottom (5 ml, 12 x 75 mm)	Falcon™ Becton Dickinson (Heidelberg, D)
quadriPERM™ cell culture vessels	Vivascience (Hannover, D)
Tissue culture plates, 6- and 24-well, „low evaporation lid“	Falcon™ Becton Dickinson (Heidelberg, D)
Tissue culture plates, 96 well, clear	Costar (VWR International, Bruchsal, D)
Tissue culture plates, 96-well, black, clear bottom	Costar (VWR International, Bruchsal, D)
Transfer pipettes, sterile	Sarstedt (Nümbrecht, D)

2.1.5 Instruments

Analysis balance AE 240	Mettler (Bergisch Gladbach, D)
Autoclave Varioklav®	H+P Labortechnik (Oberschleißheim, D)
CO_2-Incubator HERAcell 240	Heraeus (Hanau, D)
Cryogenic refrigerator, RS Series	Taylor-Wharton (Husum, D)
Digital camera DC 300FX	Leica (Wetzlar, D)
Digital camera DSC-S85	Sony (Köln, Deutschland)
Digital video camera PE 2020P	Pulnix (Alzenau, Deutschland)
Flow cytometer FACSCalibur™	Becton Dickinson (Heidelberg, D)
Fluorescent microscope DM LB	Leica (Wetzlar, D)
Fluorescent microscope DM RB	Leica (Wetzlar, D)
Freezer, -20°C, Öko Plus	Siemens (München, D)
Freezer, -70°C, Ult Freezer 994	Heraeus (Hanau, D)

Hemocytometer, Neubauer	Brand (Wertheim, D)
Hotplate SB 160 Stuart®	VWR (Darmstadt, D)
Ice flaker Scotsman® AF 100	Frimont (Milan, IT)
Incubator	Memmert (Schwabach, D)
Laminar Flow HERAsafe	Kendro (Hanau, D)
Light microscope, inverse, IM 35	Zeiss (Jena, D)
Magnet stirrer	Heidolph (Kelheim, D)
Megafuge 1.0 R	Heraeus (Hanau, D)
Microcentrifuge 5415	Eppendorf (Hamburg, D)
Microcentrifuge Biofuge fresco	Heraeus (Hanau, D)
Microplate reader, Spectrafluor Plus	Tecan Deutschland GmbH (Crailsheim, D)
Microwave	Bosch (Stuttgart, D)
Pipetboy acu	Integra Biosciences (Fernwald, D)
Pipettes (10 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf (Hamburg, D)/ Gilson (Bad Camberg, D)
Pipette, 8-channel, Discovery, 50 – 200 µl	Abimed (Langenfeld, D)
Pipette, 8-channel, Finnpipette® 5 – 50 µl,	Thermo Labsystems (Dreieich, D)
Pipette, 8-channel, multistepper, 50 - 1200 µl	Eppendorf (Hamburg, D)
pH meter CG 825	Schott (Mainz, D)
Precision balance PB 602	Mettler (Bergisch Gladbach, D)
Shaker MTS 2	IKA® Labortechnik (Staufen, D)
Thermomixer “Comfort”	Eppendorf (Hamburg, D)
Vortex “Reamix 2789”	Hartenstein (Würzburg, D)
Water bath 1083	GFL (Burgwedel, D)

2.1.6 Cell line

V79 hamster lung fibroblasts were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, D): DSMZ no. ACC 335.

2.1.7 Solutions and buffers

For the preparation of buffers and dilution of stock solutions distilled water was used in general.

2.1.7.1. Ready-for-use solutions

RPMI Medium 1640, with L-glutamine, w Phenol Red	Gibco® Invitrogen (Karlsruhe, D)
RPMI Medium 1640, with L-glutamine, w/o Phenol Red	Gibco® Invitrogen (Karlsruhe, D)
Dulbecco's PBS (10X), w/o Ca^{2+} and Mg^{2+} (10x)	Gibco® Invitrogen (Karlsruhe, D)
Fetal calf serum (FCS)	Biochrom (Darmstadt, D)
Hank's Balanced Salt Solution (HBSS)	Gibco® Invitrogen (Karlsruhe, D)
Trypsin-EDTA (0.25 % Trypsin, 0.02 % EDTA)	Gibco® Invitrogen (Karlsruhe, D)

2.1.7.2. Cell culture

Cell culture medium (complete medium)

RPMI 1640

10 % (v/v) fetal calf serum

Trypan Blue working solution, 0.05%

Trypan Blue stock solution (0.5%)

1:10 in PBS

2.1.7.3. Cytotoxicity assays

Neutral Red uptake assay

Neutral Red solution

Stock solution

4 mg/ml Neutral Red

The stock solution was shaken for at least 1h at 37°C and stored at 4°C in the dark.

Working solution

50 µg/ml Neutral Red
in cell culture medium (w/o FCS)

The working solution was prepared about 18 h before usage, loosely covered with aluminium foil and preincubated for 18 h in the incubator (37° C, 5 % CO_2). Before usage, the solution was filtered using a 0.22 µm filter Unit Millex-GP.

Neutral red fixative solution

50 % (v/v) ethanol
1 % (v/v) acetic acid

CellTiter Blue™ assay

CellTiter Blue working solution

20 % (v/v) CellTiter Blue™
in HBSS

The solution was prepared freshly before use.

BCA "Uptima" Assay

BCA working reagent

2 % (v/v) reagent B
in reagent A

Both components were elements of the BCA "Uptima" Assay Kit (see Chapter 2.1.3). The solution was prepared freshly before use.

2.1.7.4. Micronucleus assay

Standard assay

Acridine Orange solution

Stock solutions

1 mg/ml Acridine Orange

300 mM Na_2HPO_4

300 mM KH_2PO_4

Working solution

50 µg/ml Acridine Orange stock solution

21 mM Na_2HPO_4

21 mM KH_2PO_4

Methanol-acetic acid fixation solution

75 % (v/v) methanol

25 % (v/v) acetic acid

The fixation solution was prepared 24 h before use and was stored at –20° C.

KCl solution, 0.4 %

The 0.4 % (w/v) KCl solution was prepared 24 h before use, sterilized, and stored at 4° C.

CREST analysis

Soerensen buffer (pH 6.8)

Stock solutions

300 mM $Na_2HPO_4 \times 2H_2O$

300 mM KH_2PO_4

Working solution

15 mM $Na_2HPO_4 \times 2H_2O$

15 mM KH_2PO_4

CREST staining solution

Stock solutions

1 µg/ml DAPI
150 µM propidium iodide

Working solution

DAPI/propidium iodide 3:1

Antifade

Stock solution

10 mg/ml p-phenylenediamine
in PBS

Working solution

1:10 in glycerol

The antifade working solution was stored at 4° C in the dark.

2.1.7.5. Cell cycle analysis

Lysis buffer LB01

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

The pH was adjusted to 7.5 with 1 N HCl before 15 mM 2-mercaptoethanol was added. The buffer was filtered through a 0.22 µm filter Unit Millex-GP and stored at – 4° C.

Propidium iodide (PI) solution

1.5 mM propidium iodide

The PI solution was filtered through a 0.22 µm filter Unit Millex-GP and stored at -20° C as 500 µl aliquots.

RNase solution

10 mg/ml RNase

The solution was filtered through a 0.22 µm filter Unit Millex-GP and stored at -20° C as 500 µl aliquots. Before usage the solution was heated to 90° C for 15 min to inactivate DNases.

2.1.7.6. Apoptosis detection

Annexin V-FITC/PI assay

Binding buffer

Stock solution, 10 x

0.1 M Hepes/NaOH (pH 7.4)
1.4 M NaCl
25 mM $CaCl_2$

The 10x binding buffer stock solution was included in the Annexin V-FITC Apoptosis Detection Kit I® (see 2.1.3)

Working solution, 1 x

Binding buffer stock solution (10x)
1:10 in A. dest.

Caspase- 3/7 assay

Caspase-3/7 reagent

1% (v/v) Caspase-3/7 substrate Z-DEVD-R110
in Apo-ONE® Homogeneous Caspase-3/7 Buffer

Both components were elements of the Apo-ONE® Homogeneous Caspase-3/7 Assay Kit (see 2.1.3). The Caspase-3/7 reagent was prepared freshly before use.

2.1.7.7. Detection of reactive oxygen species

Carboxy -H_2DCF-DA solution

Stock solution

100 mM Carboxy-H_2DCF-DA
in DMSO

Working solution

100 µM

in cell culture medium

2.1.7.8. Test compounds

The stock solutions of the tested compounds were prepared in dimethyl sulfoxide (DMSO) and stored at –20°C in the dark.

2.1.8 Software

Advanced Chemistry Development v4.76 (ACD) software	Advanced Chemistry Development, Inc. (Toronto, CA)
CellQuest Pro®	BD Biosciences (Heidelberg, D)
Clog P 4.10	BioByte Corp. (Claremont, CA, USA)
Corina 3.20	Molecular Networks GmbH (Erlangen, D)
logS Cerius^2 4.10	Accelrys Inc. (San Diego, CA, USA)
monika 3.6	NV Organon (Oss, NL)
Sigma Plot 8.0	Systat Software Inc. (Erkrath, D)
SPSS 14.0 for Windows	SPSS Inc. (München, D)

2.2 Methods

2.2.1 Cell culture

All cell culture working steps were performed on sterile working conditions using a laminar flow bench. The liquids applied for cell culture were pre-warmed to 37° C in the water bath before usage.

2.2.1.1. Culture and subculture

For the *in vitro* experiments in the present study, the adherently growing cell line V79 derived from lung fibroblasts of the male Chinese hamster was used. The cells were cultivated as monolayer cultures in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS). An atmosphere of relative humidity of ~ 95%, 5% CO_2 in air, and a temperature of 37°C was provided in a CO_2 incubator.

The cells were grown in 75 cm^2 or 175 cm^2 cell culture flasks in 20 ml and 30 ml medium, respectively, depending on the cell number required for the experiments. Confluent cell cultures (3-4 days after seeding) were harvested and subcultured. The culture medium was aspirated and the cell monolayer was rinsed once with trypsin-EDTA (0.25% trypsin, 0.02% EDTA). Again, cells were exposed to trypsin-EDTA (3 ml/ 75 cm^2 flask; 5 ml/ 175 cm^2 flask) for 3-5 min, in which the detachment of the cells was controlled in the phase contrast microscope. By addition of medium with 20% FCS the trypsin activity was stopped, cells were transferred to sterile centrifugation tubes and centrifuged at *200 x g* for 8 min at room temperature. The pellet was resuspended in 10 ml of complete medium.

The number of viable cells was determined by means of the Trypan Blue staining. This vital stain colors dead cells blue; in viable cells with intact cell membranes the azo dye Trypan Blue is not absorbed, hence, these cells remain unstained.

100 µl of the cell suspension were mixed with 900 µl of the Trypan Blue working solution. The suspension was placed on a hemocytometer and viable (unstained) cells were counted in the 4 outer large quadrants (defined volume: 0.1 µl, i.e. chamber factor 10^4). The mean value was taken to calculate the density of viable cell in the suspension according to:

Cell number/ml = number of counted cells (mean value) x dilution factor x chamber factor

Cells were placed in new culture vessels. To maintain the culture the cells were seeded in culture flasks at a cell density of 4 x 10^5 per 75 cm^2 flask or 7 x 10^5 per 175 cm^2 flask. Continuous cultures were maintained up to a passage number of 30.

For performance of an experiment the cells were seeded in appropriate test vessels (see respective experimental procedure description).

2.2.1.2. Cryopreservation

V79 cells were stored as cryopreserved stocks. Therefore, 2 x 10^6 cells/ml were frozen in sterile cryotubes in complete medium supplemented with 10 % DMSO at –20° C. After 1 h they were transferred to –70°C. For long-term preservation the cells were stored in liquid nitrogen (-196°C).

2.2.2 Substance exposure

In each test, for substance exposure the cells were treated with the test compounds as DMSO solutions in culture medium with 10% FCS. The final concentration of DMSO in each sample was constant with 0.1 % (v/v). The presence of any precipitated material in the cultures at start and finish of the incubation period was recorded.

2.2.3 Cytotoxicity tests

Cytotoxicity of the test compounds was determined in V79 cell monolayer cultures by means of the Neutral Red uptake assay (Borenfreund and Puerner, 1985) or by means of the CellTiter Blue™ Assay (Nociari et al., 1998; O'Brien et al., 2000) to indicate a range of concentrations (at and below cytotoxicity) suitable to examine the chemicals in the micronucleus assay. Some compounds were tested in both assays.

2.2.3.1. Neutral Red uptake assay

Background

The Neutral Red uptake assay developed by Borenfreund and Puerner (1985) is used for cytotoxicity testing in monolayer cell cultures. It is based on the ability of viable cells to incorporate the weak cationic supravital dye Neutral Red (NR; 3-amino-7-dimethylamino-2-methylphenazine hydrochloride) via non-ionic diffusion and accumulate it in the lysosomes. Accumulation of NR in the lysosomes is presumed to be due to trapping of the protonated form of the dye within the acid milieu of lysosomes on one hand. Binding to fixed acidic charges in the lysosomal matrix (e.g. acidic polysaccharides) is also thought to play a role (Bulychev et al., 1978). Alterations of the cell surface or the

sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible (Bitensky, 1963). Cellular damage induced by xenobiotics results in a decreased retention and accumulation of NR in lysosomes (Filman et al., 1975). The amount of accumulated NR in the lysosomes can be detected photometrically after elution and colour intensity is indicative of the cell viability (Borenfreund and Puerner, 1985).

Experimental procedure

The Neutral Red uptake assay was performed according to the protocol of Babich and Borenfreund (1992). 1 x 10^4 V79 cells were plated in 100 µl medium per well in 96-well tissue-culture plates (rows 3-12) and allowed to grow for 24 h at 37° C. Then medium was changed and the cells were treated with test substances, medium or solvent control (DMSO 0.1 %). Each test agent concentration was supplied to 8 wells (i.e. one row of the microtiter plate) by means of a multichannel pipette, according to the following scheme (Fig. 6).

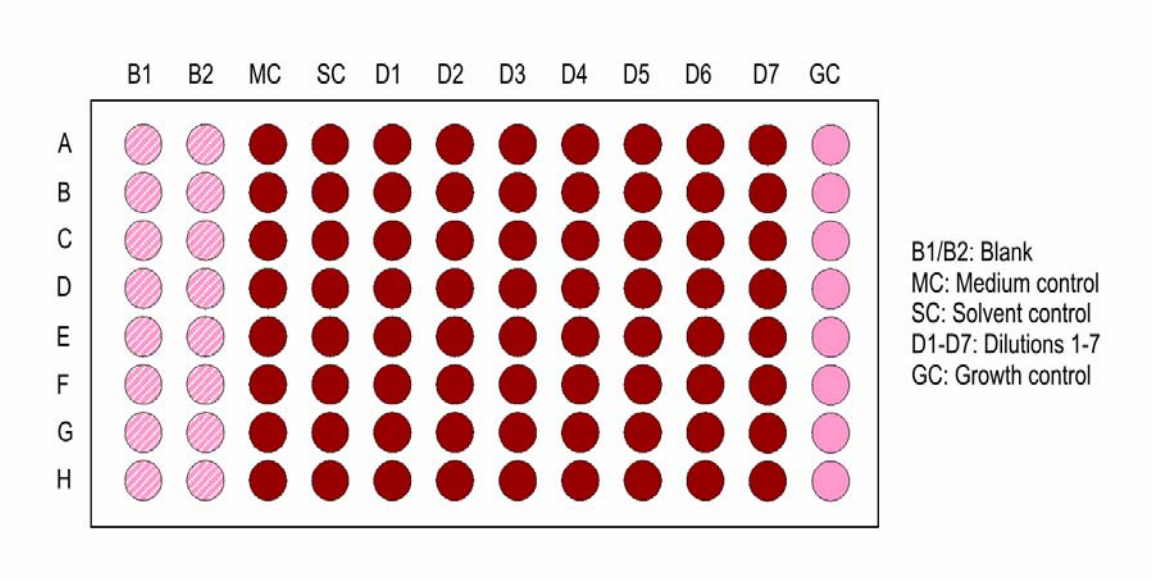


Fig. 6. Pipetting scheme for cytotoxicity test.

The final solvent concentration in the culture medium was 0.1%. Row 12 was left without medium for measurement of cell growth (Chapter 2.2.3.3). The cells were treated for 18 h (1.5 cell cycles) at 37° C in 5 % CO_2 in air. After treatment the medium was replaced by medium containing Neutral Red (50 µg/ml) and incubation was continued for 3 h at 37°C. The cells were washed five times with pre-warmed PBS (1x) and subsequently fixed with 200 µl fixative (1 % (v/v) acetic acid, 50 % (v/v) ethanol) per well upon 20 min of shaking (600 rpm) which brings neutral red into solution. The ab-

sorbance of NR was measured with a plate photometer at 540 nm. Per tested compound, at least three independent assays were performed.

Data analysis

Mean values were calculated from the 8 wells treated with one substance concentration. The results obtained in independently performed assays were combined, mean values and standard deviations were determined. NR uptake in the samples was indicated in percent of solvent control.

IC_{20} and IC_{50} values - inhibitory concentrations were cell viability was reduced by 20 % or 50 %, respectively - were taken from the concentration-response-curves.

2.2.3.2. CellTiter Blue™ assay

Background

The CellTiter Blue™ assay is a fluorometric method using the dye resazurin for measurement of cell viability. This blue and nonfluorescent dye is metabolically reduced to resorufin (pink and highly fluorescent) by living cells. The cells may either induce a reduction of the medium or enzymatically reduce resazurin intracellularly (O'Brien et al., 2000). Damaged and non-viable cells rapidly lose metabolic capacity and thus do not reduce the indicator dye. Cell viability can be measured either by colourimetry or fluorimetry; however, greater sensitivity is achieved using the fluorescent property (O'Brien et al., 2000). The fluorescence intensity is correlated with the number of viable cells.

Experimental procedure

The CellTiter Blue™ assay was performed according to the manufacturer's instructions with slight modifications. V79 cells were seeded in 96-well microtiter plates (5 x 10^3 cells/well in 100 µl) and cultured for about 24 h before substance treatment. The medium was changed and the cells were treated with different concentrations of the test substance in medium or with solvent (DMSO 0.1 %) alone: Each test agent concentration was supplied to 8 wells, i.e. one row of the microtiter plate, according to the scheme in Fig. 6. Row 12 was left without medium for measurement of cell growth (Chapter 2.2.3.3). The cells were treated with the test compounds for 18 h (about 1.5 cell cycles). As serum protein in culture medium was found to depress the reduction of resazurin (Goegan et al., 1995) the cells were – in contrast to the manufacturer's instructions - washed twice with warm HBSS after the substance treatment and subsequently incubated with 20 % (v/v) CellTiter Blue™ in HBSS for 3 h. The fluorescence intensity was read at 540 nm (ex.) and 595 nm (em.) in the microplate reader. Per tested compound, at least three independent assays were performed.

Data analysis

Mean values were calculated from the 8 wells treated with one substance concentration. The results obtained in independently performed assays were combined, mean values and standard deviations were determined. CellTiter Blue™ reduction in the samples was indicated in percent of solvent control. IC_{20} and IC_{50} values were taken from the concentration-response-curves.

2.2.3.3. Measurement of protein content

Background

The Bicinchoninic Acid (BCA) Assay for protein detection was developed by Smith et al. (1985). Bicinchoninic acid sodium salt is used for the monitoring of cuprous ion (Cu^{+}) produced during reduction of Cu^{2+} by peptidic bounds of proteins in an alkaline environment (biuret reaction). BCA chelates Cu^{+} ions with very high specificity to form a water soluble purple coloured complex. Absorbance is directly proportional to the protein concentration and can be detected photometrically.

Experimental procedure

The plates used in the cytotoxicity assays were prepared for protein detection as follows: after measurement of absorbance (NR Uptake Assay, Chapter 2.2.3.1) or fluorescence (CellTiter Blue™ Assay, Chapter 2.2.3.2), respectively, cells were washed five times with 200 µl warm (37° C) PBS. The plates were left to dry and 20 µl 0.1 % Triton X-100 were added to each well to destroy the cell membranes. The plates were kept at -70° C for at least 30 min. After thawing, the cell lysis was checked in the microscope. Then, the BCA Assay was performed, according to the manufacturer's instructions:

The BCA working reagent (reagent A : reagent B, 50:1) was prepared. Bovine serum albumin in defined concentrations (2.0, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/ml in A. dest.) was used as reference; in repeated determination, 10 µl of each concentration were given into two wells in rows 1 and 2 (no cells). Per well 200 µl of the BCA working reagent were added, the plates were shaken for 30 s (600 rpm) and incubated for 30 min at 37° C. The absorbance was measured at 570 nm.

By comparison of the values obtained in row 12 (total cellular protein amount at the beginning of incubation) and medium control (total cellular protein amount at the end of incubation time) the cell growth during the experiment could be controlled.

2.2.4 Micronucleus test

2.2.4.1. Standard assay

Background

Micronuclei (MN) are nuclear substructures containing chromosomal fragments or whole chromosomes, surrounded by a nuclear membrane. They appear in the cytosol in addition to the main cell nucleus being much smaller than this. Generation of MN can be induced by treatment with genotoxic compounds. They emerge during mitosis through two different ways of genesis (Fig. 7): after induction of chromosomal breaks (clastogenic effect) the acentric chromosomal fragments are excluded from the two daughter main nuclei and incorporated in MN at late stages of mitosis. On the other hand, micronuclei may result from an aneugenic effect, when chromosomes and chromatids are not distributed correctly during cell division, because of substance influences on the kinetochores or on the mitotic spindle. Possible mechanisms of spindle-related micronucleus formation include the reassembly of the nuclear envelope around chromosomes having failed to attach to the spindle (Sorger et al., 1997). This leads to non-disjunction (two sister chromatids migrating to a single pole) or lagging (chromatids do not migrate towards either pole).

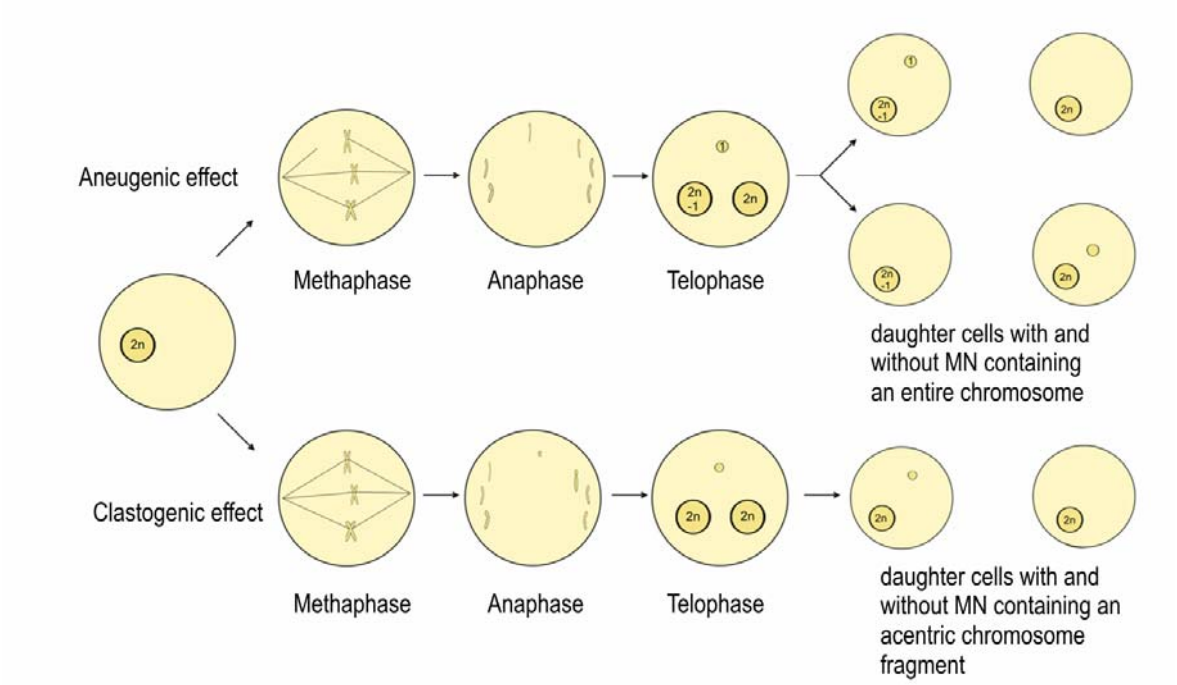


Fig. 7. Schematic of MN development (according to Andrae, 1996, modified).

For a valid detection of a genotoxic damage in the micronucleus assay cells need to fulfil a whole dividing cycle. Only after karyokinesis and the distribution of the chromosomes to the daughter nuclei, a possibly existing micronucleus becomes visible.

As positive controls, vincristine (VCR) and methylmethane sulfonate (MMS) were used. VCR is a vinca alkaloid obtained from the Madagascar periwinkle *Catharanthus roseus* (formerly *Vinca rosea* and hence its name) and used as antineoplastic drug for a variety of malignancies (Ferguson and Pearson, 1996; Johnson et al., 1963). It was shown to induce a wide spectrum of division aberrations resulting in mitotic arrest, polyploidy and aneuploidy (Miller and Adler, 1989). VCR binds to tubulin, inhibiting the tubulin polymerization and assembly of mitotic spindle microtubules.

MMS is an alkylating compound known to induce micronuclei through a clastogenic mode of action (Tao et al., 1993; Tinwell et al., 1998).

Experimental procedure

The Micronucleus assay was performed according to Matsuoka et al. (1992) with slight modifications. Initially, for each sample 2 x 10^5 V79 cells were seeded into 25 cm^2 flasks in 5 ml complete culture medium and cultured for 48 h at 37° C (5 % CO_2, relative humidity ~ 95 %) before substance

treatment. The medium was changed, and the cells were exposed to the graded concentrations of test substances, negative and positive control items; positive controls were 227 µM methylmethane sulfonate (clastogen) and 10 nM vincristine (aneugen), negative controls were cultures treated with medium alone and 0.1 % DMSO, respectively. The cells were treated with the test compounds for 18 h (about 1.5 cell cycles).

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 x g* for 8 min. Cells were then subjected to hypotonic conditions with 0.4 % KCl for swelling, which facilitates detection of micronuclei later on in the microscopic evaluation. Subsequently, the cells were fixed with ice-cold (-20° C) methanol-acetic acid mixture (4:1 (v/v); fixative). The fixative was changed four times by centrifugation in between. Cells were mounted onto slides and air-dried at ~55° C using the hotplate. It was microscopically controlled that the cell membranes stayed intact. Per culture a minimum of four slides were prepared.

For analysis, the slides were stained with Acridine Orange working solution (50 µg/ml), mounted with coverslips, and immediately analyzed by fluorescence microscope at 400-fold magnification using a filter setting providing blue excitation at 440-490 nm and emission of 520 nm.

The microscopic examination was done coded to avoid bias. Per culture 2x1000 cells with well preserved cytoplasm were examined and analyzed for MN. MN were counted 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 mix-up with apoptotic cells.

Data evaluation

The number of MN scored per slide was recorded and micronucleus rate calculated as number of micronuclei per 1000 cells for each condition/concentration. The increase of the micronucleus rate was determined relative to the concurrent negative solvent control (background MN rate). The concentration resulting in a doubling of the background MN rate (exp –log C) was taken from the concentration-response-curves to define the potency of MN induction.

2.2.4.2. CREST analysis

Background

MN are induced through an aneugenic or a clastogenic effect of genotoxic agents, respectively. By means of conventional microscopic analysis, it is not possible to discriminate MN with respect to their content, entire chromosomes or chromosomal fragments.

Distinction between aneugenic and clastogenic compounds was achieved by CREST analysis described by Renzi et al. (1996) and Russo et al. (1992). The presence or absence of kinetochore proteins in MN is utilized to distinguish MN induced by aneugenic substances from those induced by clastogens; entire chromosomes contain a centromere and bound kinetochore proteins whereas acentric chromosomal fragments do not. Indirect immunofluorescence using an human antikinetochore antibody obtained from serum of patients suffering from the CREST form of scleroderma (CREST syndrome: *c*alcinosis, *R*aynaud phenomenon, *e*sophageal dismotility, *s*clerodactyly, and *t*elangiectasia) as primary antibody allows the detection of MN containing kinetochores (CREST-positive) which derive from chromosome lagging (aneugenic effect). In contrast, MN arising after a clastogenic effect contain mainly acentric chromosomal fragments; therefore, the antikinetochore antibody is not bound (CREST-negative). Fig. 8 shows a schematic metaphase chromosome with kinetochore region.

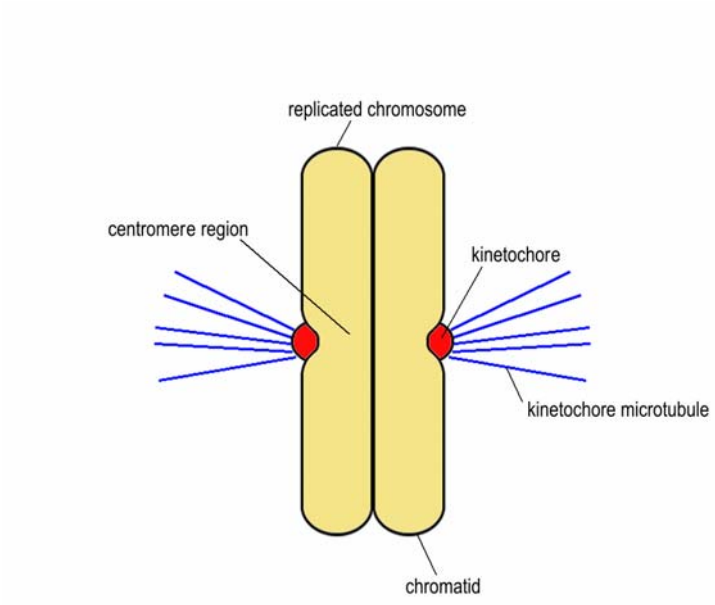


Fig. 8. Schematic of a metaphase chromosome (according to Alberts et al., 1995)

Experimental procedure

The CREST analysis was performed according to Miller and Adler (1990) and Bonacker et al. (2004b). Test conditions were as those used in the standard MN assay, except that the cells were seeded directly onto sterile slides and treated in quadriPERM™ cell culture vessels: For each sample 2.5 x 10^4 cells in 5 ml culture medium were seeded on slides lying in quadriperm dishes. Before substance treatment the cells were precultured for 48 h. Then the medium was changed, and the cells were exposed to the different test substance concentrations, negative and positive control items; vincristine (10 nM) and methylmethane sulfonate (227 µM) again served as aneugenic and clastogenic positive control agents, respectively.

The cells were treated with the test compounds for 18 h (about 1.5 cell cycles) at 37° C under 5 % CO_2, according to the standard MN assay. Then the slides were rinsed 2 min with 1x PBS and cells were treated with 0.075 M KCl for swelling for 15 min at 37° C. Subsequently, cells were fixed with ice-cold (-20° C) methanol for 30 min and acetone for another 10 min. Fixed cells were treated with cold (4° C) PBS/0.1 % Tween 20 for 5 min to be permeabilized. Slides were incubated with 50 µl of the primary antibody (CREST serum 1:50 in PBS/0.2 % Tween 20) for 24 h in the incubator, washed with PBS/0.1 % Tween 20 to eliminate the excess of primary antibody and incubated again with the second FITC-conjugated antibody (goat anti-human antibody, 1:100 in PBS/0.5 % Tween 20) for 1 h. The slides were rehydrated in Soerensen buffer, counterstained with 100 µl CREST staining solution (DAPI/PI), mounted with antifade (Johnson and Nogueira Araujo, 1981) and analyzed by fluorescence microscopy. The induction of MN was evaluated by scoring a total of 1000 cells/slide in the fluorescence microscope at 400-fold or 1000-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).

Data evaluation

Per slide, 1000 cells were scored and the micronucleus rate was calculated. The “CREST-status” of the MN was determined: 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).

Percentual distribution of CREST-positive, CREST-negative, and unclear MN was determined.

2.2.5 Cell cycle analysis

Cell cycle analysis was performed for the compounds which were detected to be positive in the MN Assay (NT, NE, Nor-C, TB, THG, MAD, NA), slightly positive (Nor-G) as well as two negative compounds (T, ETHI).

Background

The distribution of cells in the different cell cycle phases can be influenced by substance treatment. Quantitation of the total DNA per cell is a common method for analyzing the progress of cells through the cell cycle. Cells in G0/G1 phase have a relative DNA content of 2n, it increases during S phase up to 4n, which resembles DNA content of cells in G2/M phases. The DNA can be stained (after RNA digestion) with propidium iodide, which intercalates in double stranded nucleic acids. The fluorescence intensity is taken as measure of the relative DNA content of a cell.

As flow cytometry allows an analysis on single cell level the percentual distribution of cells in one population in the different cell cycle phases is determined.

Experimental procedure

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). 8 x 10^4 cells were seeded in 6-well-dishes in 2 ml complete culture medium and cultured for about 48 h before substance treatment. The medium was changed, and the cells were exposed to the test substances as DMSO solutions as well as to negative control items (medium control and 0.1 % DMSO) for 18 h.

Cells were harvested by disaggregation with trypsin/EDTA (500 µl), suspended in medium with 20 % FCS and centrifuged at 200 x g for 8 min. They were resuspended in PBS and cell density was adjusted at 1 x 10^6. After another centrifugation step cells were resuspended in 500 µl lysis buffer LB01 (Dolezel et al., 1989). 5 µl RNase solution for RNA digestion and 5 µl PI solution for DNA staining were added and the samples were incubated for 1 h at 4° C in the dark. Per sample 10,000 cells were analyzed by means of a flow cytometer using the 488 nm argon laser for excitation.

Data analysis

Data were analyzed by means of the software „CellQuest Pro“. In histograms the fluorescence intensity (reflecting DNA content) was expressed versus cell counts. The peak located at 200 relative fluorescence intensity (corresponding to a DNA content of 2n) represents cells in G0/G1 phase, the

peak located at 400 (≙ DNA content of 4n) those in G2/M phase, and that in between cells in S phase. Analysis markers were set over the single peaks to determine the percentual amount of cells in the single cell cycle phase for each sample (Fig. 9).

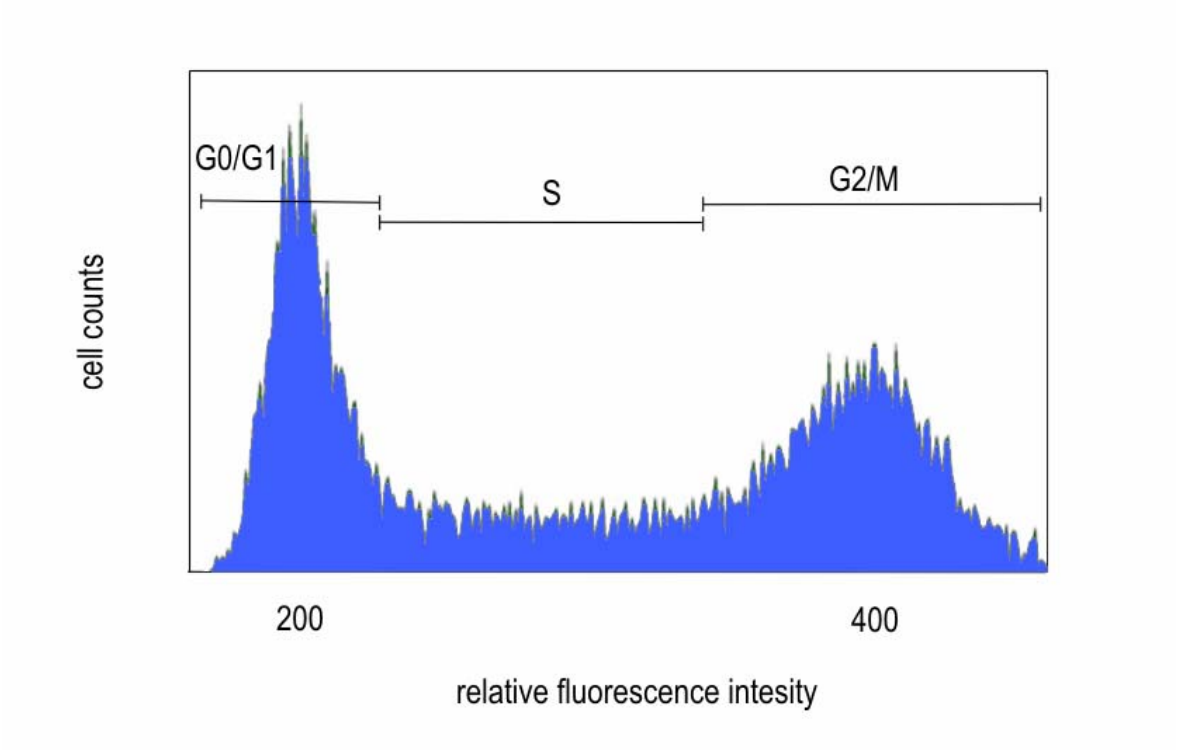


Fig. 9. Example for a histogram for determination of cell cycle phases distribution in a cell population.

2.2.6 Detection of apoptosis

Compounds which were detected positive in the Micronucleus Assay (NT, NE, Nor-C, TB, THG, MAD, NA), slightly positive (Nor-G), or two negative compounds (T, ETHI) were tested for their apoptosis-inducing potential in two different assays. The Annexin-V/PI Assay reflects typical morphological changes of apoptotic cells. In the Caspase-3/7 Assay cellular biochemical changes typical for apoptosis are detected. Camptothecin (10 µM) was used as positive control in both assays.

2.2.6.1. Annexin-V/PI assay

Background

Effects of the substances on apoptosis induction were determined by means of the Annexin V/PI method (Koopman et al., 1994). In normal cells, the membrane phospholipid phosphatidylserine (PS) is found in the inner layer of the cytoplasmic membrane. During early stages of apoptosis phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. PS is bound by the Ca^{2+} dependent phospholipid-binding protein Annexin V, which has a high affinity for PS. Fluorochrome-labelled Annexin V (here with Fluorescein isothiocyanate, FITC) is widely used as probe to detect PS externalization. Annexin V binding precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V-FITC is typically used in combination with a vital dye. Counterstaining with propidium iodide, which can only penetrate damaged membranes (typically appearing during necrosis), allows the differentiation of (early) apoptotic and late apoptotic/necrotic cells (Vermes et al., 2000; Fig. 10).

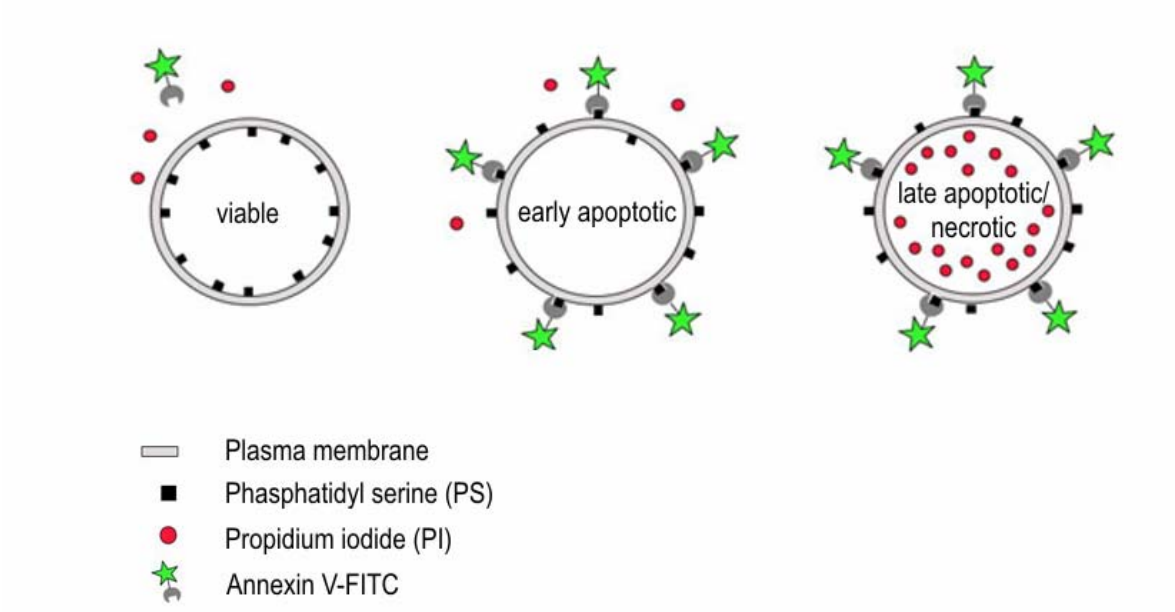


Fig. 10. Principle of the Annexin V-FITC/PI assay: viable cells remain unstained, early apoptotic cells are stained with Annexin V-FITC, whereas late apoptotic/necrotic cells are double stained with Annexin V-FITC and PI. (http://www.dundee.ac.uk/lifesciences/FACS/cell_death.htm; 11.04.2007)

Camptothecin, an extract of the Chinese tree *Camptotheca acuminata*, is a potent inhibitor of topoisomerase I, and in consequence it has been shown to induce apoptosis (Onishi et al., 1993; Solary et al., 1993). In the present study, camptothecin was used as positive control for apoptosis induction.

Experimental procedure

In 6-well tissue culture plates 8 x 10^4 cells were seeded in 2 ml of complete culture medium, allowed to attach for 48 h and treated with the test substances (applied in DMSO solution) in the culture medium for 18 h. The supernatant - containing late apoptotic cells - was transferred to centrifuge tubes, attached cells were harvested by disaggregation with trypsin/EDTA (500 µl), suspended in medium with 20 % FCS and as well transferred to the corresponding tube. The cells were centrifuged at *200 x g* for 8 min and washed once with cold (4° C) PBS. The Annexin V-FITC Apoptosis Detection Kit I® contains all components necessary for the staining procedure which was performed following the manufacturer's instructions: The cell pellet was resuspended in 1x binding buffer and cell number was adjusted to 1 x 10^6 cells/ml. 100 µl containing 1 x 10^5 cells were stained with 5 µl Annexin V-FITC and 5 µl PI for 15 min. 400 µl of binding buffer were added and 10,000 cells per sample were analyzed immediately by means of the flow cytometer using the 488 nm argon laser for excitation. Data processing was carried out using the software CellQuest Pro®.

Data analysis

The obtained data were analyzed by means of the software „CellQuest Pro". In a dot plot diagram, which quantitates percentages of cells with various properties, both fluorescences were regarded with respect to each other. This allows the differentiation between cells that express only one of the fluorescence markers, those that express neither, and those that express both.

Annexin V-FITC and PI negative ($FITC^-/PI^-$) cells are viable ones; cells that are in early apoptosis are Annexin V-FITC positive and PI negative ($FITC^+/PI^-$), and cells that are in late apoptosis or necrotic are both Annexin V-FITC and PI positive ($FITC^+/PI^+$). Only PI positive ($FITC^-/PI^+$) signals were defined as debris (Fig. 11).

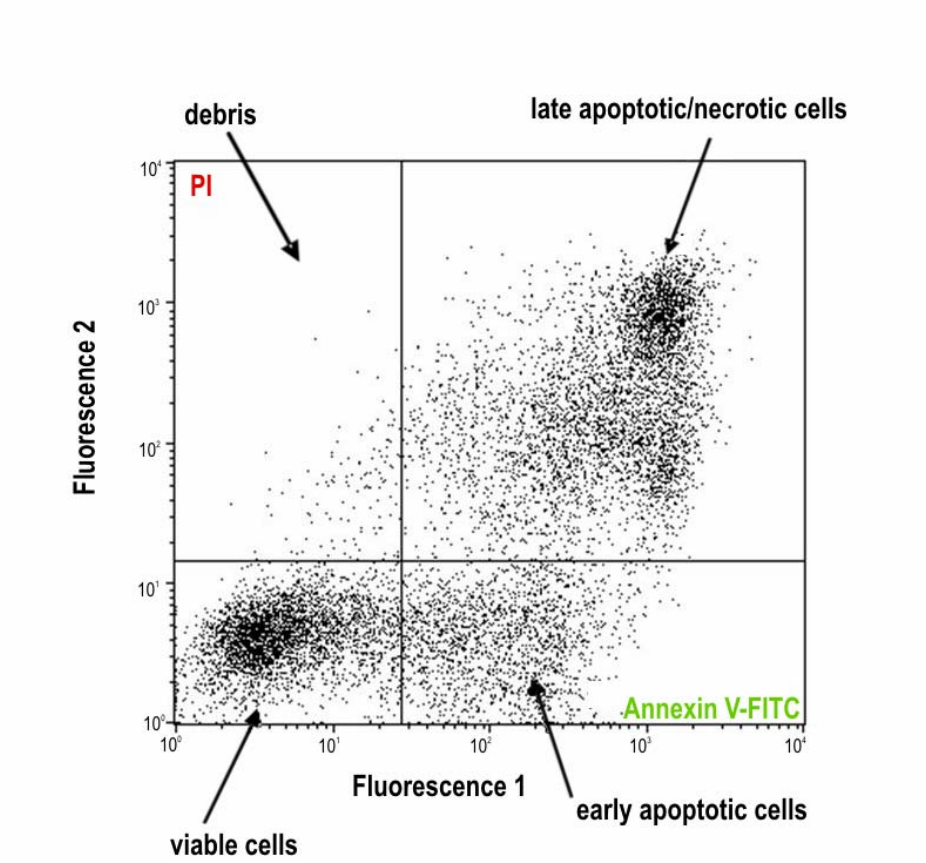


Fig. 11. Example for a dot plot for determination viable, early apoptotic, and late apoptotic/necrotic cells as well as debris.

The percentual fraction of cells in each subgroup (viable, apoptotic, late-apoptotic/necrotic, debris) was determined for each sample according to the quadrant statistics.

2.2.6.2. Caspase-3/7 assay

Background

Apoptotic cell death is mostly driven by interactions among several families of proteins; one of them is the family of *c*ysteine-dependent *asp*artate-directed prote*ases*, the caspases. Activation of caspases results in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic structural disassembly of dying cells. Two subgroups of caspases are distinguished depending on their role in apoptosis: initiator caspases, and effector caspases. Caspase-3 and -7 belong to the latter (Danial and Korsmeyer, 2004) playing a key role in apoptosis in mammalian cells; their activation usually ensures the completion of the apoptotic process (Zhang et al., 2004). Both, caspase-3 and -7 have been demonstrated to be almost synonymous in their substrates and inhibitor specificity (Fernandes-Alnemri et al., 1995).

Caspase-3-like proteases show specificity for cleavage at the C-terminal side of the aspartate residue of the sequence DEVD (Asp-Glu-Val-Asp) and are inhibited by the tetrapeptide inhibitor Ac-DEVD-CHO (Nicholson et al., 1995). Thus, the rhodamine 110-derived substrate Z-DEVD-R110 (rhodamine 110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)) was used for detection of caspase activity. It represents a profluorescent substrate added to the sample in combination with a cell lysis buffer (provided by the manufacturer). Upon sequential cleavage of Z-DEVD-R110 and removal of the DEVD peptides by caspase-3/7 activity, the rhodamine 110 leaving group becomes intensely fluorescent (Fig. 12). The amount of fluorescent product generated is proportional to the amount of caspase-3/7 cleavage activity present in the sample.

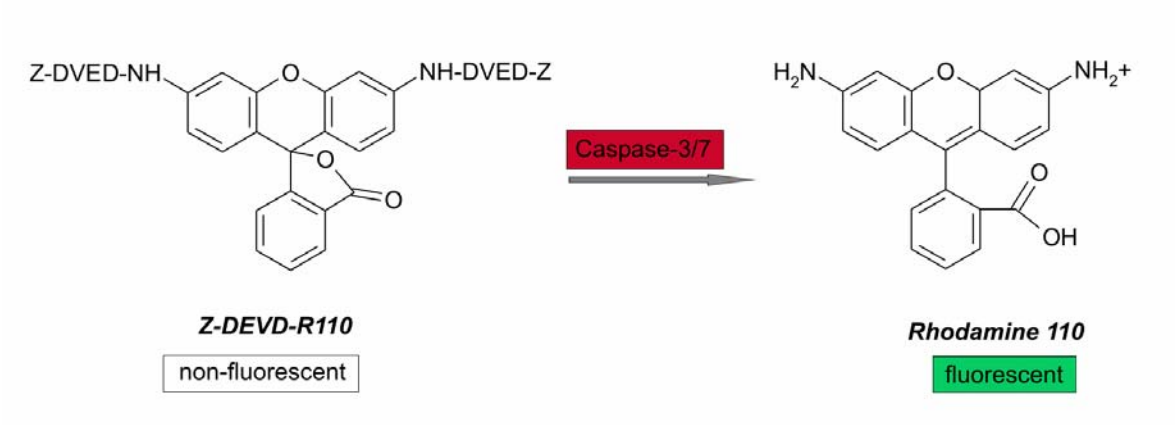


Fig. 12. Cleavage of the non-fluorescent caspase substrate Z-DEVD-R110 by caspase-3/7 to create the fluorescent rhodamine 110

Experimental procedure

The Caspase-3/7 Assay was performed using the Apo-ONE® Homogeneous Caspase 3/7 Assay kit according to the manufacturer's instructions. 5 x 10^3 V79 cells were seeded per well in 100 µl in 96-well microtiter plates and cultured for about 24 h before substance treatment. The test agent solutions were prepared in complete medium without phenol red. The medium was aspirated and the cells were treated with different concentrations of the test substance, solvent (DMSO 0.1 %) or with the positive control camptothecin (10 µM): Each test agent concentration was supplied to 8 wells, i.e. one row of the microtiter plate. The cells were treated with the test compounds for 18 h (about 1.5 cell cycles). Per well 100 µl of the caspases-3/7 reagent (Chapter 2.1.7.6) were added, the plate was shaken for 30 s (600 rpm) and incubated for another 1 h.

The fluorescence intensity was read at 485 nm (ex.) and 535 nm (em.) in the microplate reader. Per tested compound, at least three independent assays were performed.

Data analysis

Mean values were calculated from the 8 wells treated with one substance concentration. Increase of the caspase-3/7 activity was determined relative to solvent control. The results obtained in independently performed assays were combined, mean values and standard deviations were determined.

2.2.7 Detection of reactive oxygen species

The compounds which were detected to be positive in the micronucleus assay (NT, NE, Nor-C, TB, THG, MAD, NA), slight positive (Nor-G) as well as two negative compounds (T, ETHI) were tested for their potential to generate reactive oxygen species (ROS). H_2O_2 (30 µM) was used as positive control.

Background

Detection of reactive oxygen species (ROS) generation by means of a fluorometric microplate assay using dichlorofluorescein (DCF) as fluorogenic probe hearkens back to the method of Rosenkranz et al. (1992). The non-fluorescent dye 2´,7´- dichlorodihydrofluorescein diacetate (H_2DCF-DA) is membrane permeable, but once internalized, it is deacetylated by non-specific intracellular esterases in dichlorodihydrofluorescein (H2DCF; Sanchez et al., 1990), which traps the dye intracellularly. It is oxidized to the highly fluorescent compound dichlorofluorescein (DCF) by intracellular ROS (Fig. 13), primarily hydrogen peroxide and hydroxyl radicals (Rosenkranz et al., 1992).

As the carboxy derivative of fluorescein carries additional negative charges that improve its intracellular retention compared to noncarboxylated forms (De Clerck et al., 1994), 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCF-DA) was used in the present study.

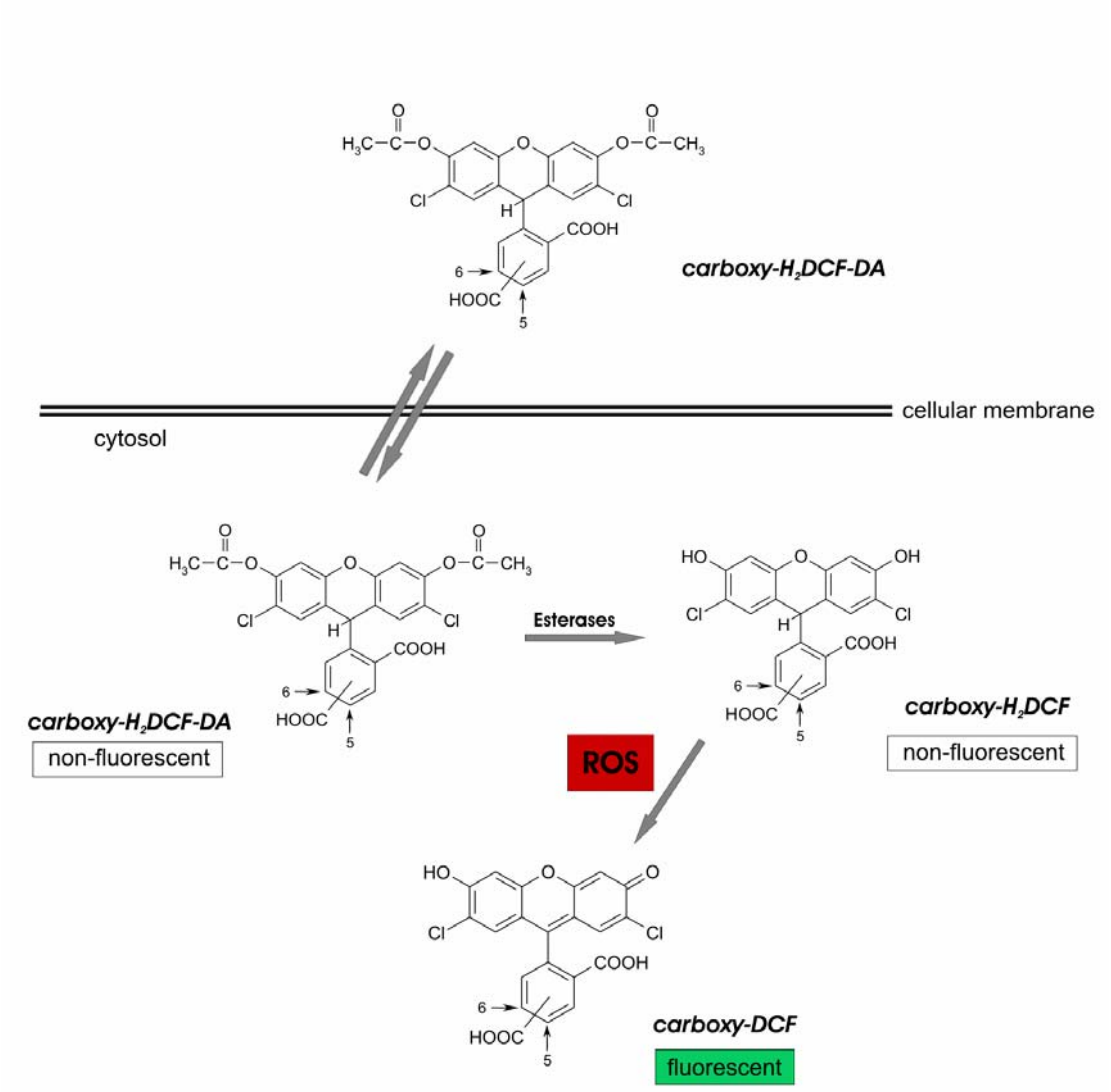


Fig. 13. Penetrance of cellular membrane of carboxy-H_2DCF-DA, deacetylation and oxidation to carboxy-DCF.

Experimental procedure

V79 cells were seeded in 96-well microtiter plates (5 x 10^3 cells/well in 100 µl) and cultured for about 24 h before substance treatment. Per well 100 µl carboxy-H_2DCF-DA solution (100 µM) were added (final carboxy-H_2DCF-DA concentration per well: 50 µM). The plate was shaken (30 s, 600 rpm) and cells were incubated for 30 min in the incubator. Then, the medium was aspirated; cells were washed once with medium without phenol red. Subsequently, the cells were treated with different concentrations of the test substance, negative or positive control (all prepared in medium without phenol red), respectively. Each test agent concentration was supplied to 6 wells ("inner" wells) in one row of the microtiter plate. The edge wells were left free to avoid evaporation effects which possibly falsify the result in these wells.

The fluorescence intensity was read at 485 nm (ex.) and 535 nm (em.) in the microplate reader after 30 min, 1 h and 18 h. Per tested compound, at least three independent assays were performed.

Data analysis

Mean values were calculated from the 6 wells treated with one substance concentration. Increase of ROS production was determined relative to solvent control. The results obtained in independently performed assays were combined, mean values and standard deviations were determined.

2.2.8 Statistical evaluation of the test results

The results obtained for the compound treated sample 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.

2.2.9 *In silico* methodologies

The experimentally obtained micronucleus data were subjected to a set of in silico procedures used in Quantitative Structure Activity Relationship (QSAR) modelling.

The relationship between lipophilicity and chromosomal genotoxicity was characterized. The statistical evaluation was performed by Dipl.-Stat. Tina Müller, Department of Statistics, Chair of Mathematical Statistics with Applications in Biometrics, University of Dortmund, Germany.

Furthermore, the influence of several physicochemical properties of compounds on their non-specific genotoxic potential was studied. The computational procedures were performed by Dr. Jos P.M. Lommerse, N.V. Organon, Department of Molecular Design & Informatics, Oss, The Netherlands.

2.2.9.1. Lipophilicity - genotoxicity relationship

Background

Chromosomal arrangements in mitosis may be disturbed by different interactions. Lipophilicity of compounds is known as an important parameter able to determine the compounds´ effects. Regarding genotoxicity, non-specific interference of organic xenobiotics with relevant processes of cytokinesis and karyokinesis may occur through lipophilic interactions.

Schultz and Önfelt (2000) have studied the influence of compounds´ lipophilicity on the induction of bi- and multi-nucleated cells *in vitro* in cultured V79 cells by a range of lipophilic chemicals such as aliphatic hydrocarbons and alcohols, as opposed to specific effectors (colcemid, cytochalasin B, diamide). They showed that a non-specific aneuploidy was generally elicited by lipophilic chemicals at concentrations related to their lipophilicity (log P). In contrast, toxicants with a specific mode of action acted at concentrations consistently lower than predicted based on their lipophilicity. Thus, they proposed to use this approach for differentiation of specific and non-specific action in the screening of potential aneugens based on lipophilicity.

In the present study, this concept was further extended, and the relationship of lipophilicity and chromosomal genotoxicity was determined for aneugenic as well as clastogenic compounds.

Lipophilicity data

The octanol-water partition coefficient log P describes a physicochemical property of a substance referring to the equilibrium distribution of the solute between the two liquid phases, the lipophilic octanol and the lipophobic water. It provides a thermodynamic measure of the lipophilicity of compounds. As log P is given by the ratio of the concentrations of the un-ionized solute in octanol versus in water, hydrophilic compounds have a negative or low log P; the higher the log P, the higher is the lipophilicity of the compound.

Lipophilicity of the tested compounds was expressed by their log P, with preference to experimental log P values as listed by Hansch et al. (1995). In cases for which no experimental log P values were available, calculated values were taken, as obtained with the BioByte Clog P programme. The source of log P data is indicated in Chapter 3.2.1, Tab. 8.

Procedure

Several data sets were regarded in combination (Tab. 2): “Data set A” was that of the original publication of Schultz and Önfelt (2000), based on the counts of binucleated V79 cells after 24 h incubation. Data sets available from our laboratory (B and C) were based on micronuclei counts in V79 cells after 18 h incubation. Data set B included compounds with some effect on the mitotic spindle (nitrobenzene and benzonitrile; Bonacker et al., 2004a), and data set C included the data for the hormonal steroids obtained in the present study as well as the phytoestrogens genistein and daidzein (Di Virgilio et al., 2004).

Tab. 2. Data sets of compounds tested in V79 cells

Data set A	Data set B	Data set C
Ethanol	1-Butanol	1-Hexanol
1-Butanol	1-Hexanol	1-Octanol
1-Pentanol	Methylmethane sulfonate (MMS)	Testosterone
1-Hexanol	Vincristine (VCR)	19-Nortestosterone
1-Heptanol	Nitrobenzene	Ethisterone
1-Octanol	Benzonitrile	19-Norethisterone
Dichloromethane		Androstenedione
Chloroform		17α-Propylmesterolone
Carbon tetrachloride		7α-Methyltestosterone
1,2-Dichloroethane		Nor-C
1,1,1,2-Tetrachoroethane		Nor-D
1,1,2,2-Tetrachoroethane		Nor-E
Pentachloroethane		Nor-F
Cytochalasin B		Genistein
Colcemid		Daidzein
Diamide		

All experimental data used were from studies in V79 cells *in vitro*. However, as the three data sets (A, B, C) have been elaborated by different investigators at different times, and as different quantitative endpoints have been used (counts of binucleated cells in data set A, micronuclei counts in data sets B and C), an internal standardization is necessary. For this purpose, some aliphatic alcohols which have been assessed in data set A were included in the experimentations for data set B and C: 1-hexanol was assessed in all 3 data sets. 1-butanol and 1-octanol were integrated in data set B and C, respectively.

Statistical analysis

The original dataset of Schultz and Önfelt (2000; Chapter 1.3, Fig. 5) included mainly non-specific agents with a mode of action merely based on lipophilicity and a small number of specific effectors (colcemide, cytochalasin B, diamide). Statistically, these specific effectors are outliers of the relationship between lipophilicity (log P) and genotoxicity (-log C). When calculating the regression line in the usual way, this is largely influenced by the outliers.

This problem is avoided by applying Least Trimmed Squares, a robust regression technique (Rousseeuw and Leroy, 1987). It computes the regression line based on minimizing a function of the smallest squared residuals (residual = distance from the respective data point to the regression line). By this procedure, the robust regression line is fitted to the non-outlying 'good' points. For analysis, the residuals are standardized by a scale estimate. The standardized residuals are considered to follow a standard Gaussian distribution; therefore, it is assumed that 98.76 % of all residuals should be located within a belt between −2.5 and +2.5 (Chapter 3.2.2, Fig. 16). All points outside of this belt are statistically considered as outliers. This belt can be displayed in the figure including all data points and the estimated regression line (Chapter 3.2.2, Fig. 17) by rescaling it accordingly to the standardized residuals.

2.2.9.2. Physicochemical parameters

Background

Lipophilicity of compounds was shown to be a pivotal physicochemical parameter determining non-specific genotoxic effectiveness (Schultz and Önfelt, 2000). So far, molecular descriptors other than log P have not been applied to distinguish between specific and non-specific chromosomal genotoxicity. In order to generate molecular descriptors for modelling non-specific chromosomal genotoxicity, and to optimize combinations thereof available data sets on aneuploidy (Schultz and Önfelt, 2000) and micronucleus formation (Dorn et al., 2007) were regarded in combination and examined using procedures used in QSAR modelling.

Procedure

Molecular structures of the regarded chemicals (Tab. 2) were converted into a single 3D model using the software Corina 3.20 before 11 descriptors of molecular properties were calculated (Tab. 3).

Tab. 3. Calculated molecular descriptors

Property/Descriptor	Abbreviation	Unit	Software
Polar surface area	polsurf	$Å^2$	monika 3.6
Number of hydrogen bond acceptor atoms	acc	-	monika 3.6
Number of hydrogen bond donor atoms	don	-	monika 3.6
Number of rotatable bonds	robo	-	monika 3.6
Molecular weight	mw	g/mol	monika 3.6
Molecular volume	vol	$Å^3$	monika 3.6
Total molecular surface	surface	$Å^2$	monika 3.6
Lipophilicity of neutralized molecules	clog P	-	ClogP 4.10
Lipophilicity of ionizable molecules at pH=7.4	clog D[a)]	-	ACD/ClogP 4.10
Solubility	log S	-	Cerius^2 4.10
Molecular dipole moment	dipole	10^{-30} C x m	Cerius^2 4.10

[a)] Clog D was calculated on basis of log D/log P values calculated using the Advanced Chemistry Development v4.76 (ACD) software and clog P values: clog D = log D(ACD)-log P(ACD)+clog P

The following compounds with a known or presumed mode of action to induce chromosomal genotoxicity were classified as “specific”: diamide (sulfhydryl blocker, c-mitotic), methylmethane sulfonate (alkylating, clastogenic), benzonitrile (tubulin effect), nitrobenzene (tubulin effect), colcemide (spindle poison), cytochalasin B (cytokinesis inhibitor), vincristine (spindle poison), genistein (topoisomerase-II poison), daidzein, and trenbolone (protein binding). The remaining compounds were classified as “non-specific”: ethanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, dichloromethane, chloroform, carbon tetrachloride, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, 1,1,1,2-tetrachloroethane, pentachloroethane, 19-nortestosterone, 19-norethisterone, Nor-C. The data of the 16 compounds assigned to a non-specific mode of action were imported into the QSAR module of the software package Cerius^2. Then, the so-called genetic function approximation (GFA) was applied. By means of this method, models having a randomly chosen proper subset of the independent variables are collected, and the collected models are "evolved'. A generation is the set of models resulting from the performance of multiple linear regression on each model; a selection of the best ones becomes the next generation. Cross-over operations are performed on these, which take some variables from each of the two models to produce an offspring. In addition, the best model from the previous generation is retained. Besides linear terms, there can also be quadratic, and quadratic

spline (not applied in this work) terms. These are added or deleted by mutation operations (Rogers and Hopfinger, 1994).

Thus, linear equations were set up relating molecular descriptors with experimental concentrations at which doubling of binucleated cells or micronuclei occurred (exp –log C), respectively. The number of variables (molecular descriptors) was limited to a maximum of three, and linear and quadratic terms were allowed.

2.2.9.3. Relationship of microtubule assembly and lipophilicity

Background

Cytokinesis relies on a multiplicity of protein-protein and protein-membrane interactions (Rappaport, 1986). An important partial process, especially critical for chromosomal segregation, is the dynamic assembly and disassembly of microtubules. Previously, it was demonstrated that mercury and lead salts interfere with this process at micromolar concentrations, organic compounds, such as nitrobenzene and benzonitrile, at millimolar concentrations, dependent on their lipophilicity (Bonacker et al., 2004a; Bonacker et al., 2004b; Bonacker et al., 2005).

In order to broaden the database, an experimental data set obtained from the Institute for Molecular Biotechnology (IMB), Jena, Germany was analyzed regarding the influence of lipophilicity on the process of microtubule assembly and disassembly. For the chemicals included in this data set there were hints from the literature pointing to the possibility of interaction with the tubulin-microtubule system. These chemicals (log P between -1.5 and +1.0) have been assessed in Jena as to their potencies to influence the dynamic processes of microtubule assembly and disassembly in a cell-free system *in vitro*, as originally proposed by Shelanski et al. (1973) and described in the Appendix (Chapter 7.1.2.1).

Procedure

The maximum microtubule assembly value (reached in the plateau) was taken to generate concentration-effect-curves, from which the no-observed-effect-concentrations (NOECs) were determined. The lipophilicity of the compounds was expressed as log P. Preference was given to experimental log P values, as listed by Hansch et al. (1995). In cases for which no experimental log P values were available, calculated values were taken, as obtained with the BioByte ClogP programme (BioByte Corp., Claremont, CA, USA). The source of log P data is indicated in Tab. 11 in Chapter 3.4.

The dependence of the experimental no-observed-effect-concentrations (-log NOEC) in the microtubule assembly assay on log P as parameter of lipophilicity was determined plotting the log P values and NOEC against each other. Regression for the “non-specific” compounds (no specific mode of action is known) was determined.

3 Results

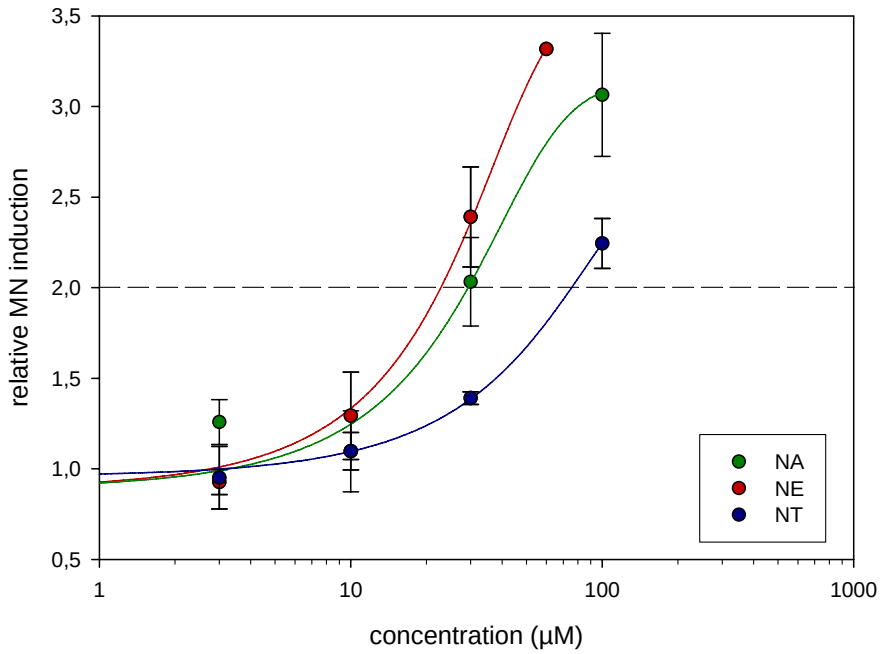
3.1 Genotoxicity

3.1.1 Standard micronucleus assay

The genotoxic potential of the steroid compounds on a chromosomal level was assessed in the micronucleus assay. In accordance with accepted toxicological procedures, compounds that lead to a doubling of the MN background rate (obtained in the solvent control) were assigned as positive. Compounds that did not induce MN or did not reach the doubling of the MN background rate in the tested concentration range were assigned as negative.

For the compounds designated “positive”, the concentration-response-curves (concentration *vs.* MN induction relative to solvent control) are given in Fig. 14 a-c.

a)



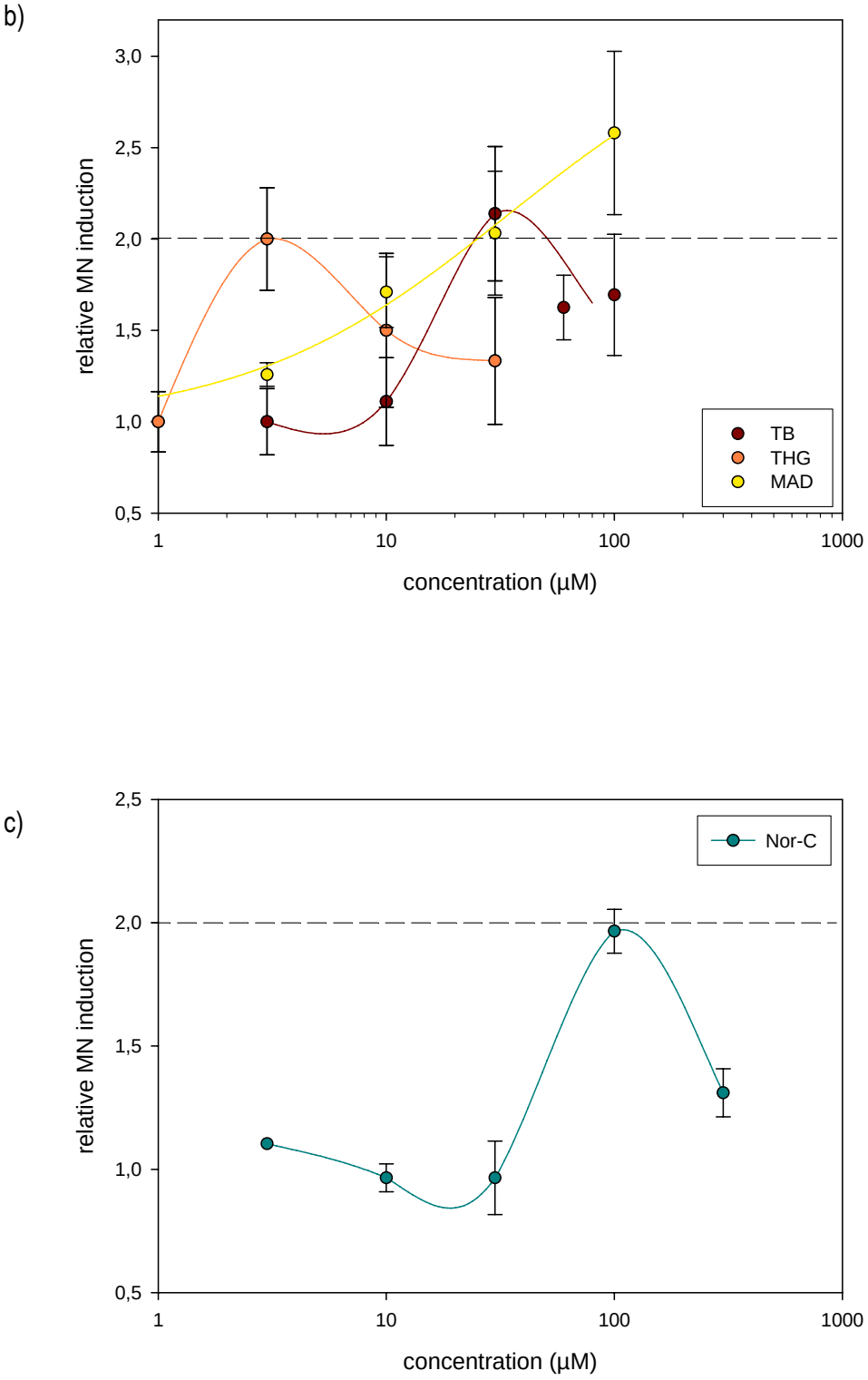


Fig. 14. Relative MN induction compared to solvent control by a) NA (19-norandrostenedione), NE (19-norethisterone), and NT (19-nortestosterone), b) MAD (madol), TB (trenbolone), and THG (tetrahydrogestrinone), and c) Nor-C; 18h treatment (the dashed line marks the 2 x increase limit).

For these positive compounds, the molar concentration leading to a doubling of the MN background rate was determined; its negative logarithm is listed (as *exp –log C*) in Tab. 4.

Tab. 4. Positive compounds with the concentrations that lead to a doubling of the MN background rate (18 h incubation).

Compounds	**exp -log C**
Trenbolone	4.64
19-Norandrostenedione	4.54
19-Nortestosterone	4.18
19-Norethisterone	4.62
Nor-C	4.00
Tetrahydrogestrinone	5.52
Madol	4.57

Steroids that yielded negative results in the micronucleus assay are listed in Tab. 5; the maximum of micronucleus increase relative to the solvent control is also given. For a detailed overview on the results obtained in the MN assay, see Appendix, Chapter 7.2.1.

Tab. 5. Hormonal steroid compounds, which did not reach doubling of MN background rate in V79 cells (18 h incubation).

Compounds	Genotoxicity
Androstenedione	1.6-fold MN increase 30 µM; no higher MN increase reached up to 100 µM
Ethisterone	No MN increase up to 100 µM
Testosterone	No MN increase up to 300 µM
Nor-E	1.2-fold MN increase 10 µM; no higher MN increase reached up to 100 µM
Nor-D	1.4-fold MN increase 30 µM; no higher MN increase reached up to 100 µM
7α-Methyltestosterone	No MN increase up to 100 µM
Nor-F	1.2-fold MN increase 10 µM; no higher MN increase reached up to 100 µM
Nor-G	1.2-fold MN increase 10 µM; no higher MN increase reached up to 100 µM
17α-Propylmesterolone	No MN increase up to 30 µM

3.1.2 CREST (kinetochore) analysis of micronuclei

In order to differentiate between aneugenic and clastogenic modes of action, a kinetochore analysis by CREST staining was performed for most of the compounds that were tested positive in the micronucleus assays.

Numbers and characteristics of MN induced by the steroids and by positive and negative controls are shown in Tab. 6.

Tab. 6. CREST analysis: number and characteristics of MN induced by the steroids NE (19-norethisterone), NT (19-nortestosterone), Nor-C, THG (tetrahydrogestrinone), and TB (trenbolone) as well as by negative (medium, 0.1% DMSO) and positive controls MMS (methylmethane sulfonate), and VCR (vincristine).

Compound	Concentration	MN/1000 cells (SD)		
		CREST-positive	CREST-negative	unclear
NE	60 µM	4.0 (+/-0.7)	23.5 (+/-0.7)	5.0 (+/-0.0)
NT	100 µM	3.5 (+/-2.8)	11.5 (+/-3.5)	1.0 (+/-0.0)
Nor-C	100 µM	2.5 (+/-0.7)	13 (+/-1.4)	3.5 (+/-0.7)
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		0.8 (+/-1.3)	3.8 (+/-1.1)	1.2 (+/-70.5)
0.1%DMSO		1.2 (+/-1.1)	4.8 (+/-0.8)	1.2 (+/-0.8)
MMS	227 µM	2.5(+/-2.7)	15.5(+/-5.3)	5.75 (+/-2.6)
VCR	10 nM	22.7 (+/-8.4)	6.7 (+/-3.2)	12.0 (+/-5.2)

The data obtained for the steroids and positive controls were corrected for background. The percentual distribution of CREST-positive, CREST-negative and unclear MN induced after 18 h substance treatment was taken to classify the compounds regarding their mode of action (Fig. 15). MN induced by TB and THG were predominantly kinetochore (CREST)-positive, pointing to an aneugenic mode of action. In contrast, NE, NT, and Nor-C acted mainly as clastogens inducing predominantly CREST-negative MN. The reference compound methylmethane sulfonate, a typical clastogen, induced mainly CREST-negative MN, the reference aneugen vincristine mainly CREST–positive MN, as expected.

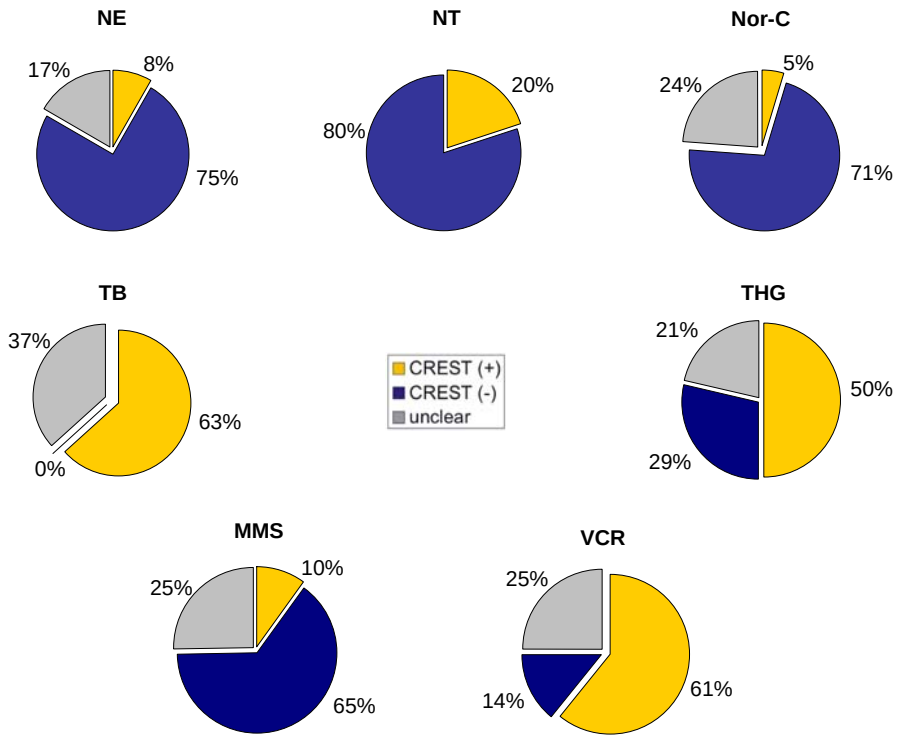


Fig. 15. Percentual distribution of CREST-positive, CREST-negative and unclear MN induced by the steroids NE (19-norethisterone), NT (19-nortestosterone), Nor-C, THG (tetrahydrogestrinone), and TB (trenbolone) as well as by the positive controls MMS (methylmethane sulfonate), and VCR (vincristine); 18 h treatment (*corrected for background*)

3.2 Genotoxicity - lipophilicity relationship

3.2.1 Characterization of data sets

Schultz and Önfelt (2000) originally proposed the concept relating compound lipophilicity and non-specific genotoxicity on a chromosomal level, which has been addressed in the Introduction section (see Chapter 1.3). In order to broaden the database of this concept, three data sets obtained from *in vitro* studies in V79 cells were regarded in combination (see Chapter 2.2.9.1). As these three data sets (A, B, C) have been elaborated by different investigators at different times, and as different quantitative endpoints have been used (counts of binucleated (BIN) cells in data set A, micronuclei (MN) counts in data sets B and C), an internal standardization was necessary. For this purpose, the aliphatic alcohol 1-hexanol was taken, which has been assessed in all three data sets. Moreover, 1-butanol was common to data sets A and B and 1-octanol to data sets A and C, respectively. For these compounds (non-specific, according to Schultz and Önfelt, 2000) the results are very similar

throughout the data sets (Tab. 7). Therefore, it appeared justifiable to combine the data of the three data sets into a common evaluation.

Tab. 7. Consistence between the data sets: exp -log C of aliphatic alcohols at the point of doubling of the background rate (A: BIN V79 cells; B,C: MN in V79 cells)

	Data set A	Data set B	Data set C
1-Butanol	2	2.4	-
1-Hexanol	3.3	3.8	3.5
1-Octanol	3.9	-	4

BIN: binucleated cells
MN: micronuclei

Data set A: Schultz and Önfelt (2000)
Data set B: Bonacker et al. (2004a)
Data set C: Di Virgilio et al. (2004) and data of the present study

3.2.2 Correlation of genotoxicity and lipophilicity

Taking all three data sets (A, B, C) together, the compounds investigated cover a lipophilicity range of log P between –0.51 (diamide) and 5.65 (17α-propylmesterolone). The entire "study data set" is listed in Tab. 8; compounds are given along with their specific log P and – where obtained - the concentrations that lead to a doubling of the background rate of BIN or MN (exp –log C), respectively.

Tab. 8. Entire study-set of compounds with their log P and concentrations that lead to a doubling of the background rate of BIN or MN (exp –log C), respectively, when obtained.

Compounds	log P	exp –log C
Diamide	-0.51**	4.1 [a,c]
Methylmethane sulfonate (MMS)	-0.48**	3.9 [b]
Ethanol	-0.31*	1 [a,c]
1-Butanol	0.88*	2.2 (mean value) [a/b,c]
Dichloromethane	1.25*	1.9 [a,c]
Colcemid	1.37*	7.9 [a]
1,2-Dichloroethane	1.48*	2.8 [a,c]
1-Pentanol	1.56*	2.2 [a,c]

Tab. 8. Continued

Compounds	log P	exp –log C
Benzonitrile	1.56*	6 [b]
Nitrobenzene	1.85*	6 [b]
Chloroform	1.97*	2.8 [a,c]
1-Hexanol	2.03*	3.5 (mean value) [a/b,c]
Daidzein	2.08**	4.2 [b]
1,1,2,2-Tetrachoroethane	2.39*	3.8 [a,c]
Genistein	2.41**	5 [b]
Vincristine (VCR)	2.57*	9.2 [b]
19-Nortestosterone	2.62*	4.18 [b]
1-Heptanol	2.72*	3.1 [a,c]
Carbon tetrachloride	2.83*	2.9 [a,c]
19-Norethisterone	2.97*	4.62 [b]
1-Octanol	3.00*	3.95 (mean value) [a/b,c]
1,1,1,2-Tetrachoroethane	3.03**	3.9 [a,c]
Nor-C	3.06**	4 [b]
Pentachloroethane	3.22*	3.4 [a,c]
Cytochalasin B	3.37*	6.3 [a,c]
Androstenedione	2.75*	n.d.
Ethisterone	3.11*	n.d.
Testosterone	3.32*	n.d.
Nor-E	3.39**	n.d.
Nor-D	3.54**	n.d.
7α-Methyltestosterone	3.54**	n.d.
Nor-F	3.8**	n.d.
Nor-G	4.3**	n.d.
17α-Propylmesterolone	5.65**	n.d.

* Experimental log P as listed by Hansch et al. (1995)

** log P (CLog P) as obtained with the BioByte ClogP programme

[a] BIN: binucleated cells

[b] MN: micronuclei

[c] Extrapolated from the data of Schultz and Önfelt (2000)

n.d. not detected

The data (log P *vs.* exp –log C) of positively tested compounds of the data set (Tab. 8) were plotted, and the result is shown in Fig. 17. The "diagnostic plot" of standardized residuals is given in Fig. 16.

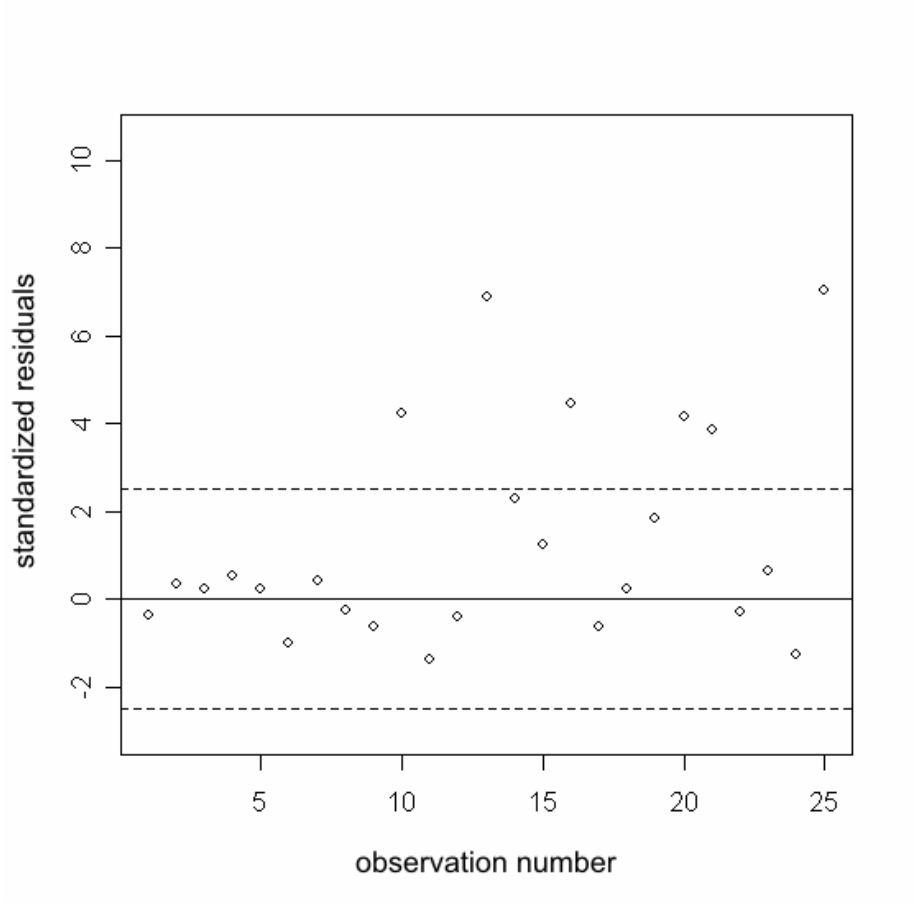


Fig. 16. "Diagnostic plot" of standardized residuals versus observation number (to calculate the robust regression as indicated in Fig. 17)

In order to separate between the cluster of the majority of compounds (falling under the Schultz and Önfelt rule of non-specific genotoxicity related to hydrophobic interactions) from statistical outliers the method of robust regression was applied (Fig. 17; "study plot"). This puts less weight on extreme observations, so that these are identified as outliers. Fig. 17 also contains the robust regression line and the borders beyond which data are identified as outliers.

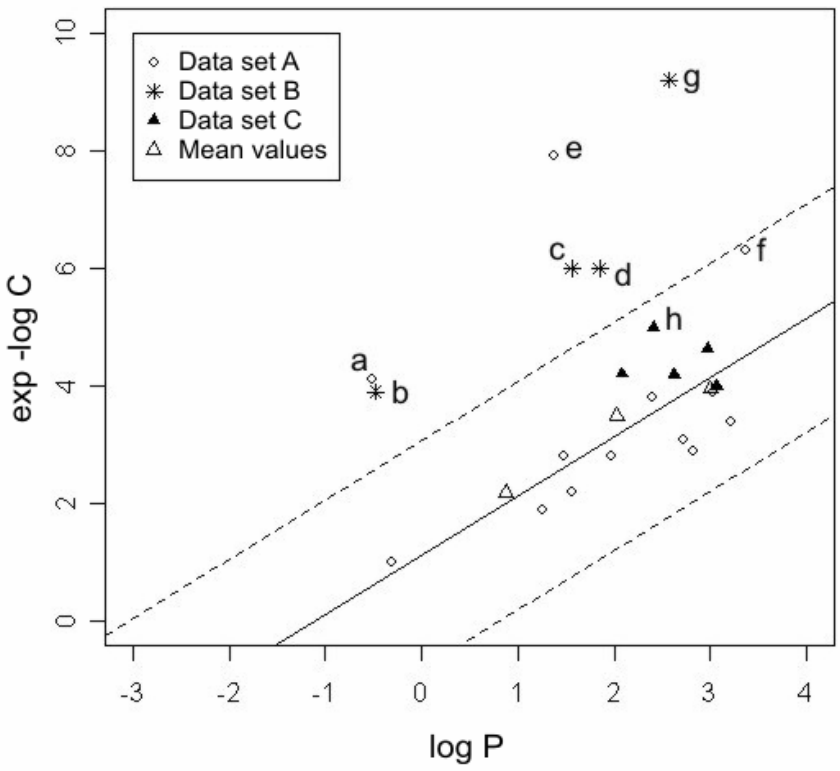


Fig. 17. "Study plot": Combined results of all data sets and robust regression: Relationship between concentrations inducing a two-fold increase in genotoxicity in V79 cells (binucleated cells or micronuclei) and the octanol/water partition coefficient (log P). Compounds with known or discussed specific effects are: a, diamide (sulfhydryl blocker, c-mitotic); b, methylmethane sulfonate (alkylating, clastogenic); c, benzonitrile (tubulin effect); d, nitrobenzene (tubulin effect); e, colcemide (spindle poison); f, cytochalasin B (cytokinesis inhibitor); g vincristine (spindle poison); h, genistein (topoisomerase-II poison).

The following linear equation describes the lipophilicity - genotoxicity relationship:

$$\exp - \log C = 1.131 + 1.008 \times \log P \qquad \text{(Equ. 1)}$$

It appears that all compounds with a specific mode of action (a - d in Fig. 17) are outliers of the Schultz and Önfelt rule. The phytoestrogen genistein, which displays weak genotoxicity within a range of 5-25 µM is clastogenic (Di Virgilio et al., 2004), is being discussed as inhibitor (poison) of topoisomerase-II (see below); this compound (designated "h" in Fig. 17) is located very closely to the border line. Cytochalasin B (point "f" in Fig. 17) is localized even closer to this border. If the borders around the regression line were changed to contain 97.86 % of all observations, cytochalasin B lies

exactly on the upper border, and is identified as an outlier if the fraction decreases further. All other observations did not change their position for percentages between 95.54 and 99.73 % (data not shown).

3.2.3 Evaluation of the genotoxicity - lipophilicity relationship

For evaluation of the genotoxicity - lipophilicity relationship the genotoxic potential of the steroids TB, THG, NA, and MAD was adducted. The compounds were included in the "study plot", plotting their experimentally determined effective concentration (exp –log C) *versus* the compound specific log P, as shown in Fig. 18.

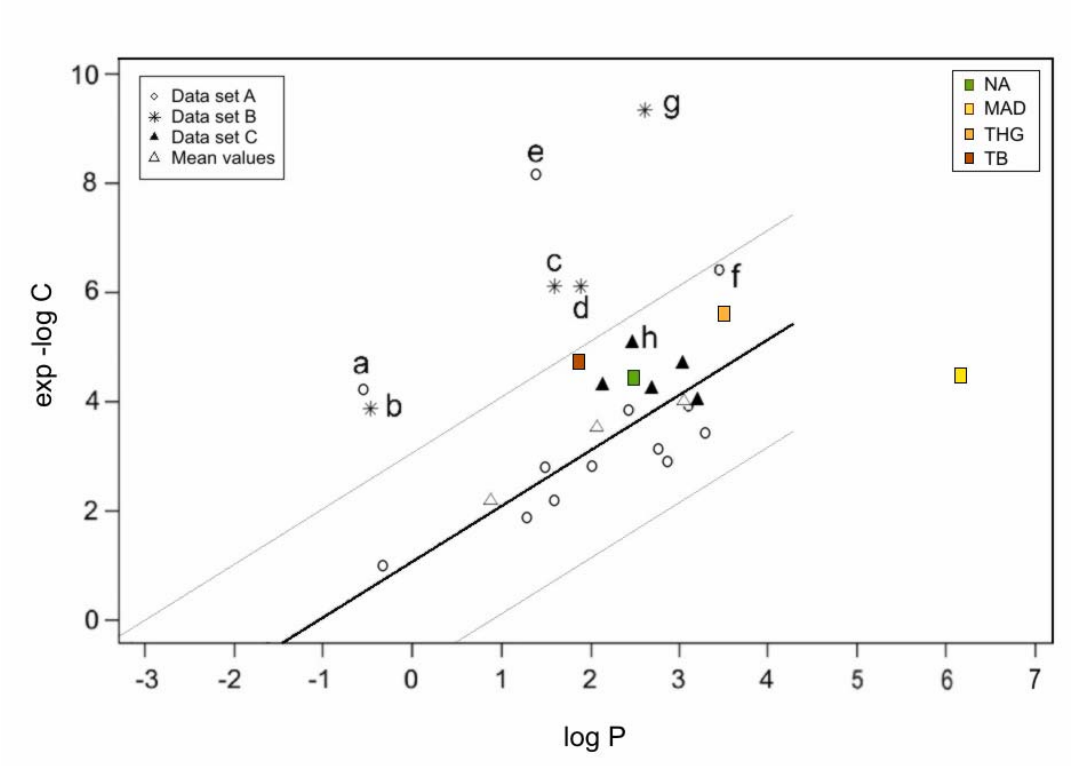


Fig. 18. Genotoxicity–lipophilicity correlation plot including the evaluation set of compounds NA (19-norandrostenedione), MAD (madol), TB (trenbolone), and THG (tetrahydrogestrinone). Compounds with known or discussed specific effects are: a, diamide (sulfhydryl blocker, c-mitotic); b, methylmethane sulfonate (alkylating, clastogenic); c, benzonitrile (tubulin effect); d, nitrobenzene (tubulin effect); e, colcemide (spindle poison); f, cytochalasin B (cytokinesis inhibitor); g vincristine (spindle poison); h, genistein (topoisomerase-II poison).

NA, TB, and THG fall into the belt of non-specific compounds, however, in doing so TB is very closely located to the border line. MAD, having a very high log P value of 6.08, is located right-hand of the so far regarded cluster of non-specific compounds.

Following the equation of the genotoxicity - lipophilicity relationship (Equ. 1) for the four steroids the theoretically effective concentration was predicted (pred -log C). The parameters log P, exp –log C, and pred -log C of the four steroids are listed in Tab. 9.

Tab. 9. Evaluation set of compounds: Positive compounds with their log P and experimentally determined concentrations that lead to a doubling of the background rate micronucleus (exp –log C) as well as the expected effective concentration following Equ. 1.

Compounds	log P	exp -log C	pred –log C
Madol	6.08**	4.57	7.26
19-Norandrostenedione	2.49**	4.54	3.64
Trenbolone	1.82**	4.64	2.97
Tetrahydrogestrinone	3.39**	5.52	4.55

**log P (Clog P) as obtained with the BioByte ClogP programme

3.3 Physicochemical parameters

3.3.1 Correlation of genotoxicity and other physicochemical parameters

In order to generate molecular descriptors - other than log P - for modelling non-specific chromosomal genotoxicity, and to evaluate optimized combinations thereof, the available data sets on aneuploidy and micronucleus formation were again regarded in combination (see Chapter 2.2.9.2). The molecular descriptors for each compound were calculated and linear equations were set up relating molecular descriptors with experimental concentrations (exp –log C) at which doubling of binucleated cells or micronuclei occurred, respectively.

The complete results of all computations obtained are listed in the Appendix, Chapter 7.2.2. Relevant results are included in graphic form in Fig. 19.

The optimum correlations that were generated by the genetic function approximation (GFA) procedure used to evolve best models to describe the relationship (see Chapter 2.2.9.2) were the following (correlation coefficient for the 16 datapoints for non-specific compounds):

Including linear terms

One variable

$$\text{pred} -\log C\ 1 = 1.38558 + 0.812685 \times \log P \qquad \text{Equ. 2}$$

$$[r^2 = 0.88]$$

Two variables

$$\text{pred} -\log C\ 2 = 1.03464 + 0.583884 \times \log D + 0.004906 \times \text{surface} \qquad \text{Equ. 3}$$

$$[r^2 = 0.94]$$

Three variables

$$\text{pred} -\log C\ 3 = 0.357691 - 6.44451 \times \text{acc} + 0.334832 \times \text{polsurf} + 0.021915 \times \text{surface} \qquad \text{Equ. 4}$$

$$[r^2 = 0.97]$$

Including linear as well as quadratic terms

One variable

$$\text{pred} -\log C = 1.38558 + 0.812685 \times \log P$$

Two variables

$$\text{pred} -\log C = 1.03464 + 0.583884 \times \log D + 0.004906 \times \text{surface}$$

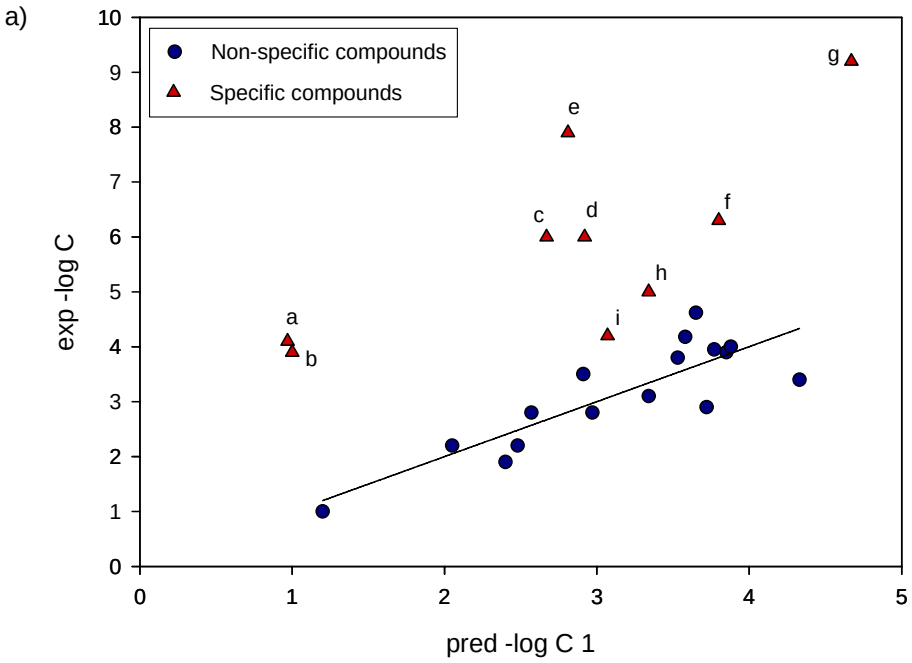
Three variables

$$\text{pred} -\log C\ 4 = 0.353887 - 0.003947 \times \text{polsurf}^2 + 0.020997 \times \text{surface} + 0.056302 \times \text{dipole} \qquad \text{Equ. 5}$$

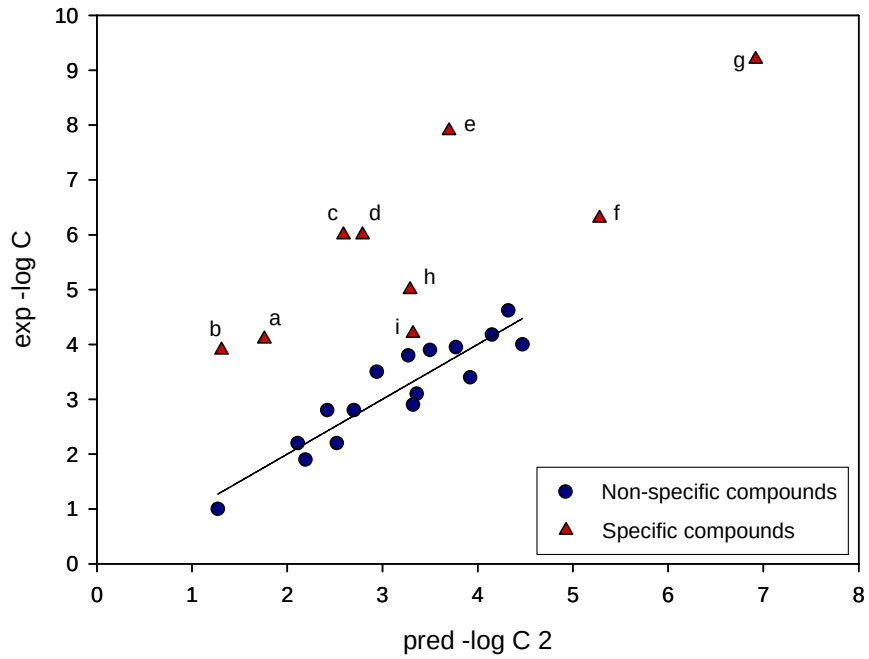
$$[r^2 = 0.97]$$

It should be noted that using one or two variables the optimum fit of calculated molecular properties with the experimental –log C values contains only linear terms, even when quadratic terms are allowed.

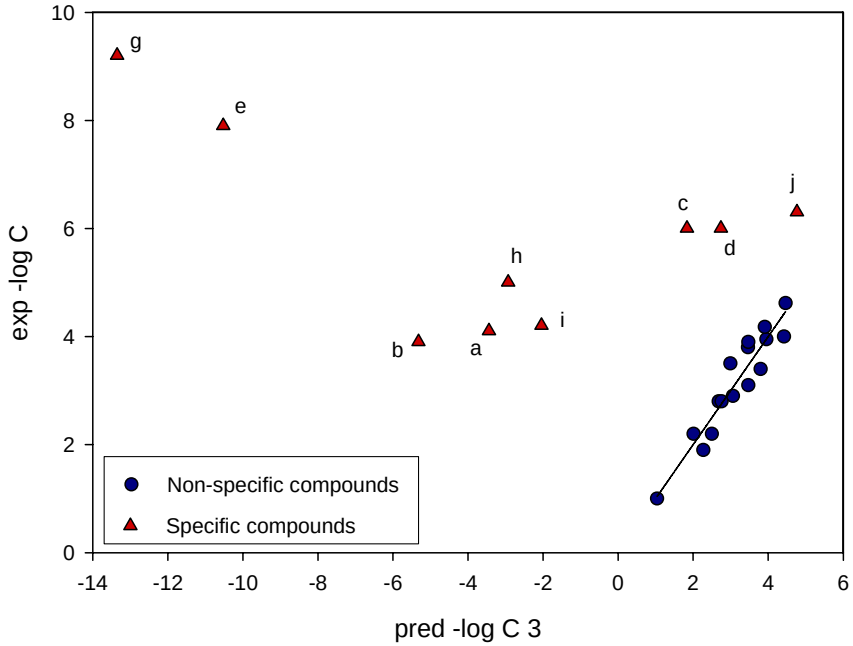
The combinations of molecular variables expressed in the four different equations (Equ. 2-5) have been plotted in Fig. 19 , i.e. the pred –log C (1-4) against the actual experimentally determined exp –log C.



b)


exp -log C
pred -log C 2
Non-specific compounds
Specific compounds
a
b
c
d
e
f
g
h
i

c)


exp -log C
pred -log C 3
Non-specific compounds
Specific compounds
a
b
c
d
e
g
h
i
j

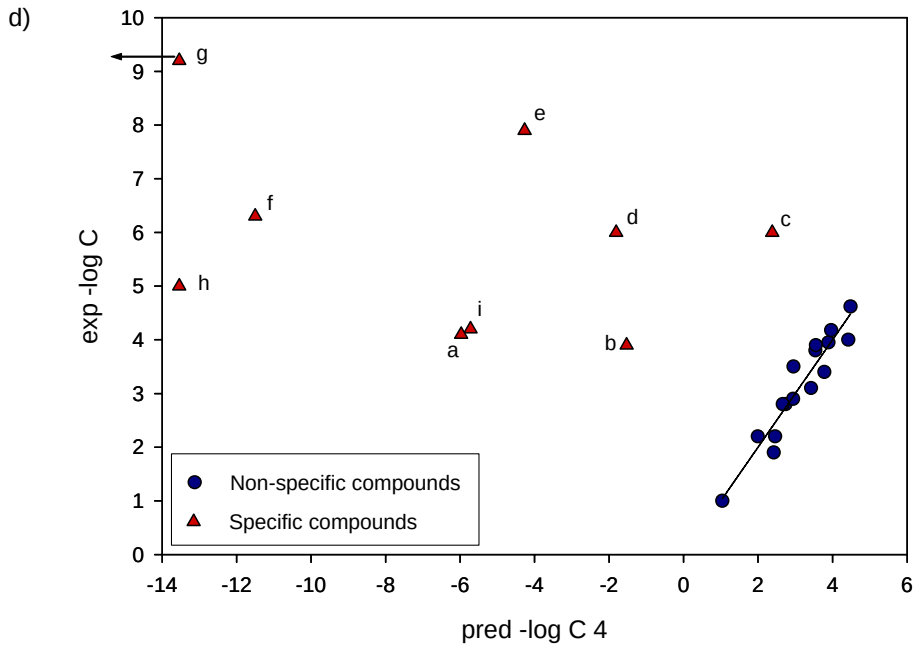


Fig. 19. Relation of experimentally detected effective concentration (exp –log C) and predicted effective concentration (pred –log C), determined through the given equations. a) single molecular parameter, Equ. 2; b) two molecular parameters, Equ. 3; c) three molecular parameters (linear term), Equ. 4; d) three molecular parameters (quadratic term), Equ. 5. Compounds with known specific modes of action were: a, diamide (sulfhydryl blocker, c-mitotic); b, methylmethane sulfonate (alkylating, clastogenic); c, benzonitrile (tubulin effect); d, nitrobenzene (tubulin effect); e, colcemide (spindle poison); f, cytochalasin B (cytokinesis inhibitor); g, vincristine (spindle poison); h, genistein (topoisomerase-II poison); i, daidzein.

Based on the descriptors provided to the GFA procedure, log P was clearly the most suitable single property to relate to the non-specific genotoxicity [r^2=0.88]. Using more descriptors, however, resulted in improved correlations (r^2 closer to 1). Using up to three descriptors in combination resulted in correlations, up to r^2=0.97 (Equ. 4 and 5).

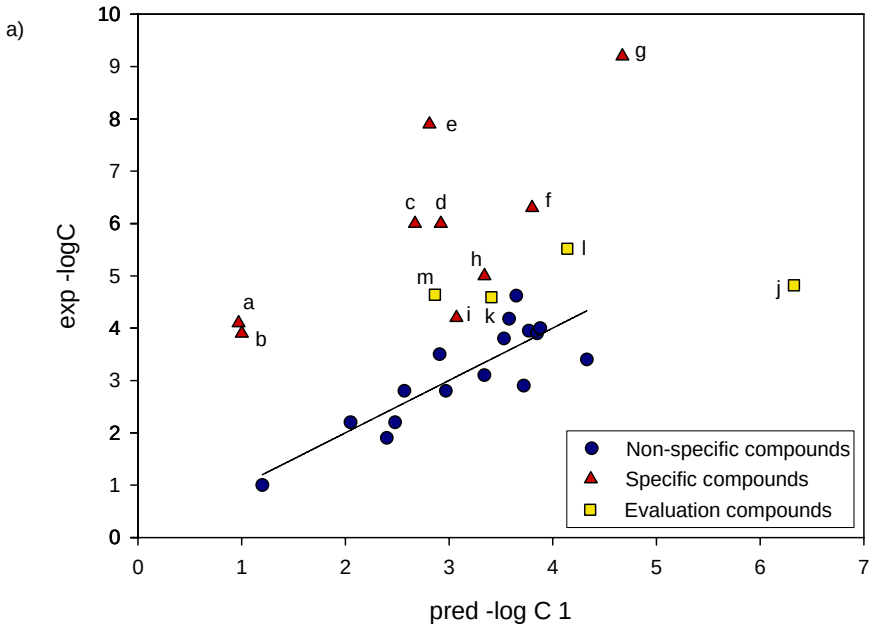
3.3.2 Evaluation of the relationship of genotoxicity and physicochemical parameters

For evaluation of the relationship between genotoxicity and different physicochemical properties the genotoxic potential of the steroids TB, THG, NA, and MAD was adducted. For the four steroids, the pred –log C values (1-4) were determined calculatively following the Equ. 2-5. The compounds were added individually to the data set and in each case correlations were determined for each single compound. The results are shown in Tab. 10.

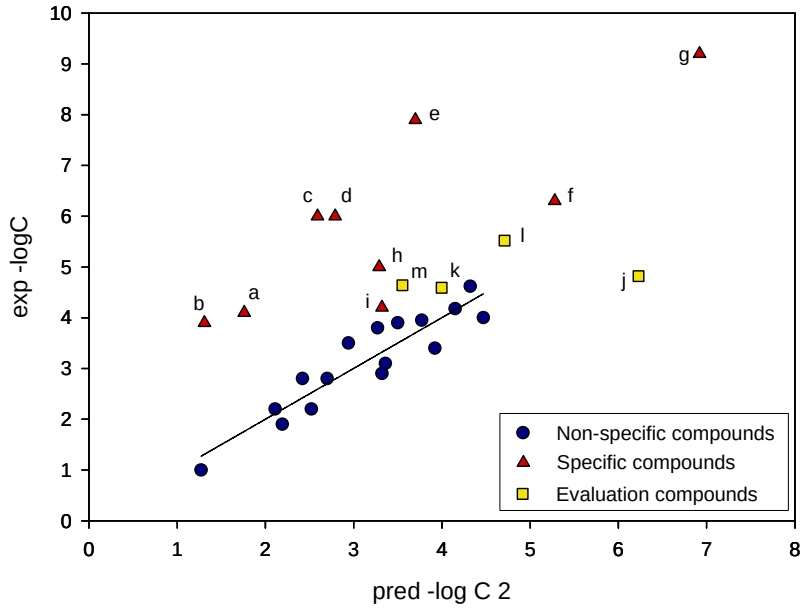
Tab. 10. Evaluation of the relationship: exp –log C and pred –log C values for MAD (madol), NA (19-norandrostenedione), THG (tetrahydrogestrinone), and TB (trenbolone), [r^2 for each single compound].

Compound	exp -log C	pred -log C 1	pred -log C 2	pred -log C 3	pred -log C 4
MAD	4.57	6.33 [r^2=0.84]	6.23 [r^2=0.90]	6.74 [r^2=0.90]	6.49 [r^2=0.91]
NA	4.54	3.41 [r^2=0.82]	4.00 [r^2=0.92]	3.87 [r^2=0.94]	3.72 [r^2=0.93]
TB	5.52	4.14 [r^2=0.76]	4.71 [r^2=0.90]	4.47 [r^2=0.95]	4.75 [r^2=0.96]
THG	4.64	2.86 [r^2=0.73]	3.55 [r^2=0.93]	3.51 [r^2=0.94]	3.65 [r^2=0.78]

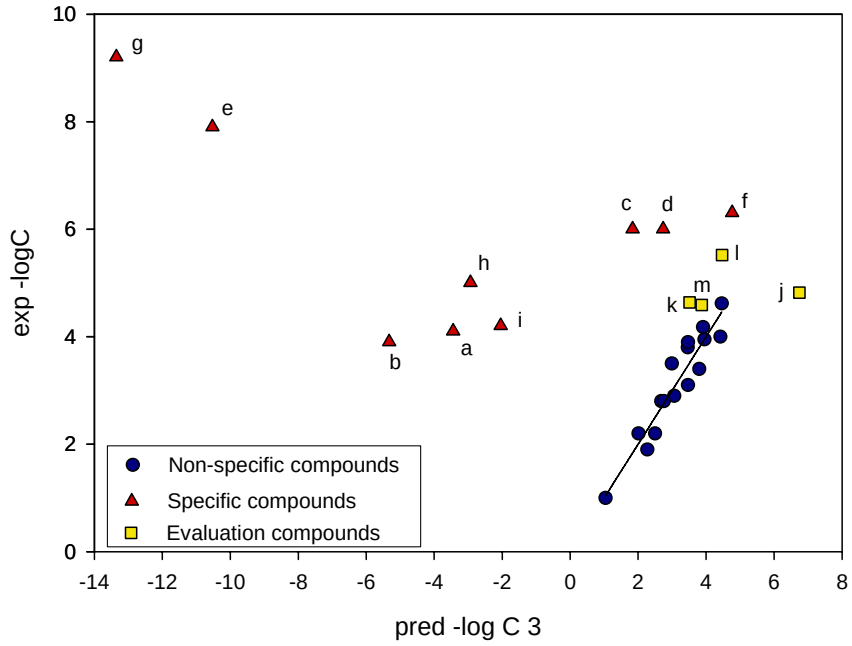
The compounds were included in the plots of Fig. 19, plotting the experimentally determined effective concentration (exp -log C) *versus* the referring pred -log C. The results are depicted in Fig. 20.



b)


exp -logC
pred -log C 2
Non-specific compounds
Specific compounds
Evaluation compounds
a
b
c
d
e
f
g
h
i
j
k
l
m

c)


exp -logC
pred -log C 3
Non-specific compounds
Specific compounds
Evaluation compounds
a
b
c
d
e
f
g
h
i
j
k
l
m

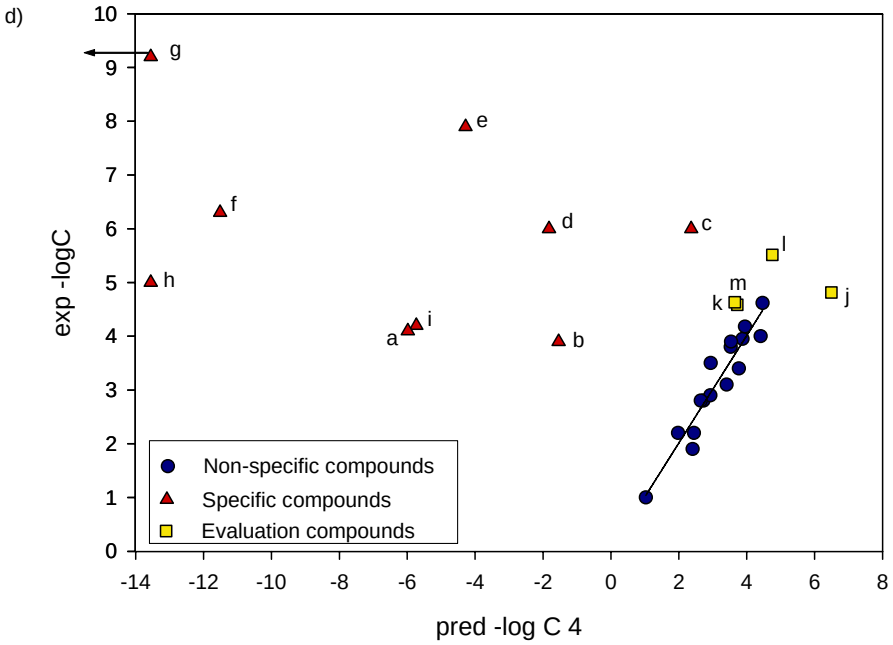


Fig. 20. Plots of correlation of exp –log C and pred –log C including the evaluation set of compounds MAD (madol; j), NA (19-norandrostenedione; k), THG (tetrahydrogestrinone; l), and TB (trenbolone; m). a) single molecular parameter, Equ. 2; b) two molecular parameters, Equ. 3; c) three molecular parameters (linear term), Equ. 4; d) three molecular parameters (quadratic term), Equ. 5.

In each plot, if one up to three physicochemical parameters was taken to describe non-specific genotoxicity, NA is located very close to the non-specific compounds. TB and THG mainly fall close but not within the cloud of non-specific compounds; in plots c) and d), TB converges the cloud (increasing r^2 to 0.96). In all cases, MAD falls outside of the cluster of non-specific compounds.

3.4 Relationship of microtubule assembly and lipophilicity

In order to further broaden the database, a data set obtained from the Institute for Molecular Biotechnology (IMB), Jena, Germany was analyzed regarding the influence of lipophilicity on the process of microtubule assembly and disassembly. The chemicals included (log P between -1.5 and +1.0) had been assessed in Jena as to their potencies to influence the dynamic processes of microtubule assembly and disassembly in a cell-free system *in vitro*. The complete results as obtained from the IMB, Jena, are listed in the Appendix, Chapter 7.2.7. The maximum microtubule assembly value that was reached at the plateau in the microtubule assembly curves was taken to generate concentration-effect-curves, from which the no-observed-effect-concentrations (NOECs) were determined.

Integrated results obtained from the microtubule assembly assay data, the no-observed-effect-concentrations (NOEC) as well as IC_{50} values and the concentrations at which aggregates were formed are given in Tab. 11, along with the log P values.

Tab. 11. Summary of the effects of organic test substances in the tubulin assembly assay (all concentrations in mM); the compounds are presented in the order of rising lipophilicity (log P). The compilation also includes results from previous publications by Bonacker et al. (2004a: benzonitrile, nitrobenzene; 2004b: colchicine, colcemid, vincristine, with a specific mode of action)

Compound	**log P**	**NOEC (mM)**	**IC_{50} (mM)**	**Formation of aggregates (mM)**
Acetamide	-1.26[a]	335	n.d.	1000
Acrylamide	-0.67[a]	15	190	300
Methylmethane sulfonate (MMS)	-0.48[b]	48	160	175
Acetonitrile	-0.34[a]	25	75	500
Acrylonitrile	0.25[a]	14	30	200
Cyclohexanone	0.81[a]	10	n.d.	20
Benzonitrile	1.56[a]	5	n.d.	15
Nitrobenzene	1.85[a]	0.5	n.d.	4
Specific compounds (action on microtubules)				
Colchicine	1.30[a]	1×10^{-3}	1.2×10^{-3}	
Colcemid	1.37[a]	4×10^{-4}	6.3×10^{-4}	
Vincristine (VCR)	2.57[a]	3×10^{-5}	8.6×10^{-5}	

[a] Hansch et al.

[b] BioByte ClogP programme (BioByte Corp., Claremont, CA, USA)

The dependence of the experimental no-observed-effect-concentrations (-log NOEC) in the microtubule assembly assay on log P as parameter of lipophilicity was determined plotting the log P values and NOEC against each other. Regression for the “non-specific” compounds (no specific mode of action is known) was determined. The results are shown in Fig. 21.

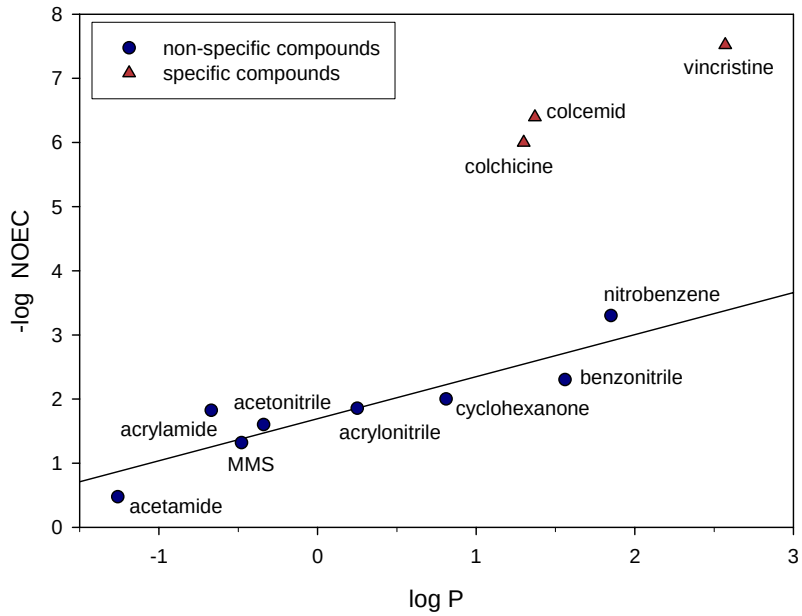


Fig. 21. Relation of microtubule assembly and lipophilicity: The ordinate shows no-observed-effect-concentrations (NOEC) in the microtubule assembly assay of acetamide, acrylaminde, acetonitrile, acrylonitrile, methylmethane sulfonate (MMS), and cyclohexanone, together with organic compounds investigated previously (Bonacker et al 2004a,b). The abscissa gives the lipophilicity values (log P) for the test compounds.
Compounds with known specific modes of action were the spindle poisons colcemide, colchicine, and vincristine.
($y = 0.655\ x + 1.694$; $R^2 = 0.81$)

For the compounds, except for those with a known specific mode of action, a clear correlation of lipophilicity and microtubule assembly is seen. This contrasts to the effects on the test system of the known specific effectors (colchicine, colcemid, vincristine), which fall apart of the regression line.

3.5 Mechanistical studies

3.5.1 Cytotoxicity

Genotoxicity assays, such as the micronucleus assay, may be compromized by interfering cytotoxic and cytostatic effects. Hence, it is important to define the cytotoxic concentration ranges, i.e. concentrations which result in strong inhibition of cell division. Cytotoxicity of the steroids was determined either using Neutral Red uptake or the CellTiter Blue™ reduction as endpoints. The limits of

cytotoxicity (IC_{20}) as well as IC_{50} values were taken from the concentration–response-curves and are given in Tab. 12.

Tab. 12. Cytotoxicity of the tested compounds: IC_{20} and IC_{50} values.

Compound	**IC_{20} (µM)**	**IC_{50} (µM)**
7α-Methyltestosterone	190(**)	n.d.(**)
17α-Propylmesterolone	35(**)	60(**)
19-Norandrostenedione	300	n.d.(**)
19-Norethisterone	55(*); 70(**)	n.d.(*); 200(**)
19-Nortestosterone	55(*); 150(**)	180(*); n.d.(**)
Androstenedione	130(**)	250(**)
Ethisterone	n.d.(**)	n.d.(**)
Madol	n.d.(**)	n.d.(**)
Testosterone	165(*); 155(**)	n.d.(**); 285(**)
Tetrahydrogestrinone	40(**)	90(**)
Trenbolone	75(**)	230(**)
Nor-C	90(**)	270(**)
Nor-D	130(**)	270(**)
Nor-E	40(**)	140(**)
Nor-F	15(**)	45(**)
Nor-G	70(**)	200(**)

n.d.: cytotoxicity not detectable

(*) Neutral Red uptake assay

(**) CellTiter™ Blue assay

NE, NT, and T were tested in two different assays, the Neutral Red uptake and the CellTiter Blue™ assay. The obtained IC_{20} and IC_{50} values in both assays were of comparable order of magnitude.

Regarding the IC_{20} values obtained by means of the CellTiter Blue™ assay the hierarchy of cytotoxicity from the most cytotoxic to the least cytotoxic compound was as follows: Nor-F > PM > THG = Nor-E > Nor-G = NE > TREN > Nor-C > Nor-D = AND > NT > T > MT > NA. No measurable cytotoxicity was seen for ETHI and MAD.

For a majority of compounds, more pronounced cytotoxicity (IC_{50} values) was reached at higher concentrations (Tab. 12) with a similar sequence of compound potency: Nor-F > PM > THG > Nor-E > Nor-G = NE > TREN > AND > Nor-C = Nor-D > T. No IC_{50} values were reached in the tested concentration range for ETHI, MAD, MT, NA, and NT.

3.5.2 Cell cycle analysis

The steroids detected as "positive" in the MN assay (NT, NE, Nor-C, TB, THG, MAD, NA), one slightly positive (Nor-G) and two negative compounds (T, ETHI) were analyzed for their effects on the cell cycle. The concentration range tested for cell cycle effects covered the concentration(s) detected positive or slightly positive in the MN assay for the positive/slight positive compounds, or the highest negative detected concentration for the negative compounds.

The results for the compounds and the corresponding concentrations leading to a significant shift of the distribution in the single cell cycle phases are summarized in Tab. 13.

The complete results of all tested compounds and concentrations are listed in the Appendix (see Chapter 7.2.4).

Tab. 13. Distribution of cells in the different cell cycle phases after 18h substance treatment

Compound	**Concentration**	**Cell cycle phases** *(%)*			
	(µM)	*G0/G1*	*S*	*G2/M*	*Sub G0*
NA	0	61.16 (+/-2.86)	18.65 (+/-1.57)	20.19 (+/-1.30)	
	30	54.33 (+/-2.67) *	21.61 (+/-1.58)	20.56 (+/-4.26)	
	100	49.54 (+/-5.77) *	24.06 (+/-4.25)	29.90 (+/-9.88)	
NT	0	61.86 (+/-4.41)	19.80 (+/-6.99)	18.34 (+/-2.75)	
	100	47.82 (+/-2.35)*	20.79 (+/-4.94)	31.38 (+/-4.08)*	
Nor-C	0	57.21 (+/-10.11)	20.95 (+/-5.79)	21.84 (+/-5.33)	
	100	42.63 (+/-4.81)*	20.55 (+/-3.37)	36.82 (+/-7.35) *	
T	0	58.88 (+/-5.28)	20.74 (+/-3.53)	20.38 (+/-3.91)	
	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)	
	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)	
	60	35.93 (+/-5.39)***	17.50 (+/-0.69)	21.13 (+/-4.06)	25.44 (+/-4.57)

*p < 0.05

**p < 0.005

***p < 0.001

No changes in cell cycle distribution were detected in V79 cells treated with ETHI (up to 100µM), NE (up to 60µM) as well as with MAD and Nor-G (up to and 30µM), respectively. THG also did not lead to cell cycle changes up to 30 µM; at 60 µM the subG0/G1 cell population was increased, thus pointing to apoptosis. Treatment with 100 µM NT, 100 µM Nor-C, and 300 µM T led to a clear increase in the fraction of cells in G2/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 G2/M, indicative of a concentration-dependent cell cycle arrest.

3.5.3 Apoptosis induction

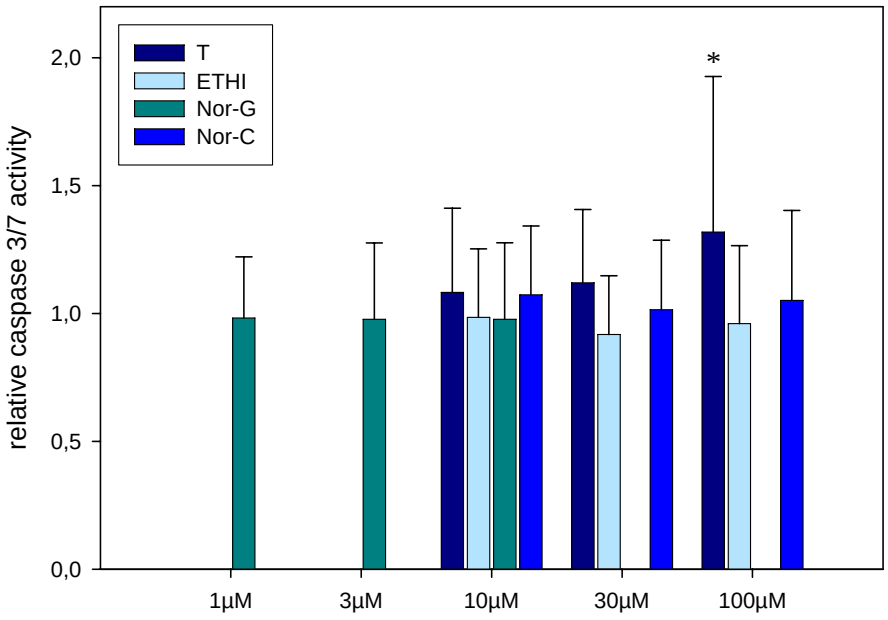
The compounds which were detected to be positive in the MN Assay (NT, NE, Nor-C, TB, THG, MAD, NA) were tested for their apoptosis inducing potential, as well as one slight positive (Nor-G) and two negative compounds (T, ETHI). The concentration range tested in the apoptosis assays covered the concentration(s) detected positive or slightly positive in the MN assay (positive/slight positive compounds), or the highest negative detected concentration (negative compounds), respectively.

The apoptosis inducing potential of the compounds was determined assaying two different endpoints, caspases-3/7 activity and Annexin V binding to phosphatidylserine (see Chapter 2.2.6). The results obtained in the Caspase-3/7 assay are depicted in Fig. 22.

For 100 µM T, a slight but statistical significant increase of caspases-3/7 activity (1.3-fold relative to solvent control) was detected. NT led to a 1.2-fold increase of caspases 3/7 activity at 10 µM, but at higher concentrations, no statistical significant increase was detectable.

No induction of caspase-3/7 activity was detected with the other tested compounds ETHI, MAD Nor-C, and TB up to 100 µM, NE up to 60 µM, and Nor-G up to and 30 µM, respectively (Fig. 22).

a)



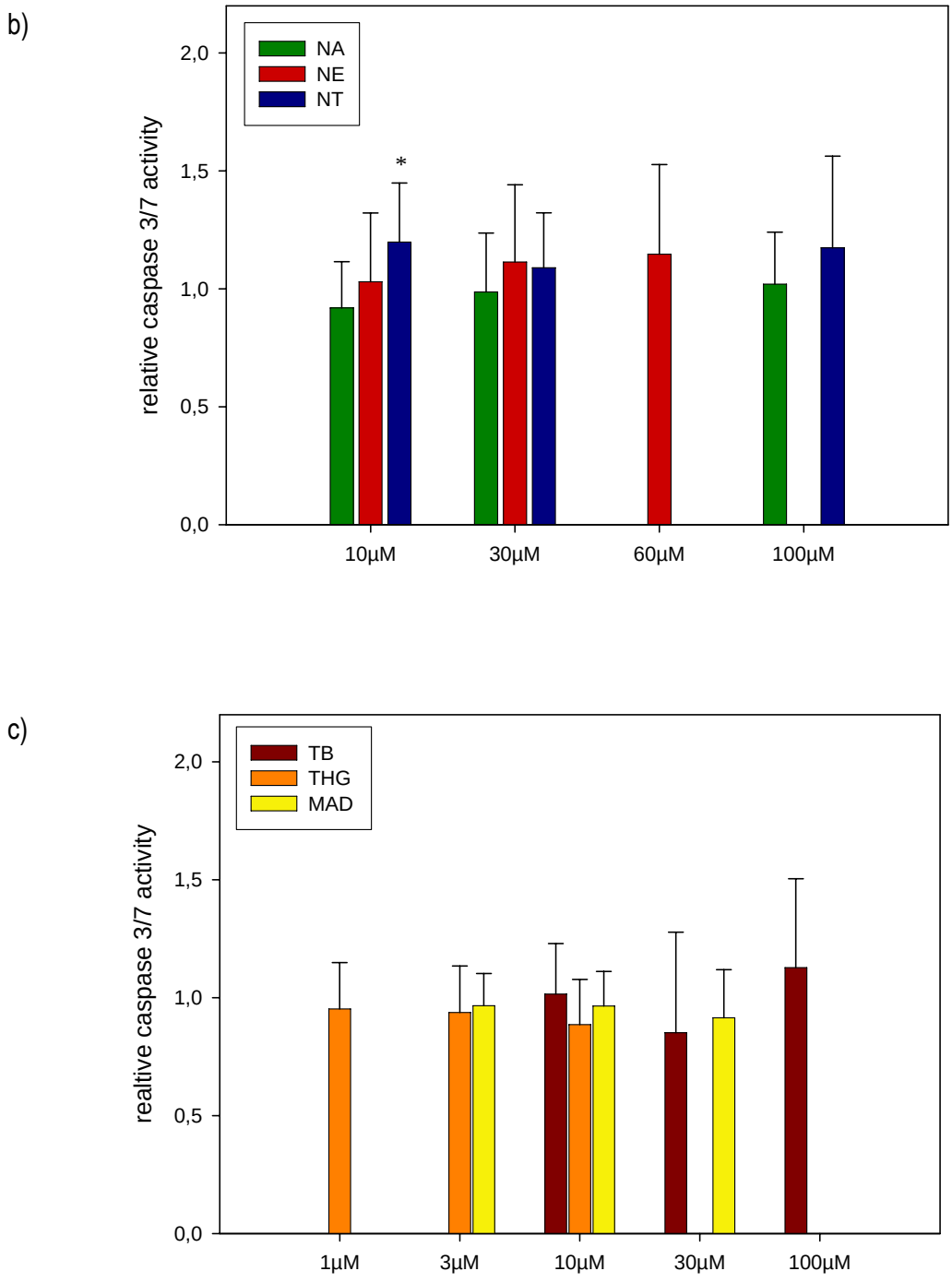


Fig. 22. Induction of caspases-3/7 activity after 18 h substance treatment relative to solvent control, a) T (testosterone), ETHI (ethisterone), Nor-G, Nor-C; b) NA (19-norandrostenedione), NE (19-norethisterone), NT (19-nortestosterone); c) TB (trenbolone), THG (Tetrahydrogestrinone), MAD (madol).

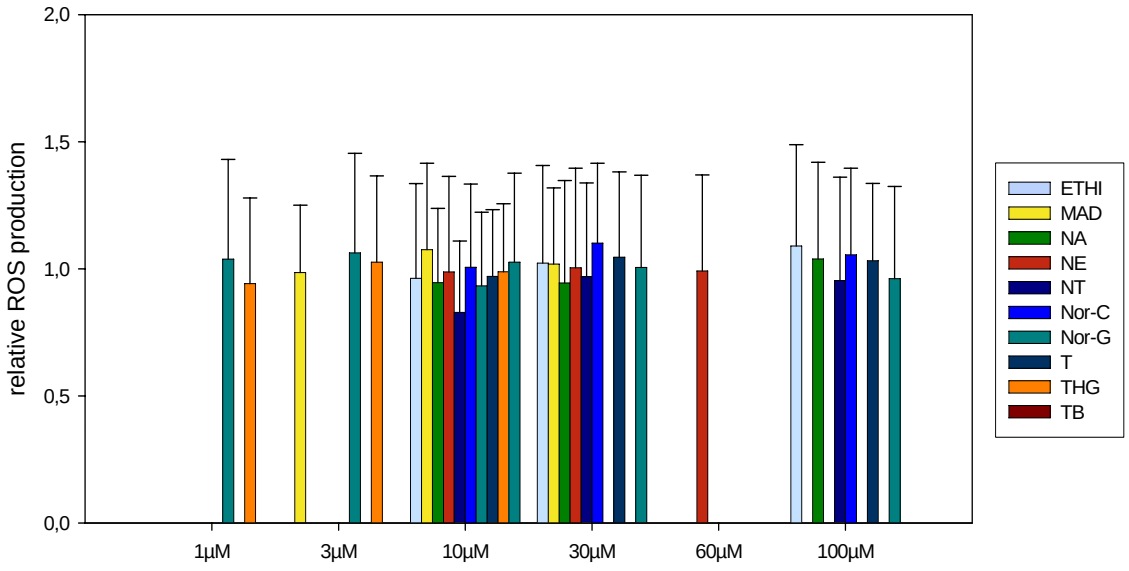
Induction of apoptosis was also measured by means of the Annexin-V/PI assay. A detailed overview on the results is given in the Appendix (see Chapter 7.2.5). With this assay, induction of apoptosis and late apoptosis/necrosis was detected at high concentrations for some of the tested compounds (T, ETHI, NT, NE, TB, and THG). But, as very high standard deviations were obtained, and because of the incongruence with the data obtained in the Caspase-3/7 assay, the Annexin-V assay appeared not suitable for apoptosis detection in the V79 cell system, under the conditions used in the present study.

3.5.4 Production of reactive oxygen species

The potential to generate reactive oxygen species (ROS) was tested for the compounds which were detected to be positive in the MN assay (NT, NE, Nor-C, TB, THG, MAD, NA), slight positive (Nor-G) as well as two negative compounds (T, ETHI).

ROS production was measured after 30 min, 1 h and 18 h substance treatment. For none of the tested steroids an increase in ROS production compared to the solvent control was detected, neither concentration-dependent, nor time-dependent (Fig. 23).

a)



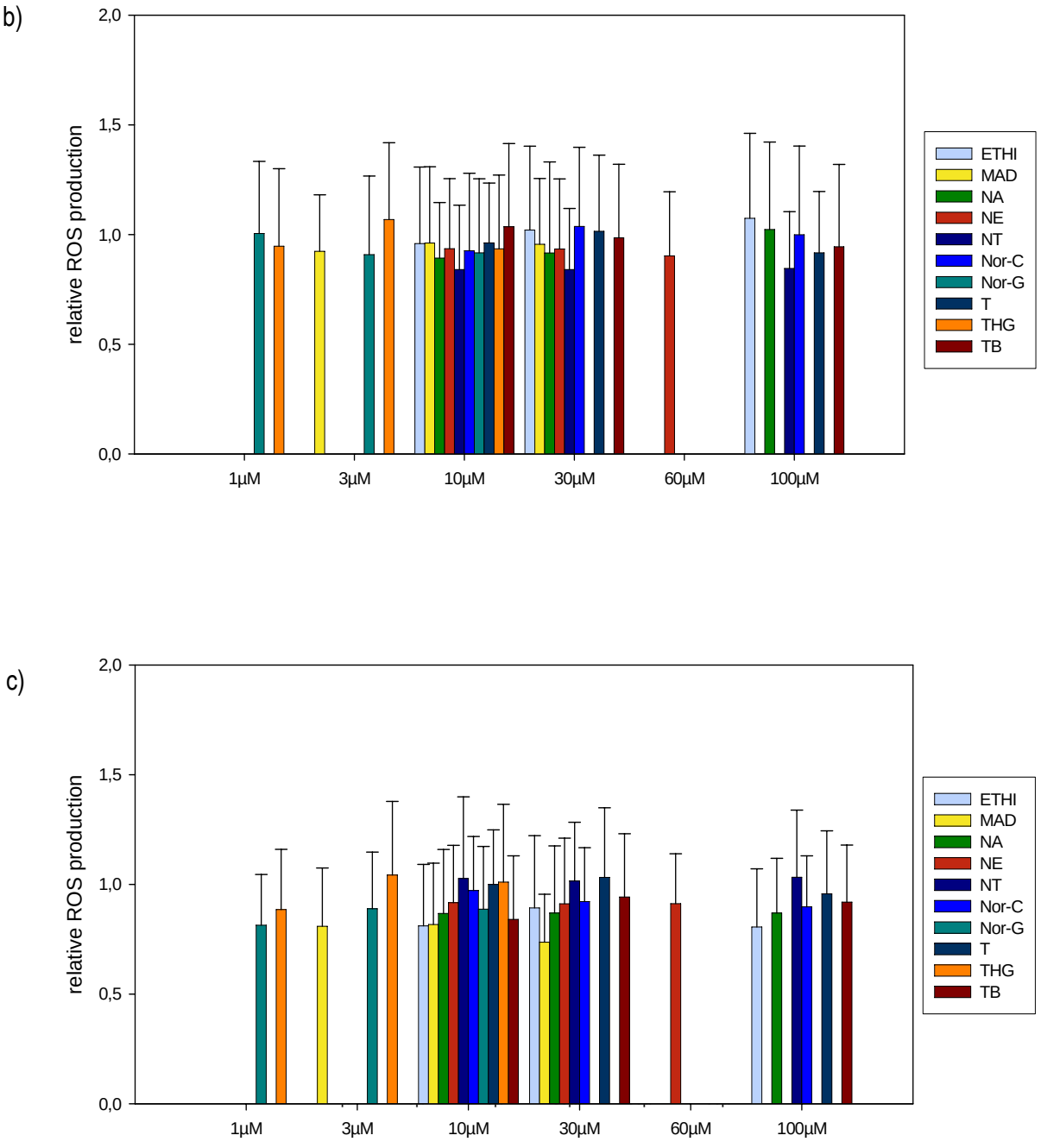


Fig. 23. ROS production of ETHI (ethisterone), MAD (madol), NA (19-norandrostenedione), NE (19-norethisterone), NT (19-nortestosterone), Nor-C, Nor-G, T (testosterone), THG (tetrahydrogestrinone), and TB (trenbolone) relative to solvent control; a) 30 min substance treatment, b) 1 h substance treatment, c) 18 h substance treatment.

4 Discussion

The present experiments were intended to apply the paradigm of “specific” versus “non-specific” genotoxicity of Schultz and Önfelt (2000) to current problems in the drug development of hormonally active steroids. This must be seen against the background of a more general scientific discourse on threshold mechanisms in genotoxicity and carcinogenicity (Bolt et al., 2004; Bolt and Degen, 2004).

4.1 General aspect: genotoxins and thresholds of effects

From a regulatory point of view, carcinogenic compounds are handled differently, according to genotoxic and non-genotoxic modes of action. A conservative strategy of carcinogenic risk evaluation is the application of a linear extrapolation model from high-dose to low-dose effects for genotoxic carcinogens (Goldman, 1996). However, there is an ongoing discussion as to whether genotoxic compounds can be divided into distinct sub-classes, considering the possibility to derive health-based “no-observed-adverse-effect-levels” (NOAELs).

4.1.1 Genotoxic mechanisms leading to a threshold

As one-base change in the DNA may theoretically lead to a mutation and DNA damage with an impact on health, it has been argued in theory that even a single molecule of a carcinogen could - at a very small, but finite probability – impose a cancer risk (Kirsch-Volders et al., 2003). The paradigm that genotoxic effects generally have a non-threshold mode of action had been accepted for years. Currently, this is increasingly being questioned (Madle et al., 2000), as a number of indirect mechanisms has been described that may result in positive results of genotoxicity testings. Chromosomal effects such as aneuploidy, chromosome loss and non-disjunction (Kirsch-Volders et al., 2003; Parry et al., 2000), but also mechanisms of clastogenicity have been repeatedly addressed (Lynch et al., 2003). Scott et al. (1991) have summarized a number of various processes and situations that could indirectly lead to genotoxicity (Tab. 14). *In vitro*, extreme culture conditions like extreme pH or osmolality are reported to be a reason of positive genotoxicity results.

Tab. 14. A number of indirect mechanisms of genotoxicity (Scott et al., 1991)

Mechanism	**Cellular target**
Enzyme inhibition	Enzymes of DNA synthesis
	Enzymes of DNA repair
	Topoisomerase I / II
	Na^+/ K^+ ATPase
Imbalance of DNA precursors	DNA precursors
Energy depletion	Energy metabolism system
ROS production	Oxygen and superoxide radicals
Lipid peroxidation	Membranes
Sulfhydryl depletion	Sulfhydryls
Nuclease release from lysosomes	Lysosomes
Protein synthesis inhibition	Nuclear proteins
Protein denaturation	Nuclear proteins
Ionic imbalance	Chromatin? Enzymes?

Processes including DNA degradation, like cytotoxicity (Kirkland et al., 2007a; Kirkland et al., 2000), and especially apoptosis (Meintières et al., 2001; Meintières and Marzin, 2004) have been described as being confounding in genotoxicity tests.

For such mechanisms biologically plausible thresholds are expected: “no-effect” levels or concentrations (NOAELs or NOECs) are likely, below which genotoxicity is not induced. When more targets have to be hit before an effect level is reached, a higher probability of a threshold response is expected (Elhajouji et al., 1997).

Now, there is consensus that thresholds in dose-response can be defined for non-DNA-reactive genotoxins, or if the dose-range between no-effect and effect is narrow (Kirsch-Volders et al., 2000a; Parry et al., 2000). In cases of interaction induced by a direct biochemical reaction between compound and DNA target (induction of DNA adducts, DNA breaks or modified bases) a threshold is not expected (Kirsch-Volders et al., 2000a).

So far, lipophilicity and other general molecular parameters of compounds have not been discussed as important contributors to indirect modes of genotoxic action. However, non-specific interactions with cellular targets may be important (see Chapter 4.3.5).

Against this background, the biological relevance of positive genotoxicity results caused by indirect mechanisms is being questioned. Often, the NOECs derived in *in vitro* tests from indirect

genotoxic chemicals may not be reached *in vivo*. In such cases, an *in vitro* genotoxicity result would not be predictive for a human hazard (Kirkland et al., 2007b). Besides indirect genotoxicity, chemicals may induce genetic damage by processes specific to the *in vitro* test system or conditions. Also, chemicals or their metabolites may induce direct damage to DNA only at concentrations above a threshold defined by protective processes.

Regulatory bodies have been involved in these scientific debates. For instance, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has proposed to evaluate positive *in vitro* test results for biological relevance, taking into account (ICH S2A, 1995):

- Is the increase in response over the negative or solvent control background regarded as a meaningful genotoxic effect for the cells?
- Is the response concentration-related? For weak/equivocal responses, is the effect reproducible?
- Is the positive result a consequence of an *in vitro* specific metabolic activation pathway/*in vitro* specific active metabolite?
- Can the effect be attributed to extreme culture conditions that do not occur in *in vivo* situations, e.g., extremes of pH; osmolality; heavy precipitates especially in cell suspensions?
- For mammalian cells, is the effect only seen at extremely low survival levels?
- Is the positive result attributable to a contaminant?
- Do the results obtained for a given genotoxic endpoint conform to that for other compounds of the same chemical class?

4.1.2 The threshold concept for genotoxic compounds in cancer risk assessment

Non-genotoxic carcinogens are characterized by the “conventional” dose-response from which a no-observed-adverse-effect-level (NOAEL) can be derived. Through insertion of an “uncertainty” or “safety” factor permissible exposure levels are anticipated, at which no relevant human cancer risks are expected.

For genotoxic chemicals, several possibilities for assessing carcinogenic risk have been suggested (Streffer et al., 2004). If positive results are only obtained for chromosomal genotoxicity, e.g. aneugenicity or clastogenicity, in the absence of mutagenicity, a non-DNA-reactive mechanism, such as topoisomerase inhibition (Lynch et al., 2003) or disturbance of spindle apparatus or associated

motor proteins (Decordier et al., 2002; Thier et al., 2003) may be assumed. For such compounds, the existence of “practical” thresholds has been supposed (Crebelli, 2000; Parry et al., 2000).

Several approaches to differentiate types of thresholds in cancer risk assessment have been proposed. Seiler (1977) distinguished between *apparent* and *real* thresholds. *Absolute, real* or *biological* versus *apparent*, and *statistical* thresholds, as well as *perfect* versus *practical* thresholds have been differentiated by Kirsch-Volders et al. (2000a) and Hengstler et al. (2003), respectively. Basically, non-genotoxic carcinogens have been connected with a *real* (Kirsch-Volders et al., 2000a) or *perfect* (Hengstler et al., 2003) threshold. A *statistical* threshold (Kirsch-Volders et al., 2000a) has been attributed to mitotic spindle poisons as the primary interaction occurs with protein(s) instead of DNA. *Apparent* (Kirsch-Volders et al., 2000a) or *practical* thresholds (Hengstler et al., 2003) address chemicals that cause no genotoxic effect due to very low or immeasurable concentrations at the target (Seiler, 1977). Such *apparent* thresholds have been connected with rapid degradation and toxicokinetics of the chemical, or other factors that limit target exposures e.g. DNA repair, apoptosis, and immune surveillance (Kirsch-Volders et al., 2000a).

In taking the different concepts and denominations together, Bolt and Degen (2004) proposed to basically distinguish between “practical” and “true” thresholds. Their “true” threshold includes perfect thresholds described by Hengstler et al. (2003) and both, real and statistical thresholds (Kirsch-Volders et al., 2000a). Apparent thresholds as defined by Kirsch-Volders et al. (2000a) were regarded equivalent to “practical” thresholds (Hengstler et al., 2003).

The described types of thresholds for carcinogens are opposed to the classical dose-response of directly acting genotoxic carcinogens for which no threshold can be defined. However, Streffer et al. (2004) suggested a further differentiation within this group of genotoxins: Clearly DNA reactive and initiating chemical carcinogens (and ionizing radiation as well) should still be classified according to the linear nonthreshold (LNT) extrapolation. For other chemicals with more uncertainty about their action, the LNT extrapolations may be used as a default procedure, backed by the precautionary principle.

In concordance to Streffer et al. (2004), Bolt and Degen (2004) proposed the general scheme to distinguish groups of carcinogens as shown in Fig. 24.

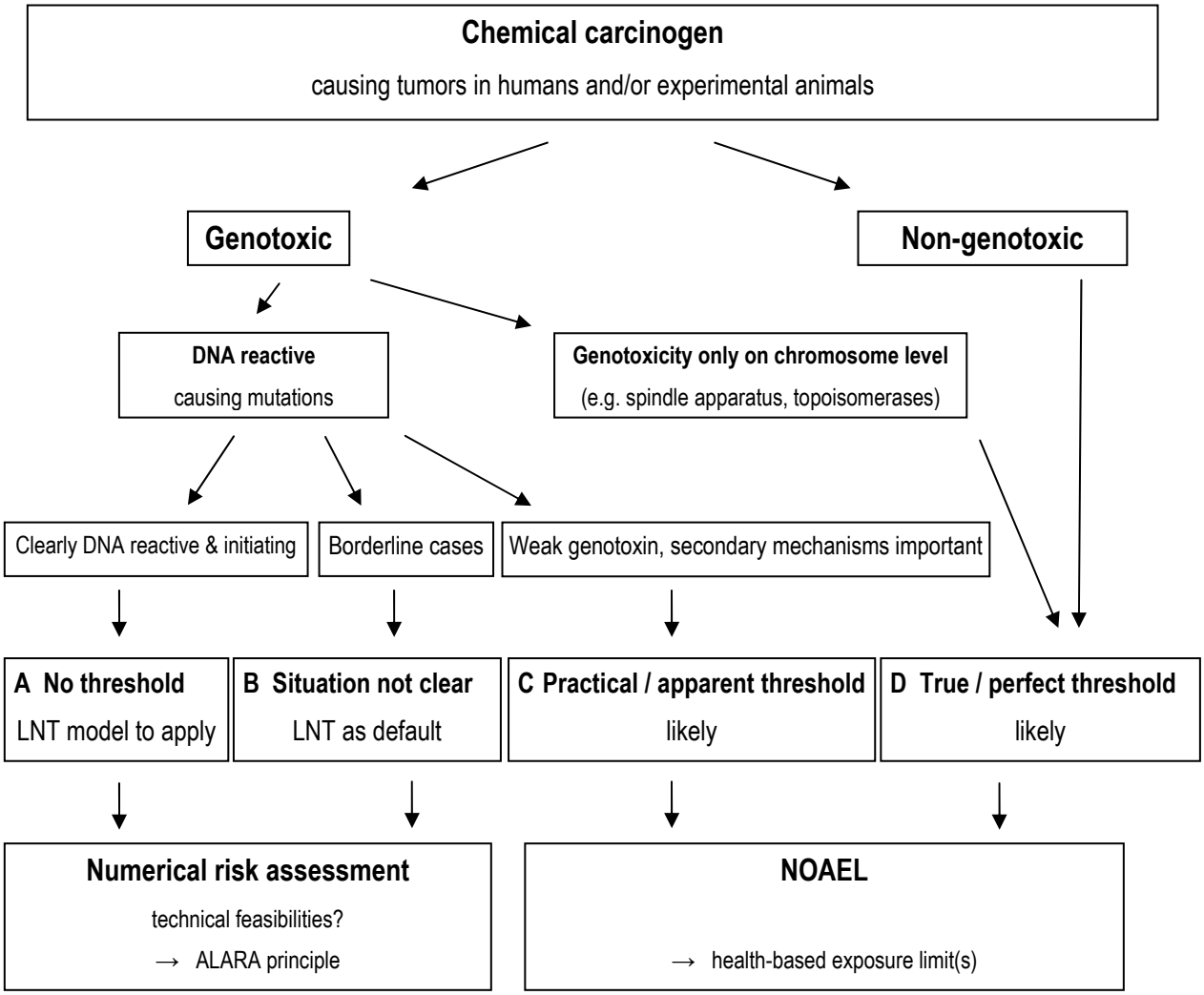


Fig. 24. Proposal for the distinction of groups of carcinogens (A–D) in view of low-dose-extrapolation for the purposes of risk assessment and standard setting (Bolt and Degen, 2004).

Bolt and Degen (2004) distinguish four basic groups of chemical carcinogens (Fig. 24):

A: **Non-threshold** genotoxic carcinogens; for low-dose risk assessment the LNT model appears appropriate. Regulations may be based on the ALARA principle ("as low as reasonably achievable"), technical feasibility, and other socio-political considerations.

B: Genotoxic carcinogens for which the existence of a threshold cannot be sufficiently-supported; **unclear situation** for which the LNT model is used as a default assumption, based on the precautionary principle.

C: Genotoxic carcinogens for which a **practical threshold** is supported by studies on mechanisms and/or toxicokinetics; health-based exposure limits may be based on an established NOAEL.

D: Non-genotoxic and non DNA-reactive carcinogens; for these compounds a **perfect threshold** is associated with a NOAEL, and health-based exposure limits are to be derived.

The proposed threshold concept for chemical carcinogens and the determination of NOAEL may also be reasonably adopted for pharmaceuticals to determine a therapeutic ratio (untoward effect / toward effect).

The scientific debate on thresholds for carcinogens is paralleled by world-wide discussions on systems of classification of carcinogens (Bolt and Degen, 2004; Seeley et al., 2001). However, so far it was only partly picked up by the according national and international organizations and transferred to the regulatory instruments. The German Senate Commission of the DFG for the investigation of health hazards in the work area ("MAK Commission") introduced a new classification system of carcinogens according to which five groups of proven and suspected carcinogens are distinguished: in addition to known (category 1), or considered (category 2) carcinogens in men, and to substances which cause concern that they could be carcinogenic for man but which cannot be assessed conclusively because of lack of data (category 3), a category for "substances with carcinogenic potential for which genotoxicity plays no or at most a minor role. No significant contribution to human cancer risk is expected, provided that the MAK value is observed" (category 4) and for "substances with carcinogenic and genotoxic potential, the potency of which is considered so low that, provided that the MAK value is observed, no significant contribution to human cancer risk is to be expected" (category 5) were included (Neumann et al., 1998). The distinction of new categories expresses the general idea that the classification of carcinogens should be based more on mechanisms by which carcinogenic effects are elicited and should more consider aspects of potency. The classification schemes of the International Agency for Research on Cancer (IARC) and the European Union (EU) are similar to the German approach as they classify chemicals according to their carcinogenic potential in humans (EC, 2001; IARC, 2007); in the Netherlands, carcinogens are grouped into two broad categories according to genotoxicity (Moolenaar, 1994; Seeley et al., 2001). However, the extrapolation and risk estimation procedures for genotoxic carcinogens vary widely among countries: the Netherlands still uses linear extrapolation procedures, whereas the EU uses a margin of exposure type of approach. Regarding determination of occupational exposure limits (OELs) for genotoxic

carcinogens, this is regulated either according to technical feasibility, as in Germany, or to acceptable risk levels, as in the EU and the Netherlands (Bolt and Degen, 2004; Seeley et al., 2001). In the United States, the Environmental Protection Agency (EPA) has published revised guidelines for carcinogen risk assessment, explicitly considering modes of action in dose – response extrapolations (U.S.EPA, 2005; Wiltse and Dellarco, 2000), which induced scientific discussions on how to use information on carcinogenic modes of action (Cohen et al., 2003; Holsapple et al., 2006; Preston, 2007; Thybaud et al., 2007).

Regarding cancer risk assessment of pharmaceuticals, unequivocally genotoxic compounds as determined in *in vitro* and *in vivo* genotoxicity studies, respecting the limitations for biological relevance (see Chapter 4.1.1.), are still presumed to be trans-species carcinogens, implying a hazard to humans (ICH S1A, 1995).

4.2 Methodological points of discussion

4.2.1 Determination of cytotoxicity

There is a wide variety of *in vitro* methods to detect cytotoxicity, which pick up a set of different endpoints. To reflect the influence of chemicals on cell proliferation, parameters such as reduction in cell count, monolayer confluency, mitotic index, and population doubling are often chosen, especially for cytotoxicity in combination with genotoxicity assessments using the CAT assay. Other ways are the measurement of cell numbers and evaluating cell protein or membrane-bound acid phosphatase activity. Cell viability is often evaluated by determination of cellular metabolic activity and mitochondrial function (e.g. in the CellTiter Blue™ (resazurin) assay), the binding capacity of supravital dyes (e.g. the Neutral Red uptake assay) or the cellular membrane integrity (e.g. lactate dehydrogenase (LDH) release).

The method of measuring cytotoxicity is important for the selection of doses/concentrations to be tested for genotoxicity (Kirkland et al., 2007a). Relative cell count, mitotic index and reduction in population doubling give quite different IC_{50} values. Mitotic index has been addressed as an inaccurate measure of cytotoxicity when mitotic activity is increased, possibly through effects of the test chemical on spindle structure and function (Kirkland et al., 2007a). It can be influenced by cell cycle disturbance, as well as by cell lethality (Armstrong et al., 1992). More recently, population doubling has also been shown to lead to a selection of lower concentrations for further testings (Greenwood et al., 2004). There is agreement that a thorough comparison of different measures of cytotoxicity is

needed, in order to find the most appropriate measures, and to make additional observations on the impact of apoptosis and necrosis on the genotoxicity result (Kirkland et al., 2007a).

In the present study, the CellTiter Blue™ assay and Neutral Red uptake assay (for some compounds) were used for determination of the cytotoxic potency of the compounds. The Neutral Red assay is a widespread method for the assessment of cell viability in monolayer cell cultures which is reported to be of high sensitivity (Babich and Borenfreund, 1991; Fotakis and Timbrell, 2006). It was performed for some steroids (testosterone, 19-nortestosterone, and 19-norethisterone) and the results were opposed to the results in the CellTiter Blue™ assay (see Chapter 3.5.1). Both assays give comparable values for cytotoxicity. The cytotoxic potency of the other steroids studied was determined in the CellTiter BlueTM assay, as this assay is simple and fast to perform and – compared to the Neutral Red uptake assay – has the advantage that the cells must not to be fixed for the examination. The manufacturer's protocol was slightly modified using HBSS instead of cell culture medium for the incubation with the dye, as serum protein in culture medium was found to depress the reduction of resazurin (Goegan et al., 1995).

4.2.2 Genotoxicity testing

The genotoxic potential of the steroids tested was determined by means of the MN assay *in vitro*. The MN test *in vitro* was found to be easily performed, providing a clear and simple scoring system, which makes the observation of MN less subjective than that of chromosomal aberrations (Matsuoka et al., 1992). The MN test allows the discrimination of modes of MN generation (aneugenic or clastogenic mode of action of the test compound). Although the differentiation of both modes of action has been done dependent on the MN size, as the induction of larger micronuclei by spindle poisons has been reported (Högstedt and Karlsson, 1985), today techniques for centromere/kinetochore detection like the fluorescence *in situ* hybridization (FISH) or CREST staining are widely used (Mäki-Paakkanen et al., 1995; von der Hude et al., 2000). In the present study, CREST staining was used to distinguish between aneugenic and clastogenic modes of action.

The *in vitro* chromosome aberration test (CAT) and the mouse lymphoma *tk* assay (MLA) are currently used methods to assess DNA and chromosomal damage for regulatory purposes. However, several comparisons of the results obtained in the *in vitro* MN test and in the *in vitro* chromosome aberration test have been performed, and results are reported to be mainly in accordance with each other (Keshava et al., 1995; Miller et al., 1998). The *in vitro* test for chromosomal aberrations (CA) and the mouse lymphoma *tk* assay (MLA) were shown to yield a four-fold higher rate of positives than that other genotoxicity tests in a standard test battery (Müller and Kasper, 2000). Von der Hude

et al. (2000) conclude from a collaborative study with ten independent laboratories for the MN assay both, a high specificity and reproducibility, in the absence of ‘‘false positive’’ results.

Taking all this together, the MN assay presents itself as a good alternative to the conventional genotoxicity screening test for chemicals, being at least as adequate as the mouse lymphoma assay or the *in vitro* chromosome aberration test (Lorge et al., 2007; Matsuoka et al., 1992; von der Hude et al., 2000).

A variant of the “conventional” MN assay is the cytokinesis-block micronucleus (CBMN) assay. In this test, cytochalasin B is used to stop dividing cells from performing cytokinesis, which allows the recognition of cells that have completed one nuclear division by their binucleate appearance. The CBMN is recommended for the assessment of genotoxicity in human lymphocytes. For cell lines, the use of cytochalasin B for cell lines is considered optional, as no clear advantage or disadvantage of its use was detectable (Kirsch-Volders et al., 2000b).

4.2.3 Methodological problems

For detection of apoptosis induction, Annexin V which detects the membrane externalization of phosphatidylserine (PS) on the cell surface during apoptosis is commonly used. It is reported that it is best applied using a single cell suspension (Vermes et al., 2000). Nevertheless, apoptosis detection by means of Annexin V was also described in adherent cell lines like V79 or CHO cells (Aberkane et al., 2001; Boersma et al., 1996; Grosicka et al., 2005). However, it has been addressed that the various treatments used for the detachment of adherent cells from culture vessels (e.g. trypsinization) could cause cell membrane damage (Zamai et al., 2001). This would make apoptosis measurement by means of Annexin V a technical problem, as the externalization of phosphatidylserine can not be measured anymore, once Annexin V can pass the membrane to the inner leaflet.

In the present study, besides measurement of caspase-3/7 activity, the Annexin-V/PI assay was performed to measure apoptosis induction. With this assay, induction of apoptosis and late apoptosis/necrosis was detected at high concentrations for some of the tested compounds (T, ETHI, NT, NE, TB, and THG). However, very high standard deviations were obtained (see Appendix, Chapter 7.2.5), also in the medium controls, which might be due to variable membrane damage in different independent experiments. Additionally, under treatment, the cell size decreased. This was seen during the flow cytometer use, which makes an analysis of the data difficult. Furthermore, the results were not consistent with the data obtained in the caspases-3/7 assay.

Taking all these points together, the Annexin-V/PI assay is regarded to be unsuitable for apoptosis detection in the V79 cell system, under the conditions used in the present study.

4.3 Mechanistic considerations regarding genotoxicity

Several indirect mechanisms are considered to exhibit “thresholds for genotoxicity”, i.e. no-effect concentrations (NOECs), below which genotoxicity would not be induced (see Chapter 4.1.1; Kirkland et al., 2007b; Scott et al., 1991). In the present study, possible mechanisms have been investigated regarding their role in the genotoxicity of hormonal steroids.

4.3.1 Cytotoxicity and genotoxicity

The influence of cytotoxicity on the outcome of genotoxicity assays and the risk of false-positive test results due to the induction of endonucleolytic DNA degradation during cell death have been addressed in a number of studies (Galloway, 2000; Scott et al., 1991; Storer et al., 1996; Vock et al., 1998). Scott et al. (1991) indicated that high levels of cell killing may indirectly lead to DNA damage. Cytotoxicity-related mechanisms of DNA (double-) strand break induction *in vitro* with a number of different agents were described by Storer et al. (1996) and Vock et al. (1998) who proposed to use differences in the shape of response-curves to obtain some distinction between cytotoxic and clastogenic/genotoxic compounds. Galloway (2000) characterized different categories of compounds in differentiating between DNA damaging compounds generally inducing aberrations without severe concomitant cytotoxicity from cytotoxicity-associated clastogens. They pointed out that a limit on cytotoxicity, and an accurate way of estimating it, would be important for reducing the frequency of non-relevant positive results in genotoxicity (especially clastogenicity) tests and thus for the increase of the accuracy of such assays.

Published official guidelines for genotoxicity testing accept the possibility that genotoxicity occurring only at highly cytotoxic concentrations may not be indicative of a relevant human hazard (Chapter 4.1.1). Nevertheless, guidelines still require *in vitro* genotoxicity tests to be performed up to cytotoxic concentrations (> 50% reduction of viability; ICH S2A, 1995).

In the present study, induction of genotoxicity, determinated as micronucleus formation, has been assessed along with cytotoxicity of hormonal steroids. Both effects have been regarded in parallel to determine the level of cytotoxicity at which a genotoxic effect occurred (Appendix, Chapter 7.2.8). From this aspect, the compounds may be grouped into the following classes:

Class I	no MN increase, but detectable cytotoxicity
Class II	increase of MN in non-cytotoxic range (cytotoxicity < 20 %) up to/above 2x MN background rate
Class IIa *(intermediate)*	increase of MN in non-cytotoxic range, then decrease, no 2x MN background rate
Class III	increase of MN only in presence of cytotoxicity (cytotoxicity ≥ 20 %)

A summarizing compilation of genotoxicity and cytotoxicity of the hormonal steroids tested is depicted in Tab. 15.

Tab. 15. Comparison of genotoxicity and cytotoxicity of the test compounds and the resulting classification

Compounds	**MN induction**	**Cytotoxicity** IC_{20} (µM)	**Class**
7α-Methyltestosterone	– (up to 100 µM)	190	
17α-Propylmesterolone	– (up to 30 µM)	35	
Ethisterone	– (up to 100 µM)	n.d.	
Testosterone	– (up to 300 µM)	155	**I**
Nor-D	– (up to 300 µM)	130	
Nor-E	– (up to 100 µM)	40	
Nor-F	– (up to 60 µM)	15	
Madol	+ (2x MN increase at 27 µM)	n.d.	
19-Norandrostenedione	+ (2x MN increase at 29 µM)	300	
19-Norethisterone	+ (2x MN increase at 24 µM)	70	**II**
19-Nortestosterone	+ (2x MN increase at 66 µM)	150	
Tetrahydrogestrinone	+ (2x MN increase at 3 µM)	40	
Trenbolone	+ (2x MN increase at 23 µM)	75	
Androstenedione	– (up to 100 µM; 1.6x MN increase at 30 µM)	130	**IIa**
Nor-G	– (up to 30 µM; 1.56x MN increase at 10µM)	53	
Nor-C	+ (2x MN increase at 100 µM)	90	**III**

It is seen from this compilation that almost all compounds (except ethisterone and madol) were cytotoxic within a relatively narrow window of about one order of magnitude. In contrast, considerable differences in the genotoxic potential of the steroids are recorded. Thus, there is no uniform picture for an influence of cytotoxicity on genotoxicity for the tested steroids; this differs for the individual compounds.

For the steroid Nor-C a genotoxic effect is seen, but only at a cytotoxic concentration (Class III). In this case, the positive outcome in the MN test may be due to a confounding influence of cytotoxicity (false-positive genotoxicity result). On the other hand, there are steroids (madol, 19-norandrostenedione, 19-norethisterone, 19-nortestosterone, tetrahydrogestrinone, and trenbolone) showing a positive genotoxicity result in sub-cytotoxic concentrations (Class II). The genotoxic effect of these compounds seems to be independent of cytotoxicity, arising at concentrations without detectable cytotoxicity. For class I-compounds, genotoxicity and cytotoxicity are to be seen as independent processes: although the steroids show cytotoxic effects, no MN increase is seen up to high, cytotoxic concentrations. Compounds in class IIa show an intermediate effect pattern: an increase of MN induction is detectable in lower concentrations, but without reaching the doubling of the MN background rate, as there is a decrease of micronucleus induction at higher concentrations.

From the latter, it is emphasized that cytotoxicity is not only a confounding factor but also a cut-off criterion for genotoxicity tests: beyond a defined cytotoxicity the detection of genotoxicity is impossible. This plays an important role for the final outcome of genotoxicity tests, if non-specific genotoxicity occurring in relatively high concentrations is to be detected, as an increase in a genotoxicity with higher concentrations might be “interrupted” by the cytotoxicity of the compound. Thus, the ratio genotoxicity over cytotoxicity determines the final outcome of a genotoxicity test (positive or negative). The stronger the cytotoxicity of a compound in relation to its genotoxic potency, the lower is the likelihood of a positive genotoxicity assay result, as the necessary concentration cannot be tested.

This coherence is true as far as cytotoxicity and non-specific genotoxicity are independent processes. When regarding the correlation of both effects, non-specific genotoxicity and cytotoxicity, with the lipophilicity of the compounds (Fig. 25) it can be seen that cytotoxicity, in contrast to (non-specific) genotoxicity, is not overtly related to lipophilicity. Hence, cytotoxicity and genotoxicity are independent of each other.

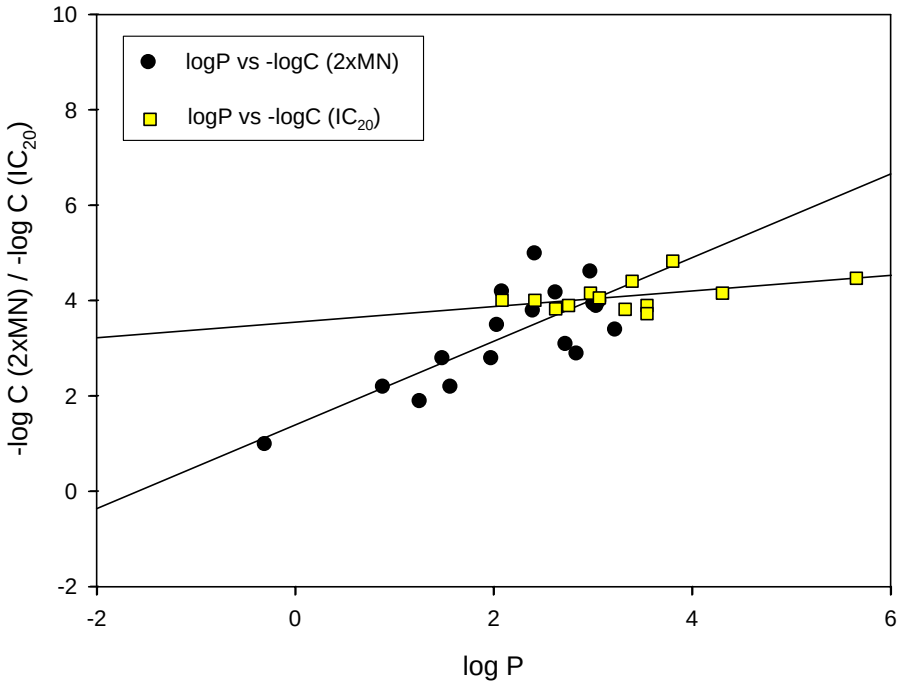


Fig. 25. Correlation of compound lipophilicity with non-specific genotoxicity (2xMN induction) and cytotoxicity (IC_{20})

4.3.2 Cell cycle progression and genotoxicity

Cells react to chromosomal insults by activating a complex response that regulates processes including cell cycle progression and apoptosis (Zhou and Elledge, 2000). Cell cycle arrest is frequently induced as a consequence of genotoxicity, to allow time for repair of DNA damage, in order to avoid fixing mutations during replication and cell division.

The basic mechanism of this process is well-known: DNA and spindle apparatus integrity is controlled through several checkpoints at different cell cycle stages (Fig. 26). The DNA damage checkpoint monitors DNA damage before the cell enters the S phase (a G1-checkpoint), as well as during S phase. Damage to DNA inhibits the progression of the cell cycle in the G1 phase so that the damage can be repaired. If the damage is so severe that it cannot be repaired, the cell undergoes apoptosis. Successful replication of DNA during S phase is controlled at the G2-M checkpoint (DNA replication checkpoint). If replication is incomplete, progress through the cell cycle is arrested before the cell enters mitosis. The spindle assembly checkpoint (M-checkpoint) detects any failure of spindle fibres to attach to kinetochores and arrests the cell in metaphase, until all the kinetochores are attached correctly. It also detects improper alignment of the spindle itself and blocks cytokinesis; if the damage is irreparable it triggers apoptosis (Elledge, 1996; Shackelford et al., 1999).

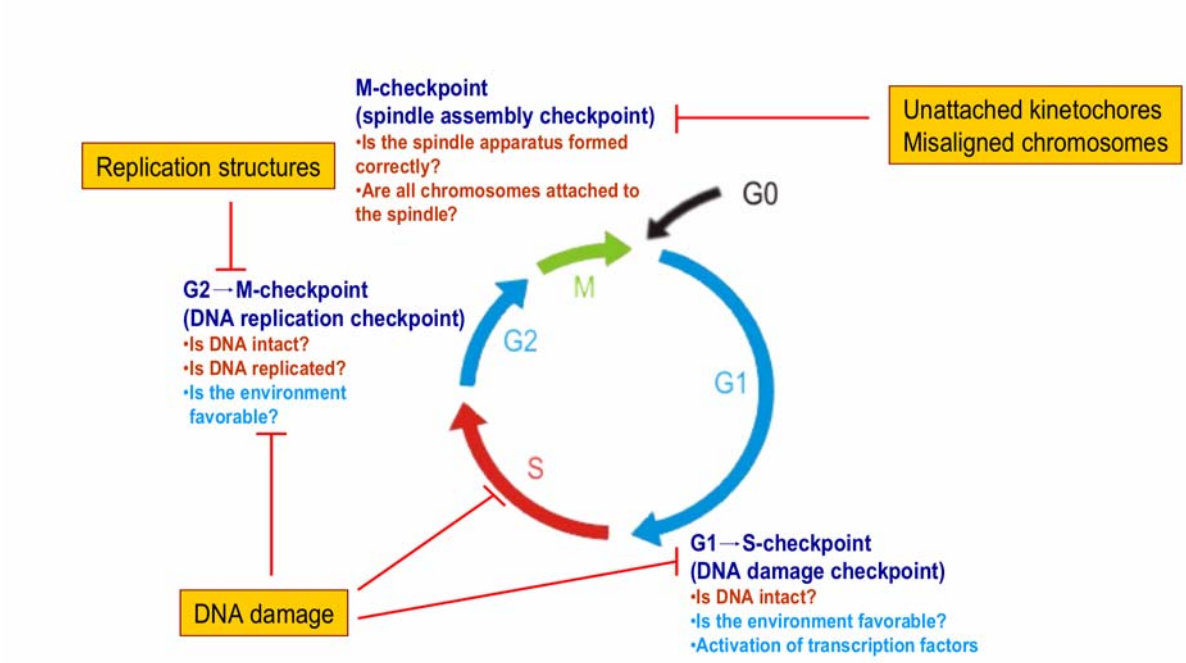


Fig. 26. A simplified scheme of the cell cycle checkpoint system: DNA damage, incomplete replication and unattached/misaligned chromosomes can lead to cell cycle arrest at different stages; G0: quiescent state, G1, G2: gap phases, S: DNA synthesis stage, M: mitosis (according to Alberts et al., 1995; Elledge, 1996, modified).

Cell cycle progression may be retarded by genotoxic carcinogens as a consequence of the DNA damage, especially at low levels (Lutz and Kopp-Schneider, 1999). Regarding cancer risk, this means, that under specific circumstances the protective influence of decreased cell division can be stronger than the deleterious influence of increased DNA damage. Thus, it is likely that the combination of both effects could result in a decreased tumor incidence. Higher levels of the same substance are described to possibly increase cell cycle progression due to cytotoxicity and regenerative cell proliferation, resulting in increased tumor incidences (Hengstler et al., 2003). The authors warn that the *in vivo* benefit of the low-dose effect should be interpreted cautiously, as a decrease in cell proliferation below baseline may interfere with normal tissue regeneration. In addition, a protective influence may be tissue or cell-type specific. Nevertheless, cell cycle progression and regenerative proliferation are seen to represent a very relevant key parameter concerning threshold mechanisms (Hengstler et al., 2003).

For the detection of the genotoxic potency of compounds by means of the MN assay, the cell cycle progression plays an important role. MN can only be expressed in dividing eukaryotic cells which have to fulfil at least one whole cell cycle for micronucleus expression. Thus, the assay cannot be used efficiently or quantitatively in non-dividing cell populations or in dividing cell populations, in which the kinetics of cell division is not well understood or controlled (Fenech, 2000). In consequence, an arrest of cell cycle may lead to underestimation of genotoxicity.

In order to identify interfering effects in the MN assay due to cell cycle arrest, in the present study, the steroids detected positive or slightly positive in the MN assay were analyzed for their effects on the cell cycle (see Chapter 3.5.2). Where an effect on the cell cycle was detected it was about an increase of the cell fraction in G2/M phase, thus pointing to a failure of passing the G2-M checkpoint or the M-checkpoint. Hence, DNA damage or defect spindle assembly can be the cause of the arrest.

As in the present study the cell cycle arrest is seen at concentrations that are clearly positive in the MN assay (see Chapter 3.5.2), the arrest in G2/M phase detected here is likely to be the consequence of the genotoxic effect of the compounds.

Cell cycle arrest may also act as a limiting factor for genotoxicity detected in the MN assay. This is seen in the example of trenbolone, which shows a “bell-shaped” concentration-response-curve in the MN assay: up to a concentration of 30 µM, there is an increase of MN detectable, but at higher concentration this decreases. This decrease might be explained by cells arrested in their replication cycle (in G2/M phase) starting at 30 µM up to 100 µM. If pronounced genotoxic damage, which might have occurred at 100µM, leads to an effective cell cycle arrest, and no further increase of MN can be detected at this concentration.

4.3.3 Apoptosis and genotoxicity

Apoptosis is a programmed form of cell death through coordinated destruction of the cell. Two major apoptotic pathways are distinguished: One is triggered by engaging so-called “death receptors” on the cell surface (‘extrinsic’ pathway), and the other by stress-inducing stimuli (‘intrinsic’ pathway). The first involves stimulation of cell surface death receptors of the tumor necrosis factor (TNF) receptor superfamily resulting in rapid activation of the initiator caspase 8, which in turn activates the caspases cascade (caspases 3,6 and 7; Danial and Korsmeyer, 2004).

In cellular reaction to stress caused by chemicals, by growth factor deprivation, or by diverse types of intracellular damage, the intrinsic apoptosis pathway results in perturbation of mitochondria and subsequent release of cytochrome *c* and other apoptosis-promoting proteins from the inter-

mitochondrial membrane space (Newmeyer and Ferguson-Miller, 2003). Once released, cytochrome *c* binds to the apoptotic protease-activating factor 1 (Apaf1), which results in formation of the Apaf1-caspase 9 apoptosome complex and activation of the initiator caspase 9. The activated initiator caspases 8 and 9 then activate the caspases cascade, i.e. the effector caspases 3, 6 and 7, which are responsible for the cleavage of important cellular substrates resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype (Danial and Korsmeyer, 2004). It is argued that in stress-induced apoptosis caspases can also be activated upstream or independently of mitochondria, so that the mitochondrial breakdown may often simply deliver the coup de grace to a cell already on the path to death (Adams, 2003). However, mitochondria are seen - additionally to their role in cellular energy metabolism – as central players in cell death.

Genotoxic damage – especially severe genomic injury – is a process to which cells react through committing suicide (Zhou and Elledge, 2000). Genotoxic events that have been reported to induce apoptosis as a consequence include formation of DNA adducts, DNA breaks and/or protein adducts (Kirsch-Volders and Fenech, 2001).

Cells are known to undergo apoptosis as a consequence of DNA damage induced by relatively high doses of genotoxic substances. *In vivo*, the control of neoplastically transformed cells via apoptosis can reduce tumor rates. However, little is known about the efficiency of apoptotic mechanisms at low doses and whether such mechanisms can lead to thresholds for carcinogenesis (Hengstler et al., 2003).

In contrast to necrosis, during apoptosis the integrity of the plasma membrane is preserved until late in the process. DNA degradation, i.e. condensation and fragmentation of nuclei, is a morphological characteristic of apoptosis. Downstream of caspase activation, degradation of DNA first occurs at A/T rich regions within the nuclear scaffold sites to produce 50–200 kb fragments (Danial and Korsmeyer, 2004). As these oligonucleosomal DNA fragments can also be detected in consequence of a genotoxic damage, apoptosis has been reported to be a confounding factor in the evaluation of the genotoxic potential of molecules in clastogenicity tests like the MN assay, thus contributing to false-positive genotoxicity test results (Meintières et al., 2001; Meintières and Marzin, 2004). The authors demonstrated this using the murine cytotoxic T cell line, CTLL-2 Bcl2, which is protected from apoptosis due to the over-expression of the apoptosis inhibitor Bcl2 in stably *bcl2*-transfected CTLL-2 cells. The apoptosis inducing and the genotoxic potentials in the MN assay were assessed in parallel in both cell lines. From a comparison between results obtained in parental CTLL-2 cells and in CTLL-2 Bcl2 cells after different treatment scenarios (treatment with non-genotoxic apoptosis inducers, with clastogens, or expressed to extreme culture conditions) apoptosis was seen to induce the formation of micronucleated cells. Thus, DNA fragmentation during apoptosis was shown to lead to

false-positive results in the MN assay and to give rise to an overestimation of the genotoxic potential of chemicals.

Fenech et al. (1999) described a method for identification of the different effects, genotoxicity and apoptosis, by means of the inclusion of apoptotic and necrotic cells in the cell counting and morphological criteria for the recognition and discrimination between necrotic, apoptotic and viable cells in the cytokinesis-block micronucleus (CBMN) assay. This variant of the "conventional" MN assay using cytochalasin B to stop dividing cells from performing cytokinesis, allows the recognition of cells that have completed one nuclear division by their binucleate appearance. The CBMN is conventionally used for the assessment of chromosome breakage, chromosome loss and frequency of dividing cells in human lymphocytes (Kirsch-Volders et al., 2000b). The proposed test system was seen to provide a procedure for discrimination between agents that primarily induce cytotoxic effects (apoptosis/necrosis) as opposed to genotoxic effects (Fenech et al., 1999), which was proposed to be helpful in *in vivo* genotoxicity studies, for example in biomonitoring (Kirsch-Volders and Fenech, 2001). However, this recommended method is based exclusively on visual evaluation of a genotoxic or apoptotic DNA damage, respectively, thus being dependent on a trained eye of the experimentator.

In the present study, apoptosis induction was assessed directly, picking up biochemical characteristics for apoptotic cells, i.e. the induction of the effector caspases 3 and 7. Thereby, apoptosis assessment is more objective than through visual determination. The results were opposed to the genotoxicity profiles of the tested steroids in order to identify possible confounding effects of apoptosis in the MN assay (see Chapter 3.5.3). As no (or only very slight) induction of caspase 3/7 activity was detected for the tested steroids, there is no indication for an interference of apoptosis and the genotoxicity detected for the steroids and for an overestimation of their genotoxic potency.

4.3.4 Reactive oxygen species and genotoxicity

Oxidative stress has been repeatedly addressed as an important mechanism of indirect genotoxicity that may be considerably increased in certain pathological conditions including cancer (Kirkland and Müller, 2000; Pratt and Barron, 2003). Oxidative DNA damage is caused by reactive oxygen species (ROS), which may be generated endogenously by cellular oxygen metabolism and exogenously by ionizing radiation, anoxia and hyperoxia, or be induced by chemicals (Dalton et al., 1999; Speit et al., 2002). ROS may cause DNA damage in the form of oxidized bases (e.g. 8-oxoG), apurinic (AP) sites, and DNA strand breaks. Other cellular macromolecules such as lipids and proteins are attacked by oxygen radicals and damaged as well. Lipid peroxidation may generate reac-

tive intermediates that couple to DNA and give rise to exocyclic etheno- and propane-adducts (Bartsch and Nair, 2000; Nair et al., 2007).

Classical stimuli producing ROS are paraquat and oxidants like hydrogen peroxide, but ROS production is also known for tobacco smoke components, ethanol, or transition metals (Bolt and Degen, 2004).

An important way for xenobiotics to lead to ROS production and oxidative DNA damage is redox cycling. For example, some steroidal estrogens are known to do so and it is discussed as the major mechanism of genotoxicity of these substances (Liehr, 2000). Estrogens are metabolically to some extent converted to catecholestrogens, which may undergo redox cycling between the hydroquinone (catechol) and quinone forms, via a semiquinone intermediate. This may react with molecular oxygen and form ROS such as the superoxide anion ($O_2^{\bullet -}$).

In principle, chemicals that give rise to excess ROS production and lipid peroxidation will cause different types of toxicity, including genotoxicity due to increased oxidative DNA damage and cell death (Collins, 1999). However, the cellular antioxidant systems, i.e. the enzymatic machineries like glutathione peroxidase, katalase and superoxiddismutase as well as the non-enzymatic antioxidants like glutathione (GSH), counteract the ROS-induced cell damage. Only when the antioxidant machinery is increasingly overloaded, oxidative DNA damage induced by ROS occurs. Thus, DNA damage due to ROS production is seen to be a thresholded genotoxicity mechanism.

For a carcinogenic risk assessment of ROS producing chemicals, a focus on genotoxicity alone may be misleading since effects observed under oxidative stress conditions (i.e. increased oxidative DNA damage) may be paralleled by pronounced epigenetic effects of ROS on signal transduction or gene expression (Epe, 2002). The balance of antioxidants and oxidants may be very complex. As ROS are described to play important roles in a large number signalling transduction pathways including, for example, apoptosis (Allen and Tresini, 2000), it is important to understand the influence if this balance is counteracted by antioxidants. Although a protective role of dietary antioxidants in cancer prevention has been reported (Collins, 1999), there are epidemiological studies that indicate a tumor-promoting effect of, for example, beta carotene supplements (Omenn, 1998). Such paradoxical effects of dietary antioxidants on cancer might be due to the fact that they protect cancer cells from oxidative stress-induced suicide and thereby accelerate cancer progression (Forsberg et al., 2001).

The glutathione content, which is often determined to control the sensitivity of cells to ROS, has been reported to be relatively low in V79 cells at 8.3 nmol/mg protein (Koberle and Speit, 1990). Hence, this cell line should be relatively sensitive to ROS. However, in the present study no induction of ROS generation was detected after steroid treatment, neither time- nor concentration-dependent.

Thus, it can be concluded, that ROS is not the relevant mechanism of genotoxicity for the steroids studied here.

4.3.5 Lipophilicity and genotoxicity

Originally, lipophilicity of compounds has not been addressed as an important contributor to (non-specific) modes of genotoxic action (ICH S2A, 1995; Scott et al., 1991). However, an influence of lipophilicity on the induction of non-specific genotoxicity was demonstrated as chromosomal arrangements in mitosis and cell division were disturbed by a number of compounds, with a reversed relationship between lipophilicity and the concentration required for an effect, but with only poor dependency on the exact chemical structures (Önfelt, 1987a; Schultz and Önfelt, 2000). In consequence, the presence of compound in cellular hydrophobic compartments was viewed to be a significant determinant (Schultz and Önfelt, 2000). As examples of such compartments, the authors mentioned membranes as well as amphiphilic pockets of proteins involved in protein-protein complex formation. In order to explain the impact of lipophilicity on non-specific genotoxic effects they argued that cytokinesis relies on a number of protein-membrane interactions (Rappaport, 1986) which may be disturbed. Rearrangement processes of actin during the cytokinesis (Fishkind and Wang, 1993) and the appearance of astral and interzonal microtubules in initiation and completion of cleavage (Cao and Wang, 1996; Rappaport, 1986) were mentioned as examples.

More recently, modelling studies have been performed on a number of partial processes involved in karyokinesis and cytokinesis, which can be disturbed by hydrophobic interactions. VanBuren et al. (2002) addressed the assembly-disassembly of microtubules, developing a stochastic model to describe of microtubule assembly dynamics for predictions of how microtubule-associated proteins and other effectors alter underlying energetics. Additional processes involved in chromosome motility, e.g. motor protein activity, and mechanical properties of the mitotic spindle (Gardner and Odde, 2006), or the impact of physicochemical factors on the mitotic spindle checkpoint and on kinetochore dynamics (Doncic et al., 2005) are also of interest in this regard. Thereby, it becomes clear that a multiplicity of protein interactions is involved in ensuring the correct division of the chromosomes and of the entire cell, whose disturbance could lead to aneugenicity.

Moreover, processes like DNA synthesis may be sensitive as well for hydrophobic disturbance, leading to clastogenicity (Galloway et al., 1998). Chemically induced lysosomal breakdown and subsequent DNase release from lysosomes is also known to lead to DNA double strand breaks and chromosomal aberrations (Bradley et al., 1987).

Thus, hydrophobic interactions between proteins and other cellular components appear as a general physicochemical principle that is basic for the multiplicity of these dynamic processes. This can be disturbed non-specifically by lipophilic substances, finally leading to a genotoxic effect.

4.4 Compounds with specific modes of action

4.4.1 Compounds studied with known specific modes of action

For a number of compounds, specific modes of genotoxic action have been identified or suspected. Basic mechanisms for inhibition of cytokinesis have been known for decades for the antimitotic drug *colcemid* (N-methyl N-deacetyl colchicine), which destroys spindle microtubules and their assembly via binding at two binding sites of tubulin (Haber et al., 1972; Ray et al., 1984; Wallin et al., 1988). The alkaloid *cytochalasin B*, a metabolite of the fungus *Helminthosporium dematioideum*, inhibits actin polymerization and the elongation at the barbed (fast growing) end of actin filaments by decreasing the rate of monomer addition during cleavage (MacLean-Fletcher and Pollard, 1980; Spudich and Lin, 1972) .

The vinca alkaloid *vincristine* (VCR) obtained from the Madagascar periwinkle *Catharanthus roseus* was shown to induce a wide spectrum of division aberrations resulting in mitotic arrest, polyploidy and aneuploidy (Miller and Adler, 1989). Like other vinca alkaloids VCR binds to tubulin, inhibiting the tubulin polymerization and assembly of mitotic spindle microtubules (Himes et al., 1976; Owellen et al., 1976).

Methylmethane sulfonate (MMS) is an alkylating compound inducing clastogenic DNA damage (Tao et al., 1993; Tinwell et al., 1998).

Diamide selectively oxidises reduced glutathione to glutathione disulfide (Kosower and Kosower, 1995). The glutathione disulfide reacts further with protein sulfhydryl groups. Agents reacting with protein sulfhydryl groups have been described to inhibit the cytokinesis process (Grace et al., 1992). Diamide is weakly c-mitotic at non-toxic concentrations (Önfelt, 1987a).

Benzonitrile was shown to disturb the tubulin assembly, and interference with the functionality of the tubulin–kinesin motor protein system was also reported for *nitrobenzene* (Bonacker et al., 2004a).

The phytoestrogen *genistein* is a naturally occurring isoflavone found in soy products. It is described as a weak clastogen causing chromosomal aberrations (Di Virgilio et al., 2004; Kulling et al., 1999) and has been found to induce topoisomerase-II mediated DNA breaks (Lynch et al., 2003;

Salti et al., 2000). Studies in mouse lymphoma cells on the induction of micronuclei by genistein support a “practical threshold” for its clastogenic activity (Lynch et al., 2003).

4.4.2 Compounds characterized in the present study

As an animal growth promoter, the genotoxicity of *trenbolone* (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., 1988; Tsutsui et al., 1995), and in assays reflecting genotoxicity at a chromosomal level, namely the mouse lymphoma assay, the chromosomal aberration test, and micronucleus 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 due to its activated system of conjugated double bonds (Ryan and Hoffmann, 1978). This raises concern of an interaction with proteins involved in karyokinesis (Bonacker et al., 2004a). So far, the genotoxicity of the structurally similar “designer drug” *tetrahydrogestrinone* (THG; Death et al., 2004) has not been investigated at all.

According to the present data, TB and THG reach the level of a doubling of the MN background rate in V79 cells only within a relatively narrow concentration window (see Chapter 3.1.1). The resulting “bell-shaped” dose-response is likely to be caused by cytotoxicity at higher concentrations (see Chapter 3.5.1). 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. As the physiological hormone *testosterone* did not induce MN, even at high concentrations up to 0.3 mM (see Appendix, Chapter 7.2.1), the MN induction by TB and THG is independent of the hormonal effect. 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 (see Chapter 3.1.2).

Covalent binding to protein structures is a long-known molecular feature of TB (Hoffmann et al., 1984; Ryan and Hoffmann, 1978). *In vitro*, this is small in magnitude, but definite. This contrasts to conditions *in vivo* where covalent protein binding of TB is relatively 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 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 disaggregation of microtubules. 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 polymerization competence (Britto et al., 2005). Taking these elements together and considering the very close structural similarity between TB and THG, 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.

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) have been observed in the present study. This may reasonably be explained by different lipophilicities of the two compounds (see Chapter 3.2.3). The introduction of the 17α-ethyl substituent that is characteristic of THG into the TB molecule leads to a log P 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.

Regarding the dependence of lipophilicity and genotoxicity of the compounds in the lipophilicity-genotoxicity-plot (see Chapter 3.2.3) it is seen that TB and THG fall into the belt of non-specific compounds. However, TB is very closely located to the border line. Following the equation of the genotoxicity – lipophilicity relationship the theoretically effective concentration was predicted for the two steroids as if they were non-specific compounds (see Chapter 3.2.3). For both steroids, the experimentally determined effective concentration in micronucleus induction is lower than predicted from their lipophilicity alone, with TB showing this in an even more pronounced manner.

4.5 The concept of specific versus non-specific genotoxins

As already mentioned initially (see Chapter 1.3), Schultz and Önfelt (2000) have advanced the concept that both mitotic spindle function and cell division show a similar sensitivity to hydrophobic interactions and that there is a general coherence of lipophilicity and “non-specific” chromosomal genotoxicity. According to this such a non-specific disturbance of cytokinesis leading to genotoxicity by disturbed chromosomal formation or distribution may simply be due to the lipophilicity of compounds. Compared to such chemicals, specific effectors should act at concentrations lower than predicted on the basis of lipophilicity (log P). Schultz and Önfelt (2000) also suggested the use of this approach for a rapid screening of potential aneugens, distinguishing between physico-chemical or chemical mechanisms of action. Such distinction could be pivotal in the evaluation of the toxicological relevance of weakly positive data on chromosomal genotoxicity of drugs and chemicals.

The present investigations substantially broaden the database of the concept of Schultz and Önfelt (2000) including hormonal steroids and basically confirm its validity. A statistical way of distinction between specific and non-specific chromosomal genotoxins can be achieved, based on the "robust regression" procedure. In the lipophilicity-genotoxicity-plot, this defines a belt of compounds with non-specific activity based on lipophilicity (see Chapter 3.2). It also identifies specific effectors - both aneugens and clastogens - as outliers (see Chapter 4.4.1).

Genistein is known as topoisomerase-II poison (Lynch et al., 2003; Yamashita et al., 1990) acting weakly clastogenic. Interestingly, in the present investigation this compound came close to the statistical borderline between specific and non-specific chromosomal genotoxicity which also points to an impact of a weak but detectable specific genotoxic effect.

The same applies to cytochalasin B, which can also be regarded as a borderline compound. This compound is an inhibitor of actin polymerization and blocks cells from undergoing cytokinesis (but not karyokinesis), thereby enabling the accumulation of all dividing cells at the binucleate stage (Fenech, 1993; Fenech, 2000). The use of cytochalasin B in micronucleus assays has been discussed in the literature: For human lymphocytes its use at appropriate concentrations is recommended, whilst application of cytochalasin B has no advantages for tests with continuously growing cells lines like V79 or CHL (Kalweit et al., 1999; Kirsch-Volders et al., 2000b; Matsushima et al., 1999).

A number of steroidal compounds tested for micronucleus induction did not reach doubling of the background micronucleus rate, even at high concentrations up to the level of cytotoxicity. Part of these compounds showed a tendency of micronucleus rate elevation (androstenedione, Nor-D, Nor-E; see Chapter 3.1.1). Others did not show such an effect at all (ethisterone, testosterone, 7α-methyltestosterone, Nor-F, 17α-propylmesterolone). These latter compounds were of high lipophilicity, with log P > 3.1. Testosterone (log P = 3.32) was negative, even when tested up to its limit of solubility in the test system.

By extending the database and including molecular descriptors other than log P into the analysis, the original approach of Schultz and Önfelt (2000) is substantially supported. Indeed, log P appears to be the most suitable single descriptor for non-specific genotoxicity [r^2=0.88], based on the results provided by the GFA procedure. Using more descriptors (up to three in combination) resulted in improved correlations, up to r^2=0.97 (Chapter 3.3.1). This correlation was reached by introducing as descriptors

a) the numbers of hydrogen bond acceptors, the polar surface and total surface areas of the molecules, or

b) the dipole moment, the polar surface and total surface areas.

In essence, the relation of polar surface to the total molecular surface appears pivotal in determining the non-specific chromosomal genotoxicity of lipophilic compounds. This is determined by the distribution of polar (hydrophilic) and non-polar (hydrophobic) areas of the chemical's surface. As the ratio of polar to total surface of a molecule has an important effect on lipophilicity, this again is in accordance with the concept of Schultz and Önfelt (2000). Nevertheless, the influence of such additional molecular properties can be important if a model is needed to discriminate better between specific and non-specific genotoxic compounds.

Although the present analysis of the influence of different molecular parameters on non-specific genotoxicity is elaborate, it should be kept in mind that the number of experimental data points and the number of calculated molecular properties are still limited for building a reliable QSAR model to predict non-specific chromosomal genotoxicity. Nevertheless, the hypothesis that lipophilicity is a most important molecular property in relation to non-specific genotoxicity holds.

This is strengthened by the results of an analysis of the influence of lipophilicity on the processes of microtubule assembly (see Chapter 3.4). A dependence of microtubule assembly on lipophilicity was shown, as for non-specific compounds the experimental no-observed-effect-concentrations (-log NOEC) correlate with the compound specific log P. The interaction pattern with microtubule assembly of the compounds, except those with a known specific mode of action, appears to be well explained based on their lipophilicity. This contrasts to the effects of the known specific effectors colchicine, colcemid, and vincristine on the test system, which are much higher than predicted from their log P values, meaning that much lower concentrations are needed to reach an effect. Comparing experimental data obtained with lipophilic xenobiotics in cellular vs. cell-free systems, it is not feasible to directly compare effect or no-effect concentrations on a molar basis. In cellular systems lipophilic compounds are much concentrated in membranes and other lipophilic structures, which is not the case to the same extent in cell-free systems. Keeping this in mind, the obvious dependence of the microtubular interaction on lipophilicity corroborates the lipophilicity concept in general. It also focuses on one particular partial process involved, which is important for the integrity of chromosomal segregation. The dynamics of assembly/disassembly of microtubules is one of the processes which have been addressed (VanBuren et al., 2002; see Chapter 4.3). In essence, this is also in accordance with the general concept that hydrophobic interactions are connected with processes contributing to a non-specific genotoxicity on a chromosomal level (Schultz and Önfelt 2000).

The four hormonal steroids madol (MAD), 19-norandrostenedione (NA), tetrahydrogestrinone (THG), and trenbolone (TB) were used for a further evaluation of the concept. Since the TB and THG molecules contain a system of activated and conjugated double bonds, which makes protein binding

likely, e.g. to tubulin, it is presumed that there is at least some specific impact on the genotoxicity of these compounds (Chapter 4.4.2.) When calculating the theoretically effective concentration (pred -log C) for the two steroids (following Equ. 1) for both steroids, the experimentally determined effective concentration was lower than predicted from their lipophilicity alone, with TB showing this in an even more pronounced manner. In the lipophilicity-genotoxicity-plot it is seen that TB and THG fall into the belt of non-specific compounds. However, TB is very closely located to the statistical border line, and NA fell directly into the belt of non-specific compounds. For this steroid, it must be presumed that it acts non-specifically. As the difference of exp and pred -log C for NA was somewhat smaller than for THG and TB, a mostly non-specific genotoxicity of this compound was underlined. MAD fell outside of the cluster of non-specific compounds and acted at very high concentrations, even higher than expected by its log P value of 6.08. This might be due to compound specific properties like strong unspecific protein binding (to serum proteins) or enrichment in lipophilic compartments like the cellular membranes; however, this can not be recorded and displayed by the present procedure and should be determined individually for the respective compounds.

In modelling non-specific genotoxicity by physicochemical parameters, and using one up to three physicochemical parameters, again MAD falls outside of the cluster of non-specific compounds. NA is located very close to the non-specific compounds (see Chapter 3.3.2). TB and THG fall close to but not within the area of non-specific compounds.

In essence, taking all these different issues together, the approach of Schultz and Önfelt (2000) can be put into a more distinct perspective. The paradigm of a lipophilicity – genotoxicity relationship is applicable for the distinction between specific and non-specific genotoxic compounds. It is valid for substances covering different classes including alcohols, aliphatic hydrocarbons, as well as steroid hormones.

4.6 Final conclusions for drug development

The current strategy of genotoxic risk assessment during drug development in general and the handling of single positive genotoxicity tests on a chromosomal level were starting points of the present study. Proposed revisions of the current guidelines for drug genotoxicity testing are intended to consider a “weight of evidence” approach that may put single positive genotoxicity results into a new perspective (see Chapter 1.1, Fig. 1).

The data presented show, that the following issues can be crucial in discussing the toxicological relevance of positive genotoxicity results:

1. There is interdependence of cytotoxicity and genotoxicity, as cytotoxicity is a cut-off criterion in genotoxicity assays. In critical cases, a more narrow concentration scaling is needed, than routinely required.
2. Lipophilic substances show a basic and non-specific genotoxicity due to hydrophobic interactions with proteins and other cellular compartments. Using molecular descriptors, this non-specific genotoxicity is predictable. For practical purposes, the log P value may be used.
3. The present data show, that for compounds of high lipophilicity (log P > 3.1) solubility problems may arise. For such compounds, a quantitative prediction of non-specific genotoxicity based on the compounds´ lipophilicity is not applicable. Conversely, for compounds of moderate lipophilicity (log P ≤ 3.1), the non-specific genotoxicity is well predictable.
4. Specific genotoxic effects must be assumed, if the quantitative range for non-specific effects is exceeded (see Chapter 3.2.2, Fig. 17).

A combination of these points leads to new views in the genotoxicity screening, which can be applied in pharmaceutical compound development. Such an application is given in Fig. 27. It shows that the prediction of non-specific genotoxicity could constitute an integral element of the genotoxicity testing strategy.

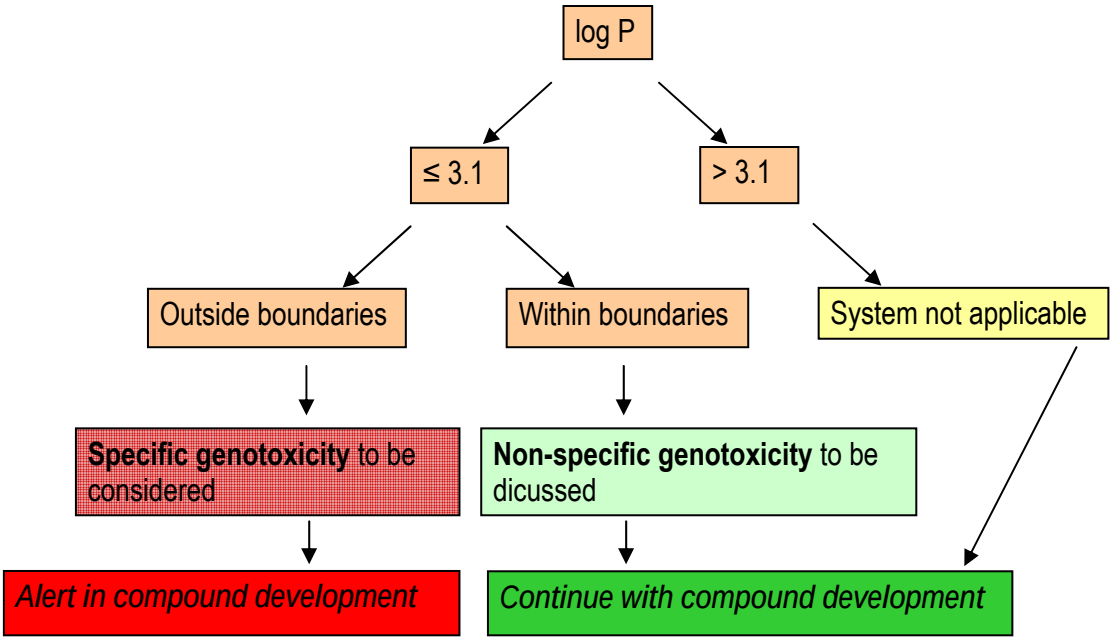


Fig. 27. General idea of a practical procedure in the toxicological assessment of specific vs. non-specific chromosomal genotoxicity

These aspects are important with respect to the "weight of evidence" (WoE) approach in the currently proposed revision of the guidelines for genotoxicity testing of drug candidates (see Chapter 1.1). As a result, improvements may be achieved in the separation of true adverse health effects on the one hand and the recognition of non-relevant effects on the other hand, which would lead to non-justified discontinuation of industrial compound developments.

5 Summary

For the safety assessment of drugs balancing beneficial versus toxic effects is pivotal. As genotoxic compounds may pose a risk of carcinogenicity, the evaluation of genotoxicity is an integral part of the toxicological assessment of drug candidates. Positive results in standard genotoxicity tests often lead to a discontinuation of drug development. In the chromosomal aberration test (CAT), positive results are often obtained *in vitro* at high, sometimes cytotoxic concentrations. The toxicological relevance of such effects must be questioned. Recently, existing guidelines for genotoxicity testing are under discussion regarding the interpretation of positive *in vitro* genotoxicity tests. In proposed revisions, the relevance is made dependent on a "weight of evidence" (WoE) approach. This must be seen against the background of current discussions in the scientific community on the relevance of threshold effects in chromosomal genotoxicity, as a number of "indirect" mechanisms have been described that may result in positive genotoxicity results, including generation of reactive oxygen species (ROS), cytotoxicity and apoptosis. Such mechanisms are characterized by no-observed-effect-concentrations (NOECs), below which no genotoxicity is induced.

Schultz and Önfelt (2000) have proposed a concept connecting the lipophilicity of chemicals with a non-specific genotoxicity on a chromosomal level. The induction of aneuploidy was related to the lipophilicity (log P) of non-specifically acting chemicals, whereas specific toxicants acted already at concentrations consistently lower than predicted based on their log P.

The present thesis aimed at investigating, whether the concept of Schultz and Önfelt (2000) and its underlying principles were applicable to hormonal steroids in the course of drug development. Quantitative Structure Activity Relationship (QSAR) modelling procedures were applied, to find additional physicochemical parameters for the modelling of non-specific chromosomal genotoxicity. Also mechanistic backgrounds of chromosomal genotoxicity, such as the induction of apoptosis, cell cycle arrest, formation of ROS, and the influence of cytotoxicity were matters of the present study.

The genotoxic potential of 16 androgenic or progestational steroids was assessed in Chinese hamster V79 cells, using the micronucleus (MN) assay *in vitro*. Thus, the original database of Schultz and Önfelt was considerably broadened. Available existing sets of MN assay data were incorporated. Applying the statistical procedure of "robust regression" it was seen that compounds with a known specific mode of action of chromosomal genotoxicity were separated as statistical outliers from the cluster of non-specific compounds, when genotoxicity was plotted against the log P. In addition, 10 other molecular descriptors were determined and equations (linear and quadratic terms) were set up relating these descriptors (up to three in combination) with effective concentrations in the MN assay. The best fitting *single* property to describe non-specific genotoxicity was again log P [r^2=0.88]. Com-

binations of (a) the numbers of hydrogen bond acceptors, the polar surface and total surface areas of molecules, and (b) the dipole moment, polar surface and total surface, resulted in an optimization of correlations up to r^2=0.97. Hence, the relation of polar surface to the total molecular surface appears pivotal to determine a non-specific chromosomal genotoxicity of lipophilic compounds.

The genotoxicity of the steroids studied was independent of ROS and of apoptosis induction. Cell cycle arrest in G2/M phase was seen for a number of steroids as consequence of the compound's genotoxic effect, or as a factor limiting genotoxicity, leading to a "bell-shaped" concentration-response in MN induction. The cytotoxic potencies of the steroids were generally comparable; this parameter was not correlated with positive genotoxicity. This indicates that cytotoxicity and genotoxicity were independent processes. Cytotoxicity may confound genotoxicity results, and is a usual cut-off criterion in genotoxicity tests.

Regarding the toxicological relevance of positive genotoxicity results, two issues appeared to be crucial: (a) the interdependence of cytotoxicity and genotoxicity, cytotoxicity being a cut-off criterion in genotoxicity assays. Thus, in critical cases, a narrow concentration scaling is indicated. (b) Lipophilic substances generally display a non-specific genotoxicity, due to hydrophobic interactions. By using molecular descriptors, this non-specific genotoxicity is well predictable. For practical purposes, the log P value is a suitable single descriptor.

Based on the results of the present thesis, a general procedure is proposed, which could be used in pharmaceutical compound development, to separate specific from non-specific modes of action of chromosomal genotoxicity.

6 References

Aberkane,H., Frank,P., Galteau,M.M., and Wellman,M. (2001) Acivicin induces apoptosis independently of gamma-glutamyltranspeptidase activity. *Biochem. Biophys. Res. Commun.* **285:** 1162-1167.

Adams,J.M. (2003) Ways of dying: multiple pathways to apoptosis. *Genes Dev.* **17:** 2481-2495.

Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K., and Watson,J.D. (1995) *Molekularbiologie der Zelle*. Weinheim: VCH.

Allen,R.G. and Tresini,M. (2000) Oxidative stress and gene regulation. *Free Radic. Biol. Med.* **28:** 463-499.

Andrae,U. (1996) Genotoxicitätstests *in vitro*. In *Toxikologie*. Greim,H. and Deml,E. (eds). Weinheim: VCH, 321-341.

Armstrong,M.J., Bean,C.L., and Galloway,S.M. (1992) A quantitative assessment of the cytotoxicity associated with chromosomal aberration detection in Chinese hamster ovary cells. *Mutat. Res.* **265:** 45-60.

Babich,H. and Borenfreund,E. (1991) Cytotoxicity of T-2 toxin and its metabolites determined with the neutral red cell viability assay. *Appl. Environ. Microbiol.* **57:** 2101-2103.

Babich,H. and Borenfreund,E. (1992) Neutral red assay for toxicology *in vitro*. In *In vitro methods of toxicology. CRC Press*. Watson,R.R. (ed). Boca Raton, FL: 237-251.

Barraud,B., Lugnier,A., and Dirheimer,G. (1984) Determination of the binding of trenbolone and zeranol to rat-liver DNA in vivo as compared to 17 beta-oestradiol and testosterone. *Food Addit. Contam* **1:** 147-155.

Bartsch,H. and Nair,J. (2000) Ultrasensitive and specific detection methods for exocylic DNA adducts: markers for lipid peroxidation and oxidative stress. *Toxicology* **153:** 105-114.

Beato,M., Chavez,S., and Truss,M. (1996) Transcriptional regulation by steroid hormones. *Steroids* **61:** 240-251.

Bitensky,L. (1963) The reversible activation of lysosomes in normal cells and the effects of pathological conditions. In *Ciba Foundation Symposium on Lysosomes*. De Reuck,A.V.S. and Cameron M.P. (eds). Ciba, London: 362-375.

Boersma,A.W., Nooter,K., Oostrum,R.G., and Stoter,G. (1996) Quantification of apoptotic cells with fluorescein isothiocyanate-labeled annexin V in chinese hamster ovary cell cultures treated with cisplatin. *Cytometry* **24:** 123-130.

Bolt,H.M. and Degen,G.H. (2004) Human carcinogenic risk evaluation, part II: contributions of the EUROTOX specialty section for carcinogenesis. *Toxicol. Sci.* **81:** 3-6.

Bolt,H.M., Foth,H., Hengstler,J.G., and Degen,G.H. (2004) Carcinogenicity categorization of chemicals-new aspects to be considered in a European perspective. *Toxicol. Lett.* **151:** 29-41.

Bonacker,D., Stoiber,T., Bohm,K.J., Prots,I., Wang,M., Unger,E., Thier,R., Bolt,H.M., and Degen,G.H. (2005) Genotoxicity of inorganic lead salts and disturbance of microtubule function. *Environ. Mol. Mutagen.* **45:** 346-353.

Bonacker,D., Stoiber,T., Böhm,K.J., Unger,E., Degen,G.H., Thier,R., and Bolt,H.M. (2004a) Chromosomal genotoxicity of nitrobenzene and benzonitrile. *Arch. Toxicol.* **78:** 49-57.

Bonacker,D., Stoiber,T., Wang,M., Böhm,K.J., Prots,I., Unger,E., Thier,R., Bolt,H.M., and Degen,G.H. (2004b) Genotoxicity of inorganic mercury salts based on disturbed microtubule function. *Arch. Toxicol.* **78:** 575-583.

Borenfreund,E. and Puerner,J.A. (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicol. Lett.* **24:** 119-124.

Bradley,M.O., Taylor,V.I., Armstrong,M.J., and Galloway,S.M. (1987) Relationships among cytotoxicity, lysosomal breakdown, chromosome aberrations, and DNA double-strand breaks. *Mutat. Res.* **189:** 69-79.

Britto,P.J., Knipling,L., McPhie,P., and Wolff,J. (2005) Thiol-disulphide interchange in tubulin: kinetics and the effect on polymerization. *Biochem. J.* **389:** 549-558.

Bulychev,A., Trouet,A., and Tulkens,P. (1978) Uptake and intracellular distribution of neutral red in cultured fibroblasts. *Exp. Cell Res.* **115:** 343-355.

Bursi,R., de Gooyer,M.E., Grootenhuis,A., Jacobs,P.L., van der,L.J., and Leysen,D. (2001) (Q) SAR study on the metabolic stability of steroidal androgens. *J. Mol. Graph. Model.* **19:** 552-558.

Cao,L.G. and Wang,Y.L. (1996) Signals from the spindle midzone are required for the stimulation of cytokinesis in cultured epithelial cells. *Mol. Biol. Cell* **7:** 225-232.

Cohen,S.M., Meek,M.E., Klaunig,J.E., Patton,D.E., and Fenner-Crisp,P.A. (2003) The human relevance of information on carcinogenic modes of action: overview. *Crit Rev. Toxicol.* **33:** 581-589.

Collins,A.R. (1999) Oxidative DNA damage, antioxidants, and cancer. *Bioessays* **21:** 238-246.

Countryman,P.I. and Heddle,J.A. (1976) The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat. Res.* **41:** 321-332.

Crebelli,R. (2000) Threshold-mediated mechanisms in mutagenesis: implications in the classification and regulation of chemical mutagens. *Mutat. Res.* **464:** 129-135.

Dalton,T.P., Shertzer,H.G., and Puga,A. (1999) Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol.* **39:** 67-101.

Danial,N.N. and Korsmeyer,S.J. (2004) Cell death: critical control points. *Cell* **116:** 205-219.

De Clerck,L.S., Bridts,C.H., Mertens,A.M., Moens,M.M., and Stevens,W.J. (1994) Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. *J. Immunol. Methods* **172:** 115-124.

Death,A.K., McGrath,K.C., Kazlauskas,R., and Handelsman,D.J. (2004) Tetrahydrogestrinone is a potent androgen and progestin. *J. Clin. Endocrinol. Metab* **89:** 2498-2500.

Decordier,I., Dillen,L., Cundari,E., and Kirsch-Volders,M. (2002) Elimination of micronucleated cells by apoptosis after treatment with inhibitors of microtubules. *Mutagenesis* **17:** 337-344.

Di Virgilio,A.L., Iwami,K., Wätjen,W., Kahl,R., and Degen,G.H. (2004) Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. *Toxicol. Lett.* **151:** 151-162.

Dolezel,J., Binarova,P., and Lucetti,S. (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. *Biologia Plantarum* **31:** 113-121.

Doncic,A., Ben Jacob,E., and Barkai,N. (2005) Evaluating putative mechanisms of the mitotic spindle checkpoint. *Proc. Natl. Acad. Sci. U. S. A* **102:** 6332-6337.

Dorn,S.B., Degen,G.H., Müller,T., Bonacker,D., Joosten,H.F., van der Louw,J., van Acker,F.A.A., and Bolt,H.M. (2007) Proposed criteria for specific and non-specific chromosomal genotoxicity based on hydrophobic interactions. *Mutat. Res.* **628:** 67-75.

EC (2001) General Classification and Labelling Requirements for Dangerous Substances and Preparations, European Commission. *Directive 67/548/EC, Annex VI* **L 225/263**.

Elhajouji,A., Tibaldi,F., and Kirsch-Volders,M. (1997) Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes. *Mutagenesis* **12:** 133-140.

Elledge,S.J. (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* **274:** 1664-1672.

Epe,B. (2002) Role of endogenous oxidative DNA damage in carcinogenesis: what can we learn from repair-deficient mice? *Biol. Chem.* **383:** 467-475.

Fenech,M. (1993) The cytokinesis-block micronucleus technique and its application to genotoxicity studies in human populations. *Environ. Health Perspect.* **101 Suppl 3:** 101-107.

Fenech,M. (2000) The in vitro micronucleus technique. *Mutat. Res.* **455:** 81-95.

Fenech,M., Crott,J., Turner,J., and Brown,S. (1999) Necrosis, apoptosis, cytostasis and DNA damage in human lymphocytes measured simultaneously within the cytokinesis-block micronucleus assay: description of the method and results for hydrogen peroxide. *Mutagenesis* **14:** 605-612.

Ferguson,L.R. and Pearson,A.E. (1996) The clinical use of mutagenic anticancer drugs. *Mutat. Res.* **355:** 1-12.

Fernandes-Alnemri,T., Takahashi,A., Armstrong,R., Krebs,J., Fritz,L., Tomaselli,K.J., Wang,L., Yu,Z., Croce,C.M., and Salveson,G. (1995) Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55:** 6045-6052.

Filman,D.J., Brawn,R.J., and Dandliker,W.B. (1975) Intracellular supravital stain delocalization as an assay for antibody-dependent complement-mediated cell damage. *J. Immunol. Methods* **6:** 189-207.

Fishkind,D.J. and Wang,Y.L. (1993) Orientation and three-dimensional organization of actin filaments in dividing cultured cells. *J. Cell Biol.* **123:** 837-848.

Forsberg,L., de Faire,U., and Morgenstern,R. (2001) Oxidative stress, human genetic variation, and disease. *Arch. Biochem. Biophys.* **389:** 84-93.

Forth,W., Henschler,D., Rummel,W., and Starke,K. (1998) *Allgemeine und spezielle Pharmakologie und Toxikologie*. Heidelberg: Spektrum Akademischer Verlag.

Fotakis,G. and Timbrell,J.A. (2006) In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol. Lett.* **160:** 171-177.

Galloway,S.M. (2000) Cytotoxicity and chromosome aberrations in vitro: experience in industry and the case for an upper limit on toxicity in the aberration assay. *Environ. Mol. Mutagen.* **35:** 191-201.

Galloway,S.M., Miller,J.E., Armstrong,M.J., Bean,C.L., Skopek,T.R., and Nichols,W.W. (1998) DNA synthesis inhibition as an indirect mechanism of chromosome aberrations: comparison of DNA-reactive and non-DNA-reactive clastogens. *Mutat. Res.* **400:** 169-186.

Gardner,M.K. and Odde,D.J. (2006) Modeling of chromosome motility during mitosis. *Curr. Opin. Cell Biol.* **18:** 639-647.

Gobinet,J., Poujol,N., and Sultan,C. (2002) Molecular action of androgens. *Mol. Cell Endocrinol.* **198:** 15-24.

Goegan,P., Johnson,G., and Vincent,R. (1995) Effects of serum protein and colloid on the alamar-Blue assay in cell cultures. *Toxicol. In Vitro* **9:** 257-266.

Goldman,M. (1996) Cancer risk of low-level exposure. *Science* **271:** 1821-1822.

Grace,K.J., Medina,M., Jacobs,R.S., and Wilson,L. (1992) Selective inhibition of cytokinesis in sea urchin embryos by the marine natural product pseudopterolide. *Mol. Pharmacol.* **41:** 631-638.

Greenwood,S.K., Hill,R.B., Sun,J.T., Armstrong,M.J., Johnson,T.E., Gara,J.P., and Galloway,S.M. (2004) Population doubling: a simple and more accurate estimation of cell growth suppression in the in vitro assay for chromosomal aberrations that reduces irrelevant positive results. *Environ. Mol. Mutagen.* **43:** 36-44.

Grootenhuis,A., de Gooyer,M., van der Louw,J., Bursi,R., and Leysen,D. (2004) Synthesis and pharmacological profiling of new orally active steroidal androgens. In *Testosterone: Action, Deficiency, Substitution*. Nieschlag,E. and Behre,H.M. (eds). Cambridge: Cambridge University Press, 665-684.

Grosicka,E., Sadurska,B., Szumilo,M., Grzela,T., Lazarczyk,P., Niderla-Bielinska,J., and Rahden-Staron,I. (2005) Effect of glutathione depletion on apoptosis induced by thiram in Chinese hamster fibroblasts. *Int. Immunopharmacol.* **5:** 1945-1956.

Haber,J.E., Peloquin,J.G., Halvorson,H.O., and Borisy,G.G. (1972) Colcemid inhibition of cell growth and the characterization of a colcemid-binding activity in Saccharomyces cerevisiae. *J. Cell Biol.* **55:** 355-367.

Hansch,C., Leo,A., and Hoekma,D. (1995) *Exploring QSAR - Hydrophobic, Electronic, and Steric Constants*. Washington DC: American Chemical Society.

Hengstler,J.G., Bogdanffy,M.S., Bolt,H.M., and Oesch,F. (2003) Challenging dogma: thresholds for genotoxic carcinogens? The case of vinyl acetate. *Annu. Rev. Pharmacol. Toxicol.* **43:** 485-520.

Himes,R.H., Kersey,R.N., Heller-Bettinger,I., and Samson,F.E. (1976) Action of the vinca alkaloids vincristine, vinblastine, and desacetyl vinblastine amide on microtubules in vitro. *Cancer Res.* **36:** 3798-3802.

Hoffmann,B., Schopper,D., and Karg,H. (1984) Investigations on the occurrence of non-extractable residues of trienbolone acetate in cattle tissues in respect to their bioavailability and immunological reactivity. *Food Addit. Contam* **1:** 253-259.

Högstedt,B. and Karlsson,A. (1985) The size of micronuclei in human lymphocytes varies according to inducing agent used. *Mutat. Res.* **156:** 229-232.

Holsapple,M.P., Pitot,H.C., Cohen,S.M., Boobis,A.R., Klaunig,J.E., Pastoor,T., Dellarco,V.L., and Dragan,Y.P. (2006) Mode of action in relevance of rodent liver tumors to human cancer risk. *Toxicol. Sci.* **89:** 51-56.

IARC (2007) Monographs on the Evaluation of Carcinogenic Risks to Humans, Preamble. *International Agency for Research on Cancer*.

ICH S1A (1995) International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Guideline on the need for carcinogenicity studies of pharmaceuticals S1A. *ICH Harmonised Tripartite Guidline*.

ICH S2A (1995) International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Guidance on specific aspects of regulatory genotoxicity test for pharmaceuticals S2A. *ICH Harmonised Tripartite Guidline*.

ICH S2B (1997) Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals S2B. *ICH Harmonised Tripartite Guidline*.

Johnson,G.D. and Nogueira Araujo,G.M. (1981) A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* **43:** 349-350.

Johnson,I.S., Armstrong,J.G., Gorman,M., and Burnett,J.P., Jr. (1963) The Vinca Alkaloids: A New Class of Oncolytic Agents. *Cancer Res.* **23:** 1390-1427.

Joosten,H.F., van den Dobbelsteen,D.J., Horbach,G.J., and Krajnc,E.I. (2004) Genotoxicity of hormonal steroids. *Toxicol. Lett.* **151:** 113-134.

Kalweit,S., Utesch,D., von der,H.W., and Madle,S. (1999) Chemically induced micronucleus formation in V79 cells--comparison of three different test approaches. *Mutat. Res.* **439:** 183-190.

Keshava,C., Ong,T., and Nath,J. (1995) Comparative studies on radiation-induced micronuclei and chromosomal aberrations in V79 cells. *Mutat. Res.* **328:** 63-71.

Kirkland,D., Pfuhler,S., Tweats,D., Aardema,M., Corvi,R., Darroudi,F., Elhajouji,A., Glatt,H., Hastwell,P., Hayashi,M., Kasper,P., Kirchner,S., Lynch,A., Marzin,D., Maurici,D., Meunier,J.R., Müller,L., Nohynek,G., Parry,J., Parry,E., Thybaud,V., Tice,R., van Benthem,J., Vanparys,P., and White,P. (2007a) How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutat. Res.* **628:** 31-55.

Kirkland,D.J., Aardema,M., Banduhn,N., Carmichael,P., Fautz,R., Meunier,J.R., and Pfuhler,S. (2007b) In vitro approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive in vitro genotoxicity results. *Mutagenesis* **22:** 161-175.

Kirkland,D.J., Hayashi,M., MacGregor,J.T., Müller,L., Schechtman,L., and Sofuni,T. (2000) Summary of major conclusions from the International Workshop on Genotoxicity Test Procedures. *Environ. Mol. Mutagen.* **35:** 162-166.

Kirkland,D.J. and Müller,L. (2000) Interpretation of the biological relevance of genotoxicity test results: the importance of thresholds. *Mutat. Res.* **464:** 137-147.

Kirsch-Volders,M., Aardema,M., and Elhajouji,A. (2000a) Concepts of threshold in mutagenesis and carcinogenesis. *Mutat. Res.* **464:** 3-11.

Kirsch-Volders,M. and Fenech,M. (2001) Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes. *Mutagenesis* **16:** 51-58.

Kirsch-Volders,M., Sofuni,T., Aardema,M., Albertini,S., Eastmond,D., Fenech,M., Ishidate,M., Jr., Lorge,E., Norppa,H., Surralles,J., von der,H.W., and Wakata,A. (2000b) Report from the In Vitro Micronucleus Assay Working Group. *Environ. Mol. Mutagen.* **35:** 167-172.

Kirsch-Volders,M., Vanhauwaert,A., Eichenlaub-Ritter,U., and Decordier,I. (2003) Indirect mechanisms of genotoxicity. *Toxicol. Lett.* **140-141:** 63-74.

Koberle,B. and Speit,G. (1990) The effect of glutathione depletion on sister-chromatid exchange induction by cytostatic drugs. *Mutat. Res.* **243:** 225-231.

Koopman,G., Reutelingsperger,C.P., Kuijten,G.A., Keehnen,R.M., Pals,S.T., and van Oers,M.H. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84:** 1415-1420.

Kosower,N.S. and Kosower,E.M. (1995) Diamide: an oxidant probe for thiols. *Methods Enzymol.* **251:** 123-133.

Krishan,A. (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* **66:** 188-193.

Kulling,S.E., Rosenberg,B., Jacobs,E., and Metzler,M. (1999) The phytoestrogens coumoestrol and genistein induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes. *Arch. Toxicol.* **73:** 50-54.

Liehr,J.G. (2000) Is estradiol a genotoxic mutagenic carcinogen? *Endocr. Rev.* **21:** 40-54.

Lorge,E., Lambert,C., Gervais,V., Becourt-Lhote,N., Delongeas,J.L., and Claude,N. (2007) Genetic toxicity assessment: employing the best science for human safety evaluation. Part II: Performances of the in vitro micronucleus test compared to the mouse lymphoma assay and the in vitro chromosome aberration assay. *Toxicol. Sci.* **96:** 214-217.

Lutz,W.K., Deuber,R., Caviezel,M., Sagelsdorff,P., Friederich,U., and Schlatter,C. (1988) Trenbolone growth promotant: covalent DNA binding in rat liver and in Salmonella typhimurium, and mutagenicity in the Ames test. *Arch. Toxicol.* **62:** 103-109.

Lutz,W.K. and Kopp-Schneider,A. (1999) Threshold dose response for tumor induction by genotoxic carcinogens modeled via cell-cycle delay. *Toxicol. Sci.* **49:** 110-115.

Lynch,A., Harvey,J., Aylott,M., Nicholas,E., Burman,M., Siddiqui,A., Walker,S., and Rees,R. (2003) Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity. *Mutagenesis* **18:** 345-353.

MacLean-Fletcher,S. and Pollard,T.D. (1980) Mechanism of action of cytochalasin B on actin. *Cell* **20:** 329-341.

Madle,S., von der,H.W., Broschinski,L., and Janig,G. (2000) Threshold effects in genetic toxicity: perspective of chemicals regulation in Germany. *Mutat. Res.* **464:** 117-121.

Mäki-Paakkanen,J., Hayashi,M., Suzuki,T., Tanabe,H., Honma,M., and Sofuni,T. (1995) Analysis by fluorescence in situ hybridization with a mouse gamma satellite DNA probe of isolated micronuclei induced in mice by two clastogens and two spindle poisons. *Mutagenesis* **10:** 513-516.

Marzin,D. (1991) Trenbolone: application of the Ames test. Recent data. *Ann. Rech. Vet.* **22:** 257-262.

Matsuoka,A., Yamazaki,N., Suzuki,T., Hayashi,M., and Sofuni,T. (1992) Evaluation of the micronucleus test using a Chinese hamster cell line as an alternative to the conventional in vitro chromosomal aberration test. *Mutat. Res.* **272:** 223-236.

Matsushima,T., Hayashi,M., Matsuoka,A., Ishidate,M., Jr., Miura,K.F., Shimizu,H., Suzuki,Y., Morimoto,K., Ogura,H., Mure,K., Koshi,K., and Sofuni,T. (1999) Validation study of the in vitro micronucleus test in a Chinese hamster lung cell line (CHL/IU). *Mutagenesis* **14:** 569-580.

Meintières,S., Biola,A., Pallardy,M., and Marzin,D. (2001) Apoptosis can be a confusing factor in in vitro clastogenic assays. *Mutagenesis* **16:** 243-250.

Meintières,S. and Marzin,D. (2004) Apoptosis may contribute to false-positive results in the in vitro micronucleus test performed in extreme osmolality, ionic strength and pH conditions. *Mutat. Res.* **560:** 101-118.

Metzler,M. and Pfeiffer,E. (2001) Genotoxic potential of xenobiotic growth promoters and their metabolites. *APMIS* **109:** 89-95.

Miller,B., Potter-Locher,F., Seelbach,A., Stopper,H., Utesch,D., and Madle,S. (1998) Evaluation of the in vitro micronucleus test as an alternative to the in vitro chromosomal aberration assay: position of the GUM Working Group on the in vitro micronucleus test. Gesellschaft fur Umwelt-Mutationsforschung. *Mutat. Res.* **410:** 81-116.

Miller,B.M. and Adler,I.D. (1989) Suspect spindle poisons: analysis of c-mitotic effects in mouse bone marrow cells. *Mutagenesis* **4:** 208-215.

Miller,B.M. and Adler,I.D. (1990) Application of antikinetochore antibody staining (CREST staining) to micronuclei in erythrocytes induced in vivo. *Mutagenesis* **5:** 411-415.

Moolenaar,R.J. (1994) Carcinogen risk assessment: international comparison. *Regul. Toxicol. Pharmacol.* **20:** 302-336.

Müller,L. and Kasper,P. (2000) Human biological relevance and the use of threshold-arguments in regulatory genotoxicity assessment: experience with pharmaceuticals. *Mutat. Res.* **464:** 19-34.

Müller,L., Kikuchi,Y., Probst,G., Schechtman,L., Shimada,H., Sofuni,T., and Tweats,D. (1999) ICH-harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. *Mutat. Res.* **436:** 195-225.

Mutschler,E., Geisslinger,G., Kroemer,H.K., and Schäfer-Korting,M. (2001) *Arzneimittelwirkungen - Lehrbuch der Pharmakologie und Toxikologie*. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

Nair,U., Bartsch,H., and Nair,J. (2007) Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans. *Free Radic. Biol. Med.* **43:** 1109-1120.

Neumann,H.G., Vamvakas,S., Thielmann,H.W., Gelbke,H.P., Filser,J.G., Reuter,U., Greim,H., Kappus,H., Norpoth,K.H., Wardenbach,P., and Wichmann,H.E. (1998) Changes in the classification of carcinogenic chemicals in the work area. Section III of the German List of MAK and BAT Values. *Int. Arch. Occup. Environ. Health* **71:** 566-574.

Newmeyer,D.D. and Ferguson-Miller,S. (2003) Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112:** 481-490.

Nicholson,D.W., Ali,A., Thornberry,N.A., Vaillancourt,J.P., Ding,C.K., Gallant,M., Gareau,Y., Griffin,P.R., Labelle,M., Lazebnik,Y.A., and . (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376:** 37-43.

Nociari,M.M., Shalev,A., Benias,P., and Russo,C. (1998) A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. *J. Immunol. Methods* **213:** 157-167.

O'Brien,J., Wilson,I., Orton,T., and Pognan,F. (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **267:** 5421-5426.

Omenn,G.S. (1998) Chemoprevention of lung cancer: the rise and demise of beta-carotene. *Annu. Rev. Public Health* **19:** 73-99.

Önfelt,A. (1983) Spindle disturbances in mammalian cells. I. Changes in the quantity of free sulfhydryl groups in relation to survival and C-mitosis in V79 Chinese hamster cells after treatment with colcemid, diamide, carbaryl and methyl mercury. *Chem. Biol. Interact.* **46:** 201-217.

Önfelt,A. (1986) Mechanistic aspects on chemical induction of spindle disturbances and abnormal chromosome numbers. *Mutat. Res.* **168:** 249-300.

Önfelt,A. (1987a) Spindle disturbances in mammalian cells. III. Toxicity, c-mitosis and aneuploidy with 22 different compounds. Specific and unspecific mechanisms. *Mutat. Res.* **182:** 135-154.

Önfelt,A. (1987b) Spindle disturbances in mammalian cells. IV. The action of some glutathione-specific agents in V79 Chinese hamster cells, changes in levels of free sulfhydryls and ATP, c-mitosis and effects on DNA metabolism. *Mutat. Res.* **182:** 155-172.

Önfelt,A. and Klasterska,I. (1983) Spindle disturbances in mammalian cells. II. Induction of viable aneuploid/polyploid cells and multiple chromatid exchanges after treatment of V79 Chinese hamster cells with carbaryl. Modifying effects of glutathione and S9. *Mutat. Res.* **119:** 319-330.

Onishi,Y., Azuma,Y., Sato,Y., Mizuno,Y., Tadakuma,T., and Kizaki,H. (1993) Topoisomerase inhibitors induce apoptosis in thymocytes. *Biochim. Biophys. Acta* **1175:** 147-154.

Owellen,R.J., Hartke,C.A., Dickerson,R.M., and Hains,F.O. (1976) Inhibition of tubulin-microtubule polymerization by drugs of the Vinca alkaloid class. *Cancer Res.* **36:** 1499-1502.

Parry,J.M., Jenkins,G.J., Haddad,F., Bourner,R., and Parry,E.M. (2000) In vitro and in vivo extrapolations of genotoxin exposures: consideration of factors which influence dose-response thresholds. *Mutat. Res.* **464:** 53-63.

Pratt,I.S. and Barron,T. (2003) Regulatory recognition of indirect genotoxicity mechanisms in the European Union. *Toxicol. Lett.* **140-141:** 53-62.

Preston,R.J. (2007) Cancer risk assessment for 1,3-butadiene: data integration opportunities. *Chem. Biol. Interact.* **166:** 150-155.

Rappaport,R. (1986) Establishment of the mechanism of cytokinesis in animal cells. *Int. Rev. Cytol.* **105:** 245-281.

Ray,K., Bhattacharyya,B., and Biswas,B.B. (1984) Anion-induced increases in the affinity of colcemid binding to tubulin. *Eur. J. Biochem.* **142:** 577-581.

Renzi,L., Pacchierotti,F., and Russo,A. (1996) The centromere as a target for the induction of chromosome damage in resting and proliferating mammalian cells: assessment of mitomycin C-induced genetic damage at kinetochores and centromeres by a micronucleus test in mouse splenocytes. *Mutagenesis* **11:** 133-138.

Richold,M. (1988) The genotoxicity of trenbolone, a synthetic steroid. *Arch. Toxicol.* **61:** 249-258.

Rogers,D. and Hopfinger,A.J. (1994) Application of Genetic Function Approximation to Quantitative Structure-Activity
Relationships and Quantitative Structure-Property Relationships. *J. Chem. Inf. Comput. Sci.* **34:** 854-866.

Rosenkranz,A.R., Schmaldienst,S., Stuhlmeier,K.M., Chen,W., Knapp,W., and Zlabinger,G.J. (1992) A microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescin-diacetate. *J. Immunol. Methods* **156:** 39-45.

Rousseeuw,P.J. and Leroy,A.M. (1987) *Robust regression and outlier detection*. New York: John Wiley & Sons, Inc.

Russo,A., Stocco,A., and Majone,F. (1992) Identification of kinetochore-containing (CREST+) micronuclei in mouse bone marrow erythrocytes. *Mutagenesis* **7:** 195-197.

Ryan,J.J. and Hoffmann,B. (1978) Trenbolone acetate: experiences with bound residues in cattle tissues. *J. Assoc. Off Anal. Chem.* **61:** 1274-1279.

Salti,G.I., Grewal,S., Mehta,R.R., Das Gupta,T.K., Boddie,A.W., Jr., and Constantinou,A.I. (2000) Genistein induces apoptosis and topoisomerase II-mediated DNA breakage in colon cancer cells. *Eur. J. Cancer* **36:** 796-802.

Sanchez,F.A., Santema,J.S., Hilhorst,R., and Visser,A.J. (1990) Fluorescence detection of enzymatically formed hydrogen peroxide in aqueous solution and in reversed micelles. *Anal. Biochem.* **187:** 129-132.

Schiffmann,D., Hieber,L., Schmuck,G., Pechan,R., Metzler,M., and Henschler,D. (1988) Trenbolone induces micronucleus formation and neoplastic transformation in Syrian hamster embryo fibroblasts but not in mouse C3H10T1/2 cells. *Arch. Toxicol.* **62:** 49-53.

Schuler,M., Rupa,D.S., and Eastmond,D.A. (1997) A critical evaluation of centromeric labeling to distinguish micronuclei induced by chromosomal loss and breakage in vitro. *Mutat. Res.* **392:** 81-95.

Schultz,N. and Önfelt,A. (2000) Sensitivity of cytokinesis to hydrophobic interactions. Chemical induction of bi-and multinucleated cells. *Chem. Biol. Interact.* **126:** 97-123.

Scott,D., Galloway,S.M., Marshall,R.R., Ishidate,M., Jr., Brusick,D., Ashby,J., and Myhr,B.C. (1991) International Commission for Protection Against Environmental Mutagens and Carcinogens. Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9. *Mutat. Res.* **257:** 147-205.

Seeley,M.R., Tonner-Navarro,L.E., Beck,B.D., Deskin,R., Feron,V.J., Johanson,G., and Bolt,H.M. (2001) Procedures for health risk assessment in Europe. *Regul. Toxicol. Pharmacol.* **34:** 153-169.

Seiler,J.P. (1977) Apparent and real threshold: a study on two mutagens. In *Progress in Genetic Toxicology*. Scott,D., Bridges,B.A., and Sobels,F.H. (eds). Amsterdam: Elsevier, 233-238.

Shackelford,R.E., Kaufmann,W.K., and Paules,R.S. (1999) Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ. Health Perspect.* **107 Suppl 1:** 5-24.

Shelanski,M.L., Gaskin,F., and Cantor,C.R. (1973) Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A* **70:** 765-768.

Smith,P.K., Krohn,R.I., Hermanson,G.T., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J., and Klenk,D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76-85.

Snyder,R.D. (2007) Assessment of atypical DNA intercalating agents in biological and in silico systems. *Mutat. Res.*

Snyder,R.D. and Green,J.W. (2001) A review of the genotoxicity of marketed pharmaceuticals. *Mutat. Res.* **488:** 151-169.

Södergård,R., Bäckström,T., Shanbhag,V., and Carstensen,H. (1982) Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. *J. Steroid Biochem.* **16:** 801-810.

Solary,E., Bertrand,R., Kohn,K.W., and Pommier,Y. (1993) Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by DNA topoisomerase I and II inhibitors. *Blood* **81:** 1359-1368.

Sorger,P.K., Dobles,M., Tournebize,R., and Hyman,A.A. (1997) Coupling cell division and cell death to microtubule dynamics. *Curr. Opin. Cell Biol.* **9:** 807-814.

Speit,G., Dennog,C., Radermacher,P., and Rothfuss,A. (2002) Genotoxicity of hyperbaric oxygen. *Mutat. Res.* **512:** 111-119.

Spudich,J.A. and Lin,S. (1972) Cytochalasin B, its interaction with actin and actomyosin from muscle (cell movement-microfilaments-rabbit striated muscle). *Proc. Natl. Acad. Sci. U. S. A* **69:** 442-446.

Storer,R.D., McKelvey,T.W., Kraynak,A.R., Elia,M.C., Barnum,J.E., Harmon,L.S., Nichols,W.W., and DeLuca,J.G. (1996) Revalidation of the in vitro alkaline elution/rat hepatocyte assay for DNA damage: improved criteria for assessment of cytotoxicity and genotoxicity and results for 81 compounds. *Mutat. Res.* **368:** 59-101.

Streffer,C., Bolt,H.M., Føllesdal,D., Hall,P., Hengstler,J.G., Jacob,P. et al. (2004) *Environmental Standards—Dose-Effect Relations in the Low Dose Range and Risk Evaluation*. Berlin: Springer-Verlag.

Tao,K.S., Urlando,C., and Heddle,J.A. (1993) Mutagenicity of methyl methanesulfonate (MMS) in vivo at the Dlb-1 native locus and a lacI transgene. *Environ. Mol. Mutagen.* **22:** 293-296.

Thier,R., Bonacker,D., Stoiber,T., Böhm,K.J., Wang,M., Unger,E., Bolt,H.M., and Degen,G. (2003) Interaction of metal salts with cytoskeletal motor protein systems. *Toxicol. Lett.* **140-141:** 75-81.

Thybaud,V., Aardema,M., Casciano,D., Dellarco,V., Embry,M.R., Gollapudi,B.B., Hayashi,M., Holsapple,M.P., Jacobson-Kram,D., Kasper,P., MacGregor,J.T., and Rees,R. (2007) Relevance and follow-up of positive results in in vitro genetic toxicity assays: An ILSI-HESI initiative. *Mutat. Res.* **633:** 67-79.

Tinwell,H., Lefevre,P.A., and Ashby,J. (1998) Relative activities of methyl methanesulphonate (MMS) as a genotoxin, clastogen and gene mutagen to the liver and bone marrow of MutaMouse mice. *Environ. Mol. Mutagen.* **32:** 163-172.

Tsutsui,T., Komine,A., Huff,J., and Barrett,J.C. (1995) Effects of testosterone, testosterone propionate, 17 beta-trenbolone and progesterone on cell transformation and mutagenesis in Syrian hamster embryo cells. *Carcinogenesis* **16:** 1329-1333.

U.S.EPA (2005) Guidelines for Carcinogen Risk Assessment, U.S.Environmental Protection Agency, EPA/630/P-03/001F. Washington, DC.

van Acker,F.A.A., Joosten,H.F., and van den Dobbelsteen,D.J. (2007) Strategies in industrial genotoxicity testing of drugs. *Toxicol. Lett.* **172, Supplement 1:** S18.

VanBuren,V., Odde,D.J., and Cassimeris,L. (2002) Estimates of lateral and longitudinal bond energies within the microtubule lattice. *Proc. Natl. Acad. Sci. U. S. A* **99:** 6035-6040.

Vermes,I., Haanen,C., and Reutelingsperger,C. (2000) Flow cytometry of apoptotic cell death. *J. Immunol. Methods* **243:** 167-190.

Vock,E.H., Lutz,W.K., Hormes,P., Hoffmann,H.D., and Vamvakas,S. (1998) Discrimination between genotoxicity and cytotoxicity in the induction of DNA double-strand breaks in cells treated with etoposide, melphalan, cisplatin, potassium cyanide, Triton X-100, and gamma-irradiation. *Mutat. Res.* **413:** 83-94.

von der Hude,W., Kalweit,S., Engelhardt,G., McKiernan,S., Kasper,P., Slacik-Erben,R., Miltenburger,H.G., Honarvar,N., Fahrig,R., Gorlitz,B., Albertini,S., Kirchner,S., Utesch,D., Potter-Locher,F., Stopper,H., and Madle,S. (2000) In vitro micronucleus assay with Chinese hamster V79 cells - results of a collaborative study with in situ exposure to 26 chemical substances. *Mutat. Res.* **468:** 137-163.

Wallin,M., Friden,B., and Billger,M. (1988) Studies of the interaction of chemicals with microtubule assembly in vitro can be used as an assay for detection of cytotoxic chemicals and possible inducers of aneuploidy. *Mutat. Res.* **201:** 303-311.

Wiltse,J.A. and Dellarco,V.L. (2000) U.S. Environmental Protection Agency's revised guidelines for carcinogen risk assessment: evaluating a postulated mode of carcinogenic action in guiding dose-response extrapolation. *Mutat. Res.* **464:** 105-115.

Yamashita,Y., Kawada,S., and Nakano,H. (1990) Induction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. *Biochem. Pharmacol.* **39:** 737-744.

Zamai,L., Canonico,B., Luchetti,F., Ferri,P., Melloni,E., Guidotti,L., Cappellini,A., Cutroneo,G., Vitale,M., and Papa,S. (2001) Supravital exposure to propidium iodide identifies apoptosis on adherent cells. *Cytometry* **44:** 57-64.

Zhang,A., Wu,Y., Lai,H.W., and Yew,D.T. (2004) Apoptosis - A brief review. *Neuroembryology* **3:** 47-59.

Zhou,B.B. and Elledge,S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408:** 433-439.

7 Appendix

7.1 Additional information

7.1.1 Physicochemical parameters

7.1.1.1. Detailed description of some molecular properties

Definition of hydrogen bond donors and acceptors

The establishment of hydrogen bond donors and acceptors was performed in two steps:

1. A simple default rule is applied to determine the donors and acceptors.
2. Then, a list of exceptions to the default rules is applied.

Default Rule

- Any nitrogen or oxygen atom with at least one pair of free electrons is considered as an acceptor.
- Any hydrogen atom that is bonded to a nitrogen or oxygen atom is considered as a donor.

Exceptions

- The nitrogen atoms of amides, sulfonamides, and equivalent structures are not considered as acceptors.
- Only one nitrogen atom of guanidines, amidines, and equivalent groups is considered as an acceptor.
- The nitrogen atoms of aromatic amines are not considered as acceptors.
- The nitrogen atoms of five-membered aromatic rings like pyrrole are not considered as acceptors.
- The sulfur atoms of thiocarbonyl compounds are considered as acceptors.

Definition of rotatable bonds

A bond was considered as being rotatable, if:

- It is not in a ring, or if it is in a ring larger than 8;
- it is a single or a triple bond;

- none of the two atoms is terminal;
- it is not adjacent to a triple bond;
- it is not an amide, amidine, guanidine bond.

7.1.2 Microtubule assembly turbidity assay

The microtubule assembly turbidity assay is based on the observation that - under defined conditions - tubulin assembles and disassembles, dependent on the temperature. Using microtubule protein (MTP; i.e., tubulin containing microtubule associated proteins, MAPs) in a cell free environment, physiological temperatures lead to polymerisation of tubulin dimers and to microtubule formation. This process is reversible at lower temperature (below 10°C).

7.1.2.1. Procedure (experiments performed at the IMB, Jena)

Microtubule protein (MTP) was isolated from porcine brain by two cycles of temperature-dependent assembly-disassembly (Shelanski et al. 1973), with slight modifications (Bonacker et al. 2004a,b). Microtubule assembly was induced by addition of 10 mM GTP to 1 mg/ml MTP and shifting temperature to 37°C.

The microtubule assembly was recorded spectrophotometrically at 360 nm as described by Gaskin et al. (1974). During temperature cycles from 4ºC to 37ºC and back to 4ºC, the assembly and disassembly of microtubule was observed.

Due to the formation of microtubules, the turbidity increases upon heating to 37°C. Approximately twenty minutes after inducing MTP polymerisation at 37°C, the turbidity curve reaches a plateau level (assembly/disassembly steady-state). The corresponding absorbance within the plateau phase is taken as a reference value to quantify the effects of the test substances. If tubulin assembly is partly inhibited by an effector, the turbidity at the plateau (37ºC) is reduced compared to the control sample. The assembly may be completely inhibited, with no rise in absorbance due to turbidity. Assembly antagonists or disassembly effectors (e.g. cold) prevent microtubule formation or cause microtubule degradation. Denaturation of tubulin by the test chemical, leading to aggregation and/or precipitation is reflected by a change in the curve shape during the period of warming, compared to the control sample.

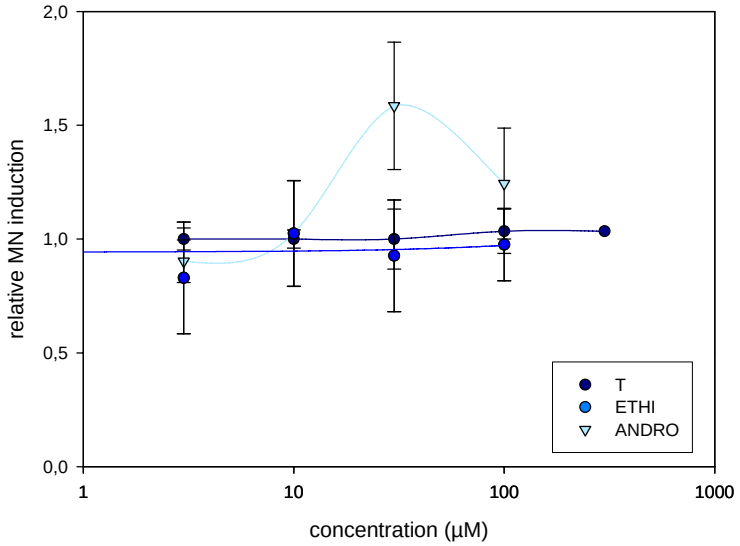
To prove the reversibility of microtubule assembly process, the measurement is continued for additional 20 min decreasing the temperature to 4°C. An increased absorption due to denaturation is not reversible, resulting in a higher absorbance at the final stage of the test (4ºC), compared to the respective untreated control.

7.2 Results in detail

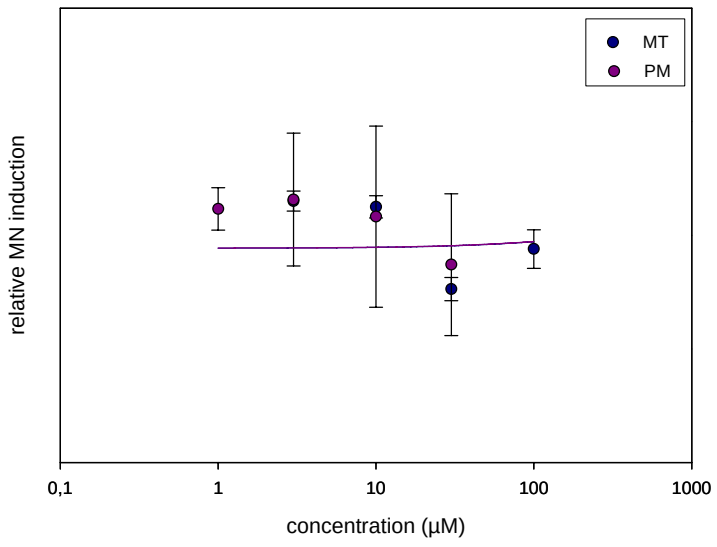
7.2.1 MN results

The MN assay results obtained for the compounds that did not reach the doubling of the MN background rate ("negative" compounds) are shown in Fig. 28.

a)



b)



c)

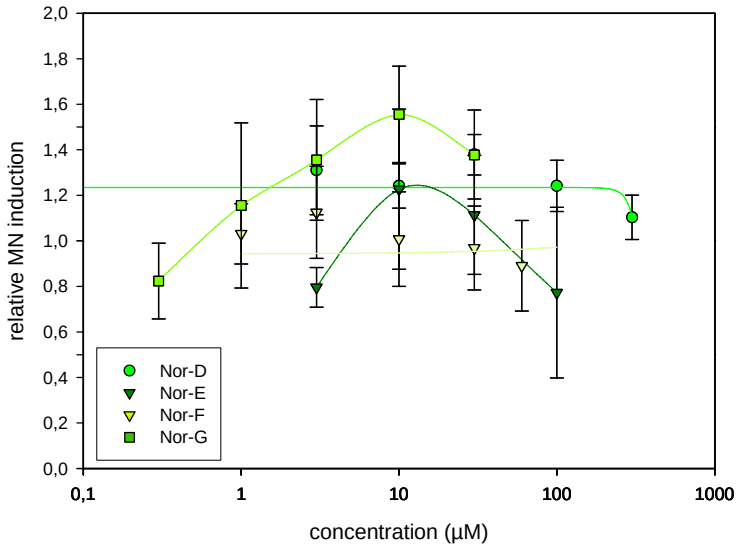


Fig. 28. MN assay results for the negative compounds: relative MN induction compared to solvent control by: a) T (testosterone), ETHI (ethisterone), and ANDRO (androstenedione), b) MT (7α-methyltestosterone), PM (17α-propylmesterolone), and c) Nor-D, Nor-E, Nor-F, and Nor-G

7.2.2 Physicochemical parameters

In order to generate molecular descriptors for modelling non-specific chromosomal genotoxicity, and to evaluate optimised combinations thereof, a number of molecular descriptors were calculated for the compounds of the combined data sets. A detailed overview on the calculated molecular properties is given in Tab. 16.

Tab. 16. Overview on the molecular parameters calculated for the compounds.

Compound	*polsurf*	*robo*	*acc*	*don*	*mw*	*log P*	*logD*	*dipole*	*logS*	*vol*	*surface*	*class*	*exp –log C*[a]	*eq1*[e]	*eq2*[e]	*eq3*[e]	*eq4*[e]	*Tox*
Ethanol	16.31	0	1	1	46.07	-0.23	-0.24	2.51	0.55	47.33	76.20	alkanol	1	1.20	1.27	1.04	1.04	clean[c]
1-Butanol	16.31	2	1	1	74.12	0.82	0.83	2.77	-0.27	79.07	120.30	alkanol	2.20	2.05	2.11	2.01	1.99	clean
1-Pentanol	16.31	3	1	1	88.15	1.35	1.35	3.03	-0.65	94.90	142.39	alkanol	2.20	2.48	2.52	2.50	2.46	clean
1-Hexanol	16.31	4	1	1	102.18	1.88	1.88	3.22	-1.03	111.08	164.85	alkanol	3.50	2.91	2.94	2.99	2.95	clean
1-Heptanol	16.31	5	1	1	116.20	2.41	2.41	3.45	-1.40	126.77	186.80	alkanol	3.10	3.34	3.36	3.47	3.42	clean
1-Octanol	16.31	6	1	1	130.23	2.94	2.94	3.66	-1.78	142.43	208.60	chlorHC[b]	3.95	3.77	3.77	3.95	3.89	clean
Dichloromethane	0.00	0	0	0	84.93	1.25	1.25	4.09	-0.98	61.16	87.39	chlorHC	1.90	2.40	2.19	2.27	2.42	clean
Chloroform	0.00	0	0	0	119.38	1.95	1.96	2.94	-1.58	78.27	105.94	chlorHC	2.80	2.97	2.70	2.68	2.74	clean
Carbon tetrachloride	0.00	0	0	0	153.82	2.88	2.87	0.00	-3.48	95.11	123.12	chlorHC	2.90	3.72	3.32	3.06	2.94	clean
1,2-Dichloroethane	0.00	1	0	0	98.96	1.46	1.45	$6.08e^{-5}$	-1.35	77.09	109.78	chlorHC	2.80	2.57	2.42	2.76	2.66	clean
1,1,2,2-Tetrachloroethane	0.00	1	0	0	167.85	2.64	2.64	3.79	-2.39	110.67	141.51	chlorHC	3.80	3.53	3.27	3.46	3.54	clean
1,1,1,2-Tetrachloroethane	0.00	0	0	0	167.85	3.03	3.04	3.81	-2.57	110.66	142.17	chlorHC	3.90	3.85	3.50	3.47	3.55	clean
Pentachloroethane	0.00	0	0	0	202.29	3.63	3.62	2.32	-3.08	127.25	156.92	chlorHC	3.40	4.33	3.92	3.80	3.78	clean
Benzonitrile	15.16	0	1	0	103.12	1.57	1.57	3.36	-2.04	104.91	130.49	other	6	2.67	2.59	1.85	2.38	tox[d]
Colcemid	57.52	5	6	1	371.43	1.75	1.29	4.56	-4.22	323.82	389.67	other	7.90	2.81	3.70	-10.51	-4.27	tox
Cytochalasin B	75.97	13	5	3	479.61	2.97	2.97	3.35	-5.27	434.60	511.55	other	6.30	3.80	5.28	4.78	-11.50	tox
Daidzein	53.69	1	4	2	254.24	2.08	1.84	2.14	-3.04	209.54	246.84	other	4.20	3.07	3.32	-2.03	-5.72	tox
Diamide	52.06	2	4	0	172.19	-0.51	-0.51	0.05	-1.43	149.82	208.17	other	4.10	0.97	1.76	-3.43	-5.97	tox
Genistein	70.00	1	5	3	270.24	2.40	1.75	3.05	-2.74	213.37	251.26	other	5	3.34	3.29	-2.92	-13.54	tox

Tab. 16. Continued

Compound	*polsurf*	*robo*	*acc*	*don*	*mw*	*log P*	*logD*	*dipole*	*logS*	*vol*	*surface*	*class*	*exp –log C*[a]	*eq1*[e]	*eq2*[e]	*eq3*[e]	*eq4*[e]	*Tox*
Methyl methanesulfonate	33.33	1	3	0	110.13	-0.48	-0.48	1.64	-0.01	79.06	114.54	other	3.90	1.00	1.31	-5.31	-1.53	tox
Nitrobenzene	36.91	0	2	0	123.11	1.89	1.89	7.25	-2.27	104.33	133.55	other	6	2.92	2.79	2.75	-1.81	tox
Vincristine	139.52	16	12	3	824.97	4.04	3.59	4.79	-5.60	693.14	772.23	other	9.20	4.67	6.92	-13.34	-59.99	tox
19-Nortestosterone	28.57	0	2	1	274.40	2.70	2.70	4.30	-4.43	257.57	313.81	steroid	4.18	3.58	4.15	3.91	3.96	clean
19-Norethisterone	28.57	0	2	1	298.42	2.78	2.78	4.08	-5.39	285.76	339.04	steroid	4.62	3.65	4.32	4.46	4.48	clean
Nor-C	28.57	1	2	1	300.44	3.06	3.06	3.68	-4.86	283.64	337.15	steroid	4	3.88	4.47	4.42	4.42	clean
Trenbolone	28.57	0	2	1	270.37	1.82	1.83	5.45	-4.00	247.78	295.66	steroid	4.64	2.87	3.55	3.51	3.65	tox

[a] negative logarithm of the concentration at which doubling of the number of micronuclei occurs

[b] chlorinated hydro carbon

[c] non-specific genotoxicity observed,

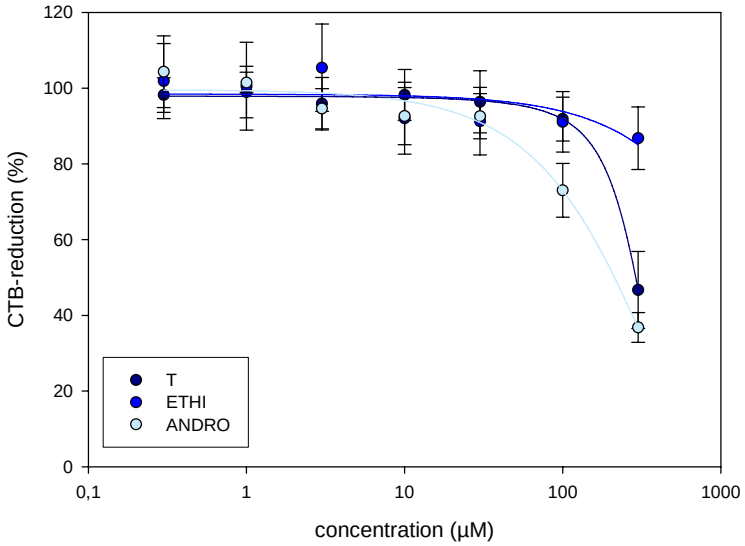
[d] specific genotoxic mode of action of the compound

[e] predicted -log C values that result from fitting linear equations with the experimental -log C.

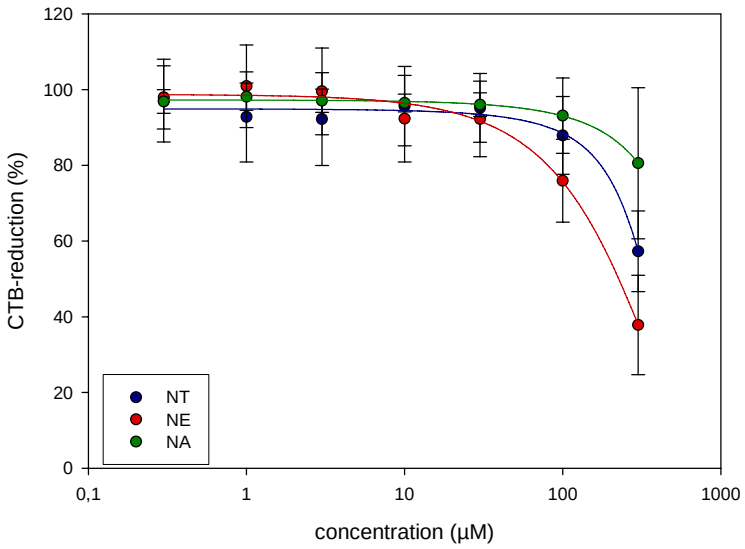
7.2.3 Cytotoxicity

The concentration-response-curves obtained in the CellTiter Blue assay are depicted in Fig. 29. From these, the limits of cytotoxicity (IC_{20}) and the IC_{50} values were taken.

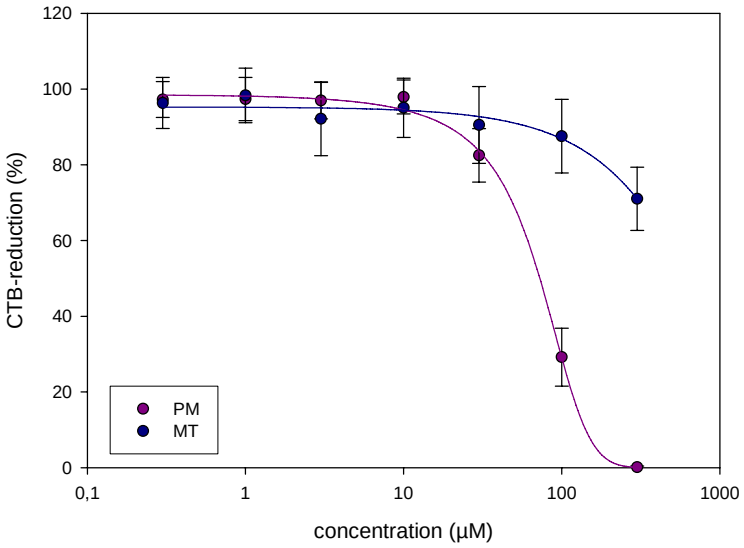
a)



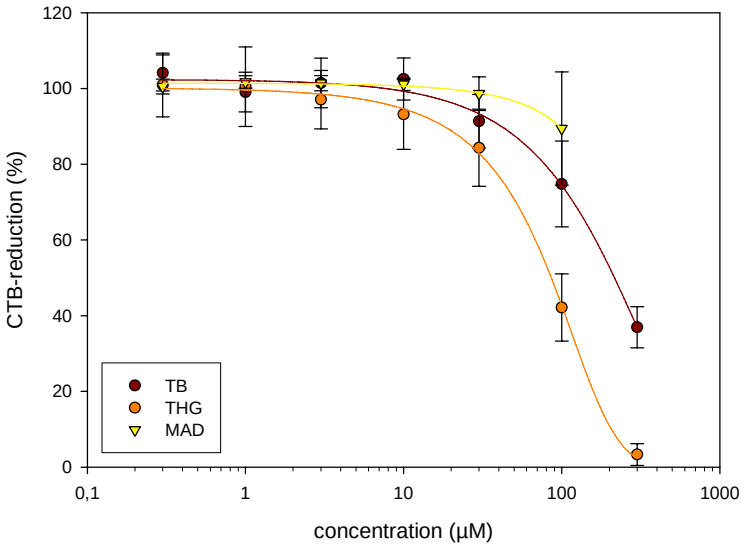
b)



c)


120
100
80
60
40
20
0
CTB-reduction (%)
PM
MT
0,1
1
10
100
1000
concentration (µM)

d)


120
100
80
60
40
20
0
CTB-reduction (%)
TB
THG
MAD
0,1
1
10
100
1000
concentration (µM)

e)

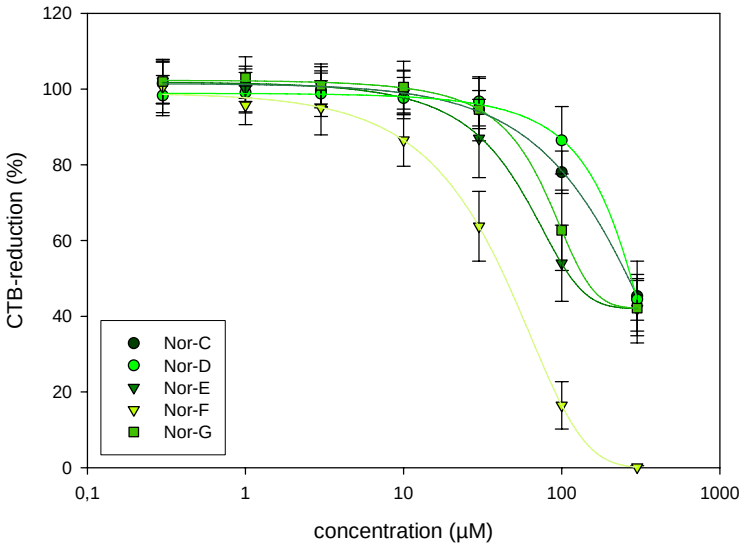
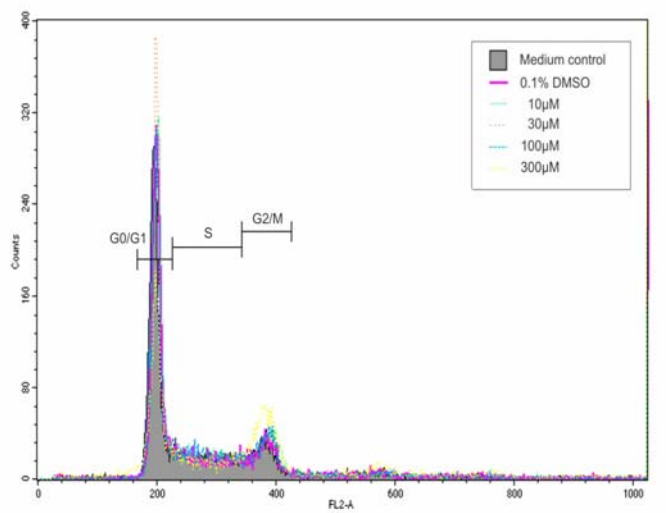


Fig. 29. Cytotoxicity of a) T (testosterone), ETHI (ethisterone), ANDRO (androstenedione); b) NA (19-norandrostenedione), NE (19-norethisterone), NT (19-nortestosterone); c) PM (17α-propylmesterolone), MT (7α-methyltestosterone); d) MAD (madol), THG (tetrahydrogestrinone), TB (trenbolone); e) Nor-C, Nor-D, Nor-E, Nor-F, and Nor-G, relative to solvent control

7.2.4 Cell cycle analysis

Exemplarily, typical profiles of the FACS analysis for testosterone (as example for a negative compound in the micronucleus assay) and trenbolone (as example for a positive compound in the micronucleus assay) are shown in Fig. 30.

a)



b)

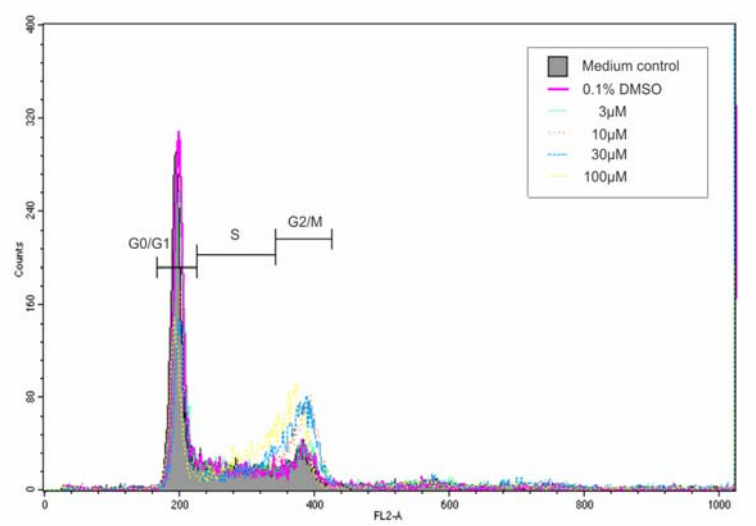


Fig. 30. Cell cycle analysis: a typical experiment profile for a) testosterone and b) trenbolone

The results of the cell cycle analysis for the compounds and the tested concentrations and the distribution in the single cell cycle phases are summarized in Tab. 17.

Tab. 17. Distribution of cells in the different cell cycle phases after 18h substance treatment

Compound	**Concentration**	**Cell cycle phases** *(%)*			
	(µM)	*G0/G1*	*S*	*G2/M*	*Sub G0*
ETHI	0	61.72 (+/-4.49)	18.39 (+/-6.19)	19.89 (+/-2.07)	
	0.1	66.38	10.45	23.17	
	10	65.04	15.75	19.21	
	30	56.63 (+/-1.16)	22.32 (+/-3.94)	21.04 (+/-4.02)	
	100	56.94 (+/-1.61)	21.42 (+/-3.39)	21.64 (+/-2.84)	
MAD	0	60.17 (+/-1.16)	18.58 (+/-1.70)	21.26 (+/-0.60)	
	3	61.76	17.47	20.77	
	10	59.42 (+/-2.01)	20.76 (+/-2.67)	19.82 (+/-4.36)	
	30	60.67 (+/-2.10)	20.26 (+/-1.92)	19.07 (+/-2.36)	
NA	0	61.16 (+/-2.86)	18.65 (+/-1.57)	20.19 (+/-1.30)	
	10	57.22	24.99	17.79	
	30	54.33 (+/-2.67) *	21.61 (+/-1.58)	20.56 (+/-4.26)	
	100	49.54 (+/-5.77) *	24.06 (+/-4.25)	29.90 (+/-9.88)	

Tab. 17. Continued

Compound	Concentration	Cell cycle phases *(%)*			
	(µM)	*G0/G1*	*S*	*G2/M*	*Sub G0*
NE	0	61.86(+/-4.41)	19.80 (+/-6.99)	18.34 (+/-2.75)	
	3	57.88	24.44	17.68	
	10	54.50 (+/-9.39)	19.57 (+/-7.51)	25.93 (+/-6.80)	
	30	53.35 (+/-4.82)	24.69 (+/-3.07)	21.57 (+/-2.37)	1.52
	60	55.25 (+/-6.78)	22.15 (+/-4.07)	21.75 (+/-2.41)	2.57
NT	0	61.86 (+/-4.41)	19.80 (+/-6.99)	18.34 (+/-2.75)	
	3	60.01	20.60	19.39	
	10	50.58 (+/-4.80	25.59 (+/-4.86	23.83 (+/-0.06	
	30	57.84 (+/-7.24	18.67 (+/-6.82	23.70 (+/-2.47	
	100	47.82 (+/-2.35)*	20.79 (+/-4.94)	31.38 (+/-4.08)*	
Nor-C	0	57.21(+/-10.11)	20.95(+/-5.79)	21.84 (+/-5.33)	
	10	53.10 (+/-4.60)	24.87 (+/-5.47)	22.03 (+/-0.88)	
	30	54.68 (+/-6.52	23.18 (+/-8.82	22.15 (+/-3.03	
	100	42.63 (+/-4.81)*	20.55 (+/-3.37)	36.82 (+/-7.35) *	
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)	
		58.87(+/-1.92)	21.48 (+/-2.87)	19.65 (+/-0.99)	
		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)	
	1	56.46	23.89	19.65	
	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)

7.2.5 Detection of apoptosis

7.2.5.1. Annexin-V/PI assay

The apoptosis inducing potential of the compounds studied was determined assaying two different endpoints, caspases 3/7 activity and Annexin V binding to phosphatidylserine. The results obtained in the Annexin-V/PI assay are depicted in Fig. 31.

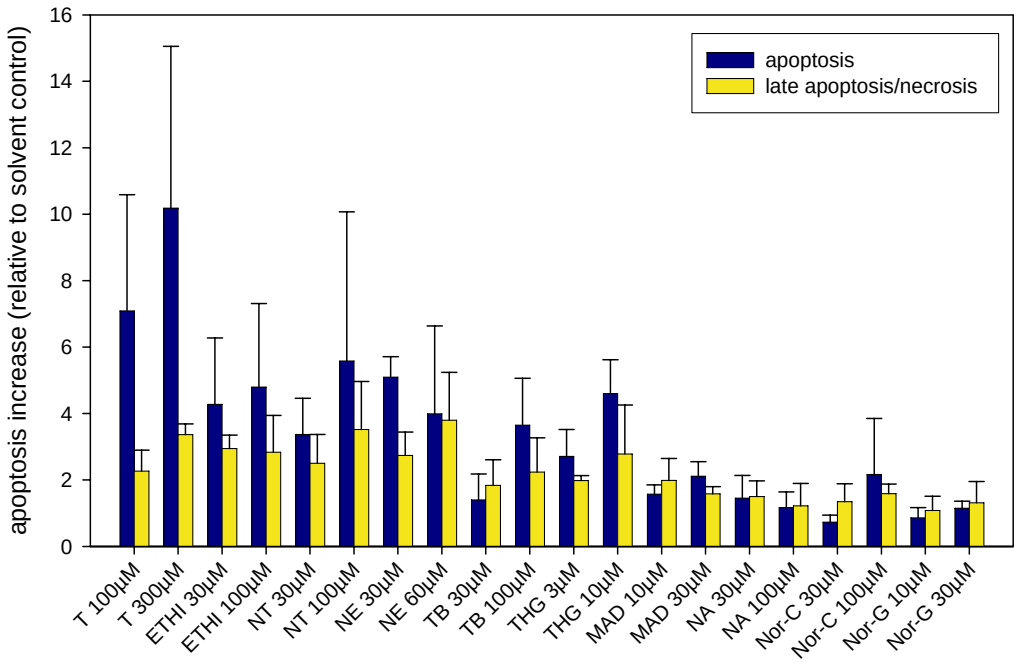


Fig. 31. Induction of apoptosis and late apoptosis/necrosis by T (testosterone), ETHI (ethisterone), NT (19-nortestosterone), NE (19-norethisterone), TB (trenbolone), THG (tetrahydrogestrinone), MAD (madol), NA (19-norandrostenedione), Nor-C, and Nor-G, relative to solvent control

7.2.6 Detection of reactive oxygen species

The potential to generate reactive oxygen species (ROS) was tested for the compounds which were detected to be positive in the MN assay (NT, NE, Nor-C, TB, THG, MAD, NA), slight positive (Nor-G) as well as two negative compounds (T, ETHI). The increase of ROS generation induced by the tested compounds relative to solvent control is depicted in Tab. 18.

Tab. 18 Increase in ROS generation relative to solvent control

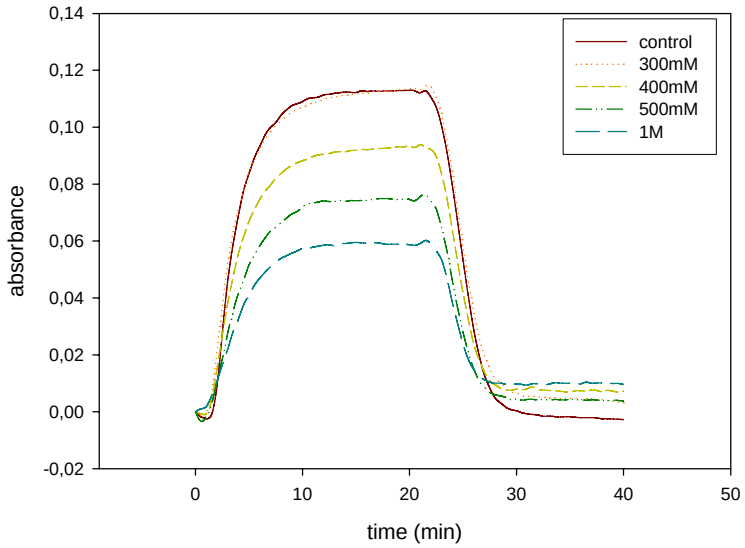
Compound	Concentration	ROS production after		
	(µM)	*30 min*	*1 h*	*18 h*
ETHI	10	0.96 (+/- 0.37)	0.96 (+/- 0.35)	0.81 (+/- 0.28)
	30	1.02 (+/- 0.38)	1.02 (+/- 0.38)	0.89 (+/- 0.33)
	100	1.09 (+/- 0.40)	1.07 (+/- 0.39)	0.81 (+/- 0.27)
MAD	3	0.99 (+/- 0.27)	0.92 (+/- 0.26)	0.81 (+/- 0.27)
	10	1.08 (+/- 0.34)	0.96 (+/- 0.35)	0.82 (+/- 0.28)
	30	1.02 (+/- 0.30)	0.96 (+/- 0.30)	0.74 (+/- 0.23)
NA	10	0.95 (+/- 0.29)	0.89 (+/-0.25)	0.87 (+/- 0.29)
	30	0.94 (+/- 0.40)	0.92 (+/-0.42)	0.87 (+/- 0.31)
	100	1.04 (+/- 0.38)	1.02 (+/-0.40)	0.87 (+/- 0.25)
NE	10	1.00 (+/- 0.38)	0.94 (+/-0.32)	0.92 (+/- 0.26)
	30	1.00 (+/- 0.39)	0.93 (+/-0.32)	0.91 (+/- 0.30)
	60	0.99 (+/- 0.38)	0.90 (+/-0.29)	0.91 (+/- 0.23)
NT	10	0.95 (+/- 0.41)	0.84 (+/-0.29)	1.03 (+/- 0.37)
	30	0.83 (+/- 0.28)	0.84 (+/-0.28)	1.02 (+/- 0.27)
	100	0.97 (+/- 0.37)	0.85 (+/-0.26)	1.03 (+/- 0.31)
Nor-C	10	1.01 (+/- 0.33)	0.93 (+/- 0.35)	0.97 (+/- 0.25)
	30	1.10 (+/- 0.31)	1.04 (+/- 0.36)	0.92 (+/- 0.25)
	100	1.06 (+/- 0.34)	1.00 (+/- 0.40)	0.90 (+/- 0.23)
Nor-G	1	1.04 (+/- 0.39)	1.01 (+/- 0.33)	0.81 (+/- 0.23)
	3	1.06 (+/- 0.39)	0.91 (+/- 0.36)	0.89 (+/- 0.26)
	10	0.93 (+/- 0.29)	0.92 (+/- 0.34)	0.89 (+/- 0.29)
T	10	0.97(+/- 0.26)	0.96 (+/- 0.27)	1.00 (+/- 0.25)
	30	1.05 (+/- 0.34)	1.01 (+/- 0.35)	1.03 (+/- 0.32)
	100	1.03 (+/- 0.30)	0.92 (+/- 0.28)	0.96 (+/- 0.29)
TB	10	1.03 (+/- 0.35)	1.04 (+/- 0.38)	0.84 (+/- 0.29)
	30	1.01 (+/- 0.36)	0.99 (+/- 0.34)	0.94 (+/- 0.29)
	100	0.96 (+/- 0.36)	1.00 (+/- 0.37)	0.92 (+/- 0.26)
THG	1	0.94 (+/- 0.34)	0.97 (+/- 0.35)	0.89 (+/- 0.28)
	3	1.03 (+/- 0.34)	1.07 (+/- 0.35)	1.04 (+/- 0.34)
	10	0.99 (+/- 0.27)	0.94 (+/- 0.34)	1.01 (+/- 0.36)

7.2.7 Microtubule assembly turbidity assay

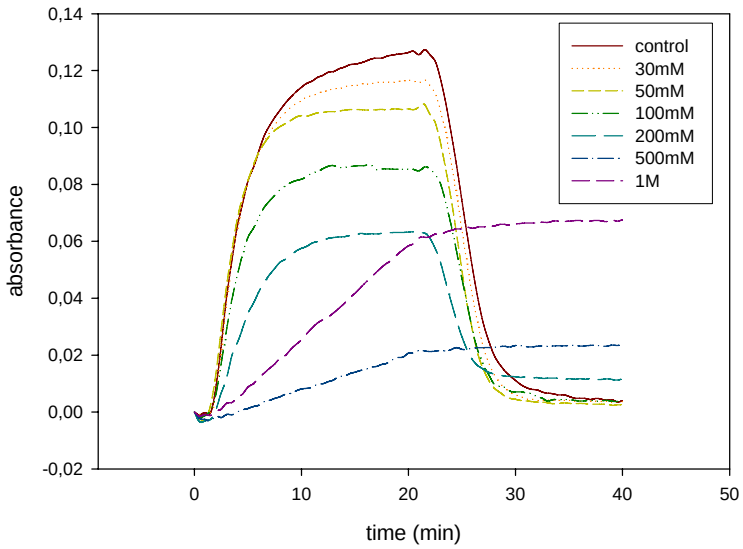
7.2.7.1. Results

The influence of a set of compounds (IMB, Jena) on the assembly-disassembly of microtubules was tested. The resulting absorbance curves resemble the dynamic microtubule assembly-disassembly process (Fig. 32 a-e).

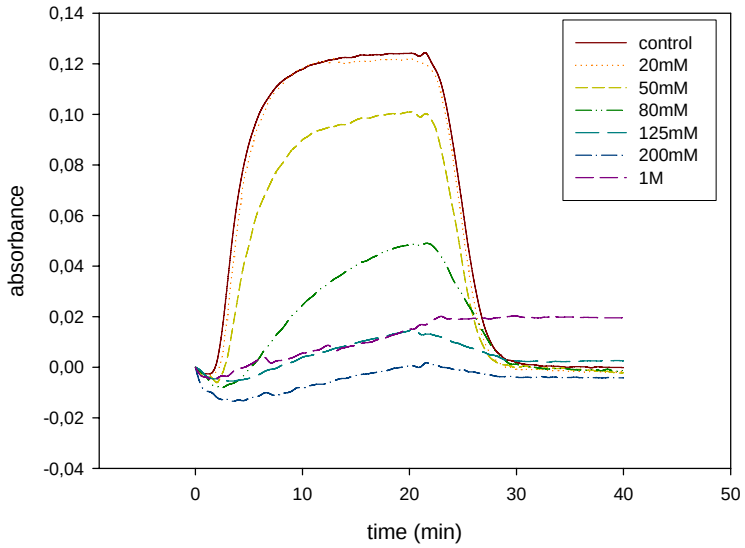
a)



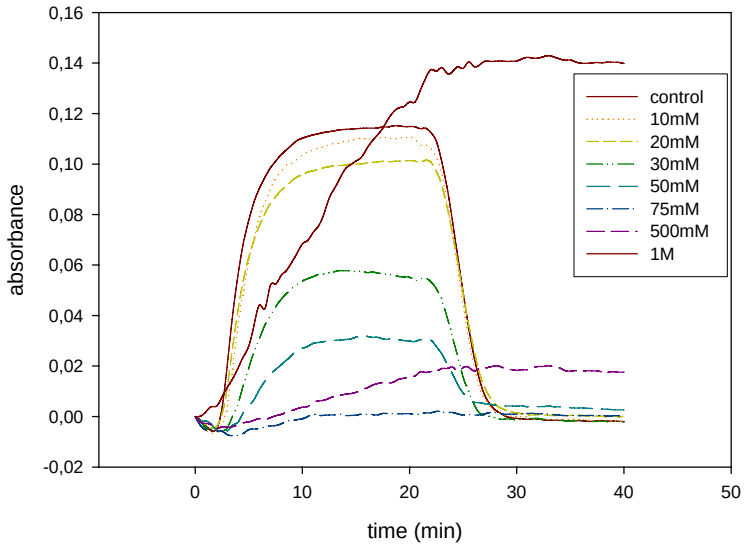
b)



c)


0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
-0,02
-0,04
absorbance
0
10
20
30
40
50
time (min)
control
20mM
50mM
80mM
125mM
200mM
1M

d)


0,16
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
-0,02
absorbance
0
10
20
30
40
50
time (min)
control
10mM
20mM
30mM
50mM
75mM
500mM
1M

e)

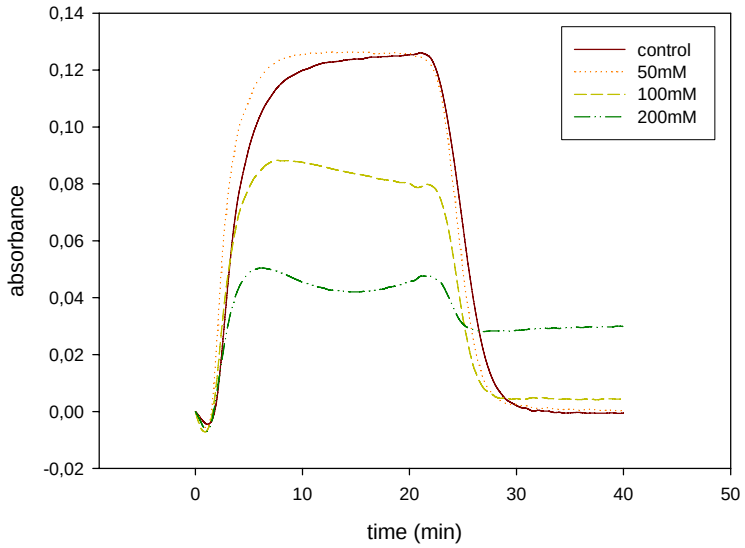


Fig. 32. Microtubule assembly assay with a) acetamide, b) acrylamide, c) acetonitrile, d) acrylonitrile, and e) MMS

The maximum microtubule assembly value (reached in the plateau) was taken to generate concentration-effect-curves (Fig. 33), from which the no-observed-effect-concentrations (NOECs) were determined.

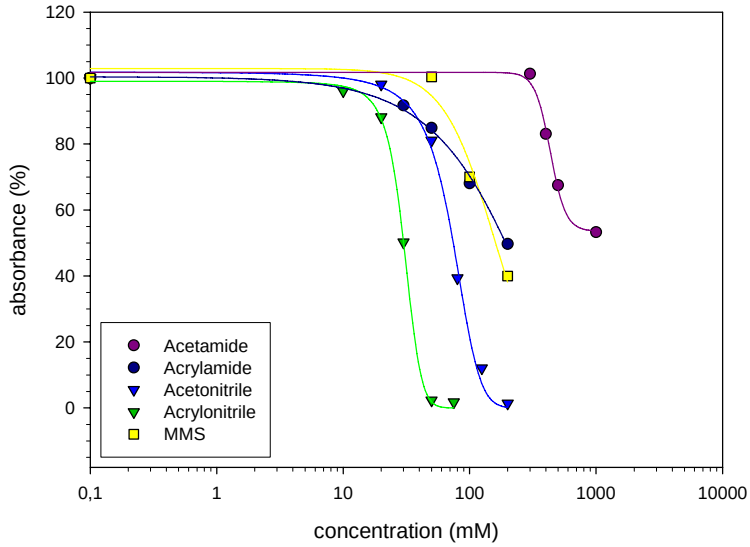
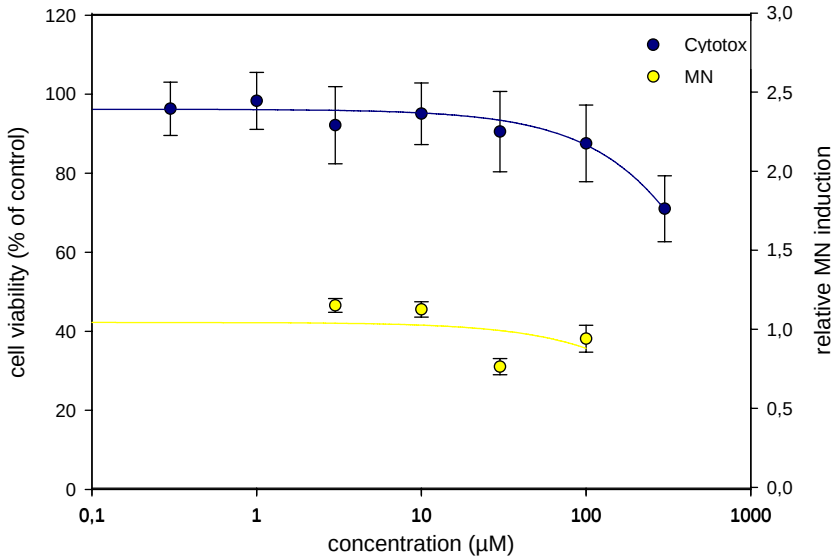


Fig. 33. Concentration-response-curves for microtubule assembly inhibition by acetamide, acrylamide, acetonitrile, acrylonitrile, and MMS.

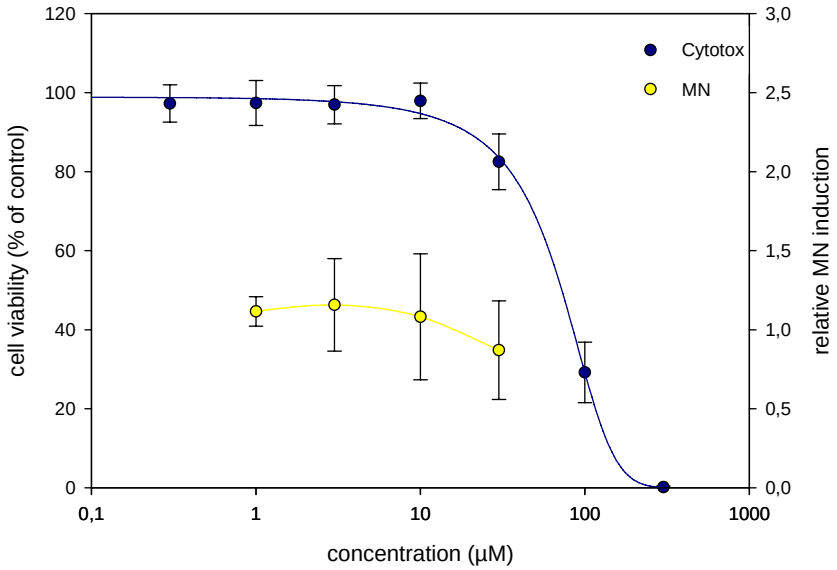
7.2.8 Cytotoxicity and genotoxicity – a comparison

A comparison of genotoxicity and genotoxicity of the steroids tested was done to deduce a classification, in which the compounds were grouped regarding their genotoxicity in presence or absence of cytotoxicity, respectively (see Chapter 4.3.1). The concentration-response-curves for class I compounds (no micronuclei increase, but detectable cytotoxicity) is given in Fig. 34 a-g. Those for class II compounds (increase of micronuclei in non-cytotoxic range (cytotoxicity < 20%) up to/ above 2x micronucleus background rate) are shown in Fig. 35 a-f; concentration-response-curves for class II a compounds (increase of micronuclei in non-cytotoxic range, then decrease, no 2x micronucleus background rate) are given in Fig. 36 a-b. Fig. 37 shows the curves for the class III compound Nor-C (increase of micronuclei only in presence of cytotoxicity (cytotoxicity ≥ 20%)).

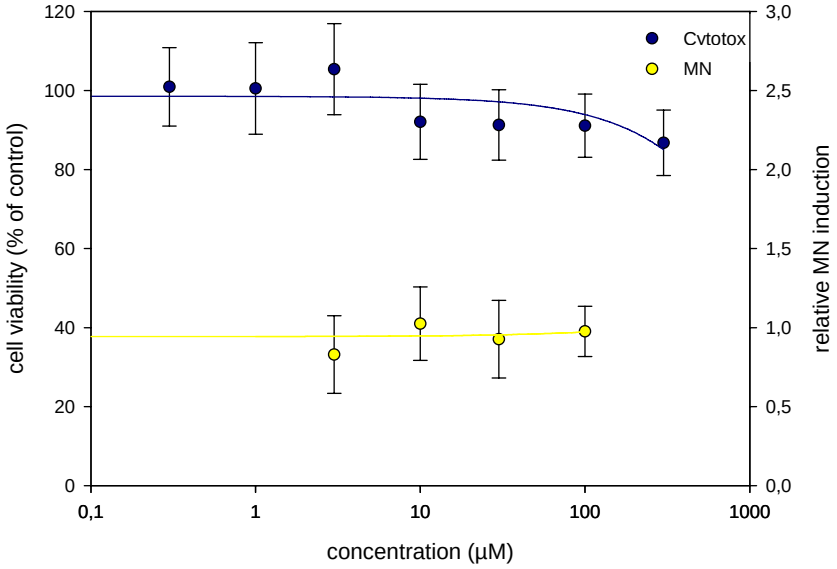
a)



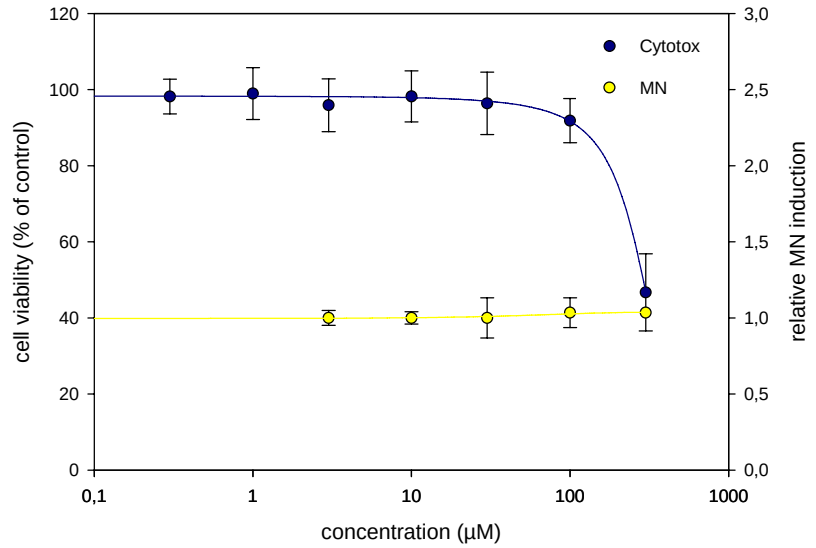
b)


Cytotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
0
20
40
60
80
100
120
0,0
0,5
1,0
1,5
2,0
2,5
3,0
0,1
1
10
100
1000

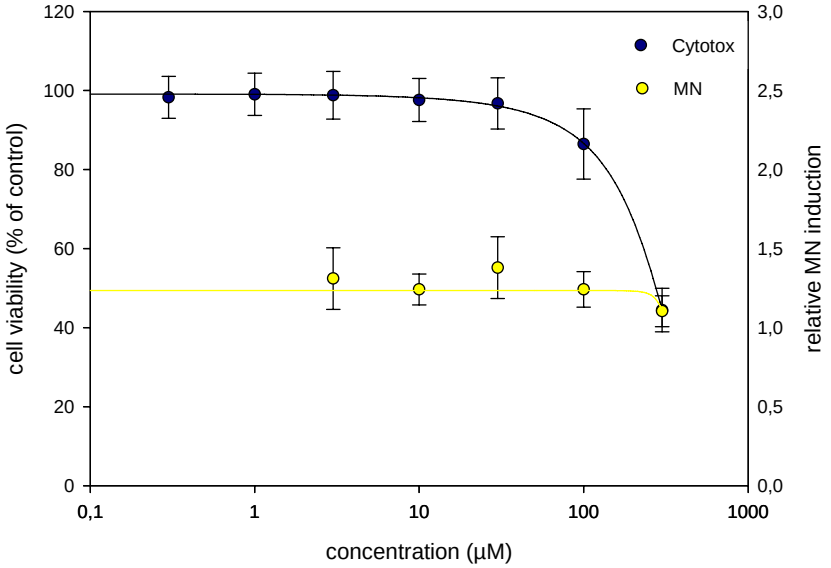
c)


Cvtotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
0
20
40
60
80
100
120
0,0
0,5
1,0
1,5
2,0
2,5
3,0
0,1
1
10
100
1000

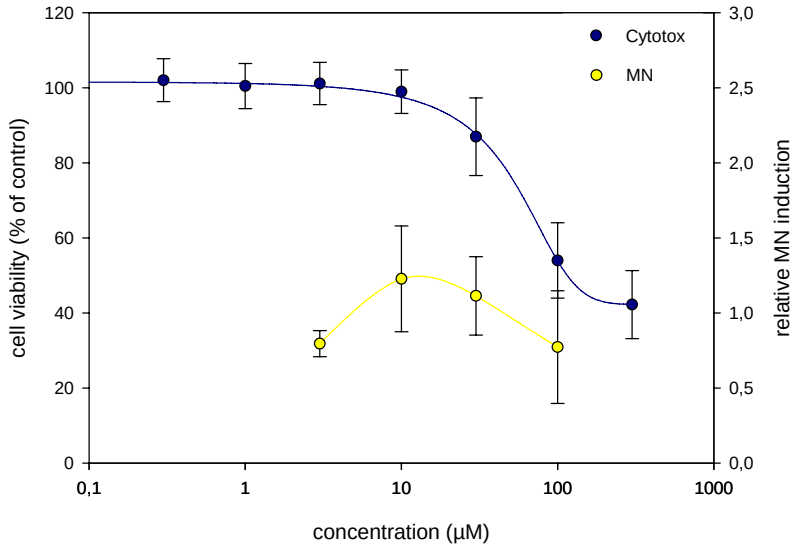
d)


Cytotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
120
100
80
60
40
20
0
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,1
1
10
100
1000

e)


Cytotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
120
100
80
60
40
20
0
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,1
1
10
100
1000

f)



g)

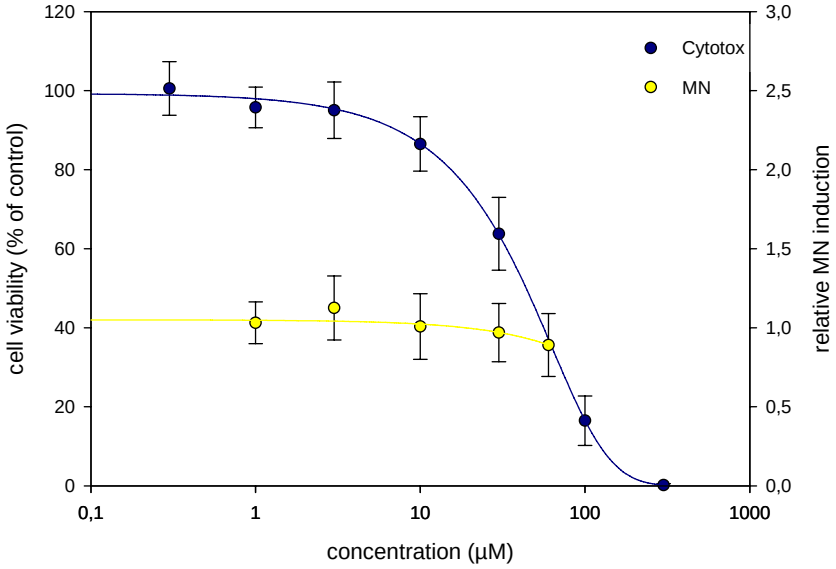
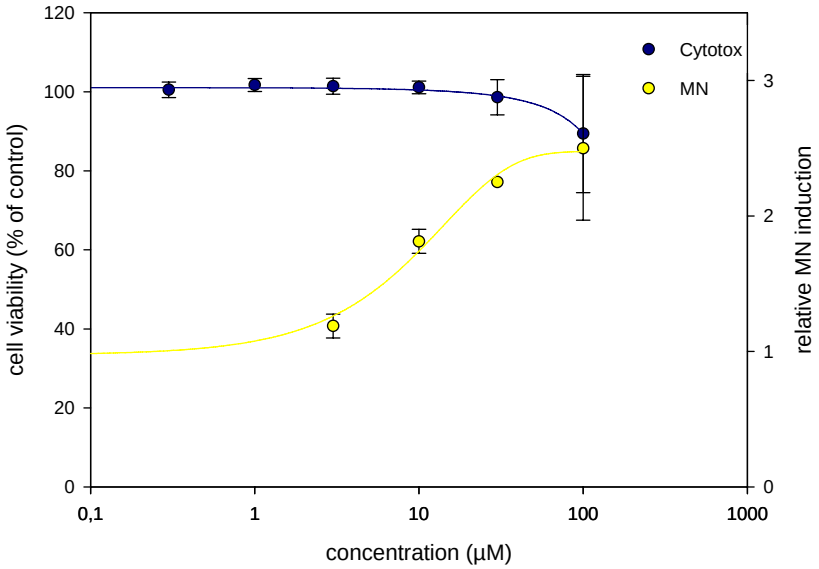
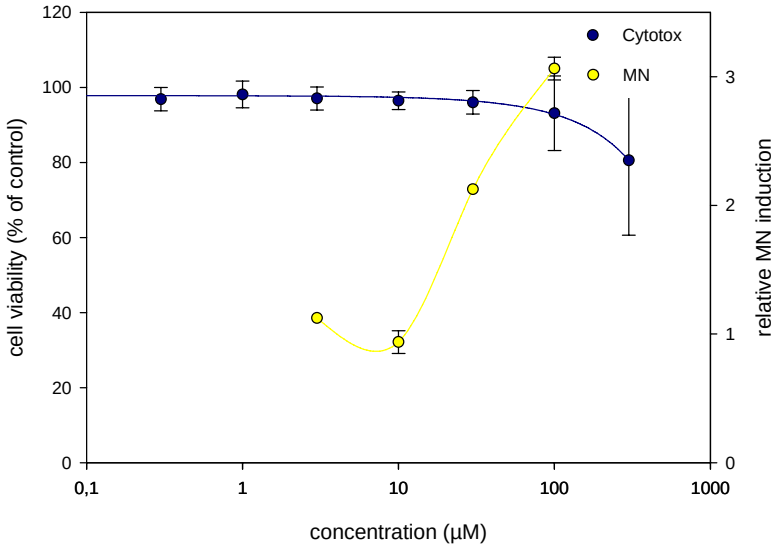


Fig. 34. Comparison of cytotoxicity and genotoxicity of class I compounds: a) MT (7α-methyltestosterone); b) PM (17α-propylmesterolone); c) ETHI (ethisterone); d) T (testosterone); e) Nor-D; f) Nor-E; g) Nor-F

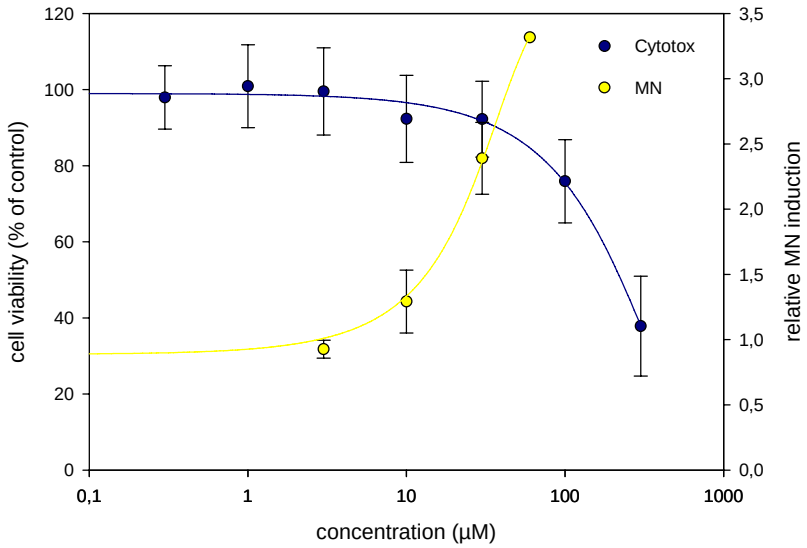
a)


Cytotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
120
100
80
60
40
20
0
3
2
1
0
0,1
1
10
100
1000

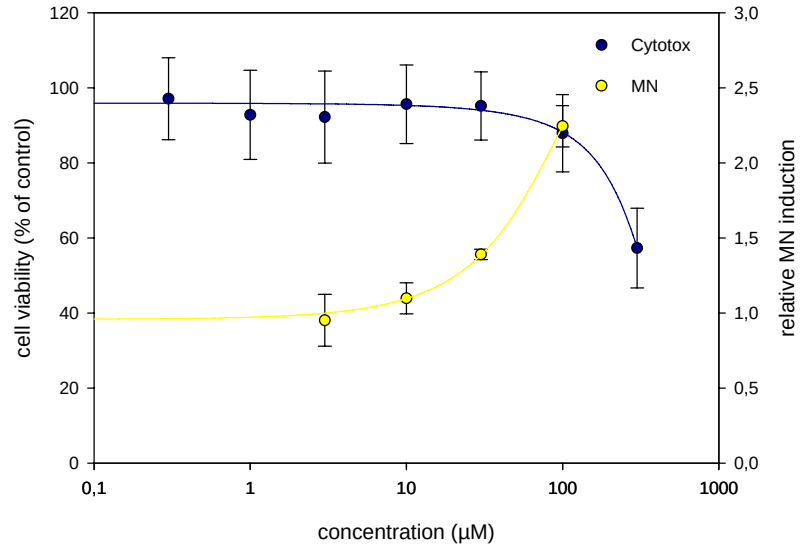
b)


Cytotox
MN
cell viability (% of control)
relative MN induction
concentration (µM)
120
100
80
60
40
20
0
3
2
1
0
0,1
1
10
100
1000

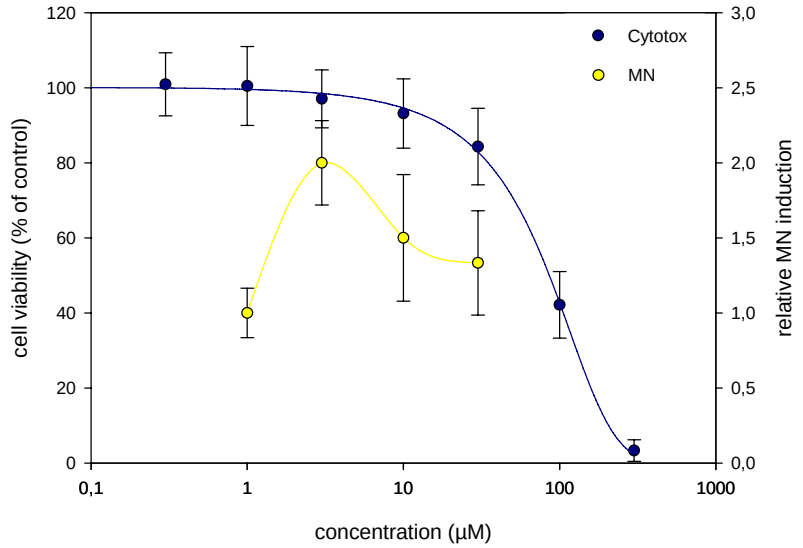
c)


cell viability (% of control)
relative MN induction
concentration (µM)
Cytotox
MN
120
100
80
60
40
20
0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,1
1
10
100
1000

d)


cell viability (% of control)
relative MN induction
concentration (µM)
Cytotox
MN
120
100
80
60
40
20
0
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,1
1
10
100
1000

e)



f)

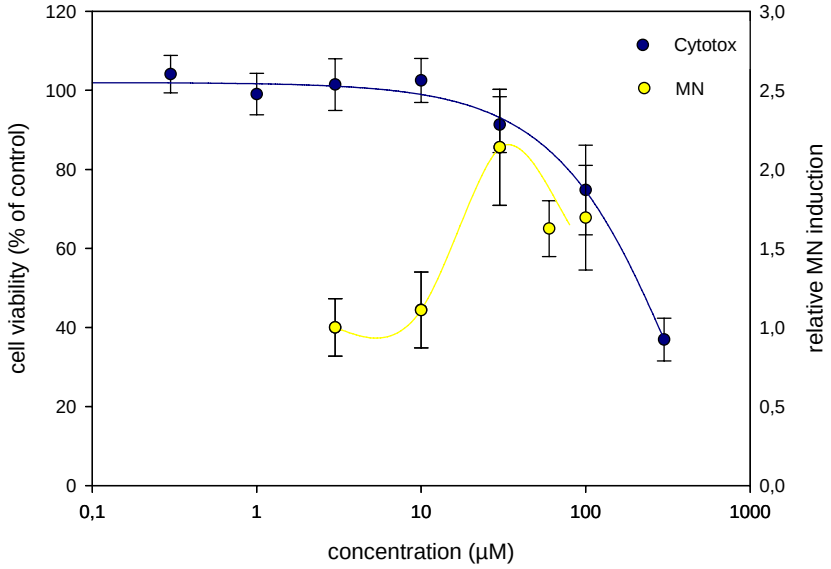
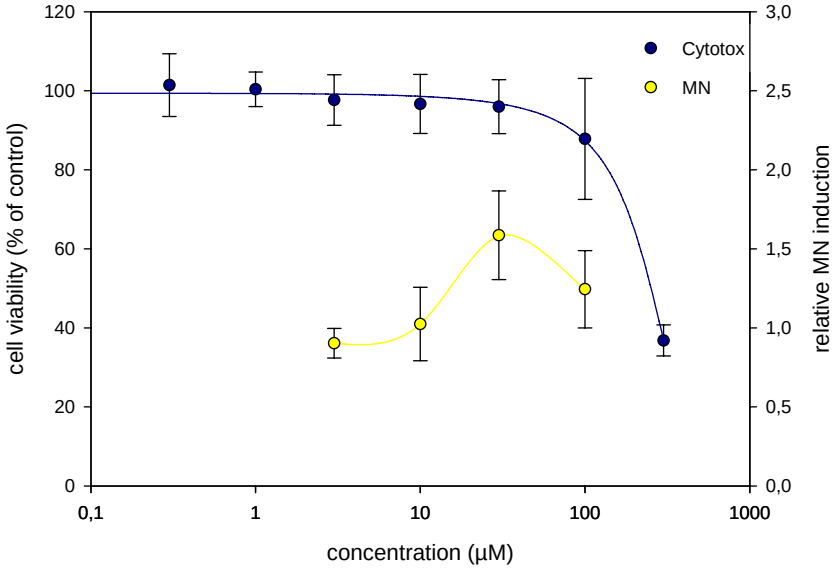


Fig. 35. Comparison of cytotoxicity and genotoxicity of class II compounds: a) MAD (madol); b) NA (19-norandrostenedione); c) NE (19-norethisterone); d) NT (19-nortestosterone); e) THG (tetrahydrogestrinone); f) TB (trenbolone)

a)



b)

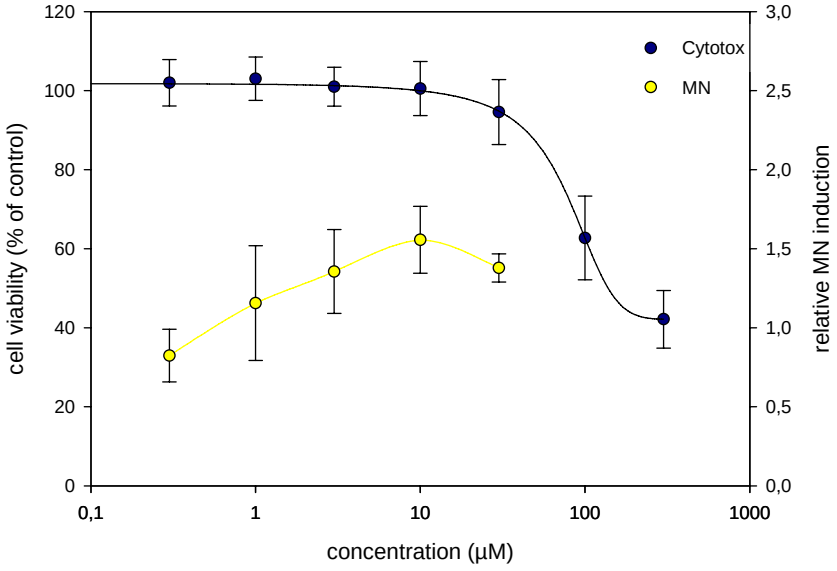


Fig. 36. Comparison of cytotoxicity and genotoxicity of class IIa compounds: a) ANDRO (androstenedione); b) Nor-G

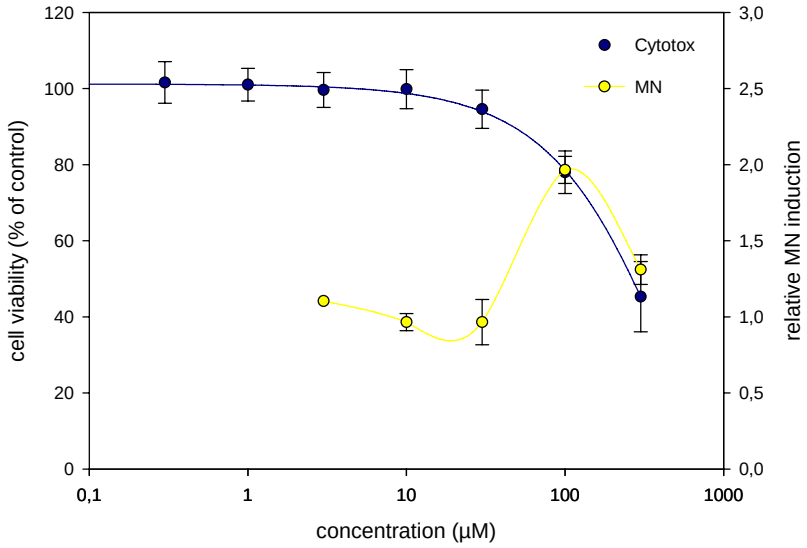


Fig. 37. Comparison of cytotoxicity and genotoxicity of the class III compound Nor-C

Acknowledgements

I wish to convey my special and sincere thanks to Prof. Dr. Dr. Hermann M. Bolt and Prof. Dr. Dr. Gisela H. Degen for giving me the possibility of pursuing this project at the Institut für Arbeitsphysiologie, University of Dortmund, for their incessant support, countless discussions, comments and ideas, and for their perpetual help.

Thanks are also due to Prof. Dr. Frank Wunderlich for the supervision of the thesis in the frame of the International Graduate College „Molecular Mechanisms of Food Toxicology“ at the Heinrich-Heine University of Düsseldorf.

I thank Prof. Dr. Hartmut Greven for his review of the thesis.

Thanks are due to Prof. Dr. Regine Kahl for affiliating me as collegiate of the International Graduate College „Molecular Mechanisms of Food Toxicology“ at the Heinrich-Heine University of Düsseldorf and for the dedicated hours, giving valuable motivation during the Graduate College Lectures.

The research work presented was financially supported by Organon, Oss, The Netherlands. The critical scientific input and numerous valuable discussions of Dres. Arijan Grootenhuis, Harrie F.P. Joosten, Jos P.M. Lommerse, Frederique A.A. van Acker, Diels van den Dobbelsteen, Jaap van der Louw, Tjong Yih (NV Organon) to this work are greatly acknowledged.

My very special thanks go to Anke Jung for her great help in the laboratory. To her, to Ingrid Ester and Martina Koch I am grateful for their support and for giving me a good time at the institute.

I am indebted to my IfADo (ex-)colleagues Alexander Bauer, Dr. Daniela Bonacker, Dr. Marc Brulport, Dr. Ana Laura Di Virgilio, Dr. Matthias Hermes, Christina Köhler, Juri Lektar, Dr. Stefan Moors, Jeannette Niestroy, Dr. Sabine Plöttner, Claudia Rudolph, Wiebke Schormann, Markus Schug, Sebastian Tschirbs, and Anna Zimmermann, who shared with me months and years giving support and help in different circumstances and facets.

Special thanks to Dr. Sabine Plöttner, as well as to my friends Nadine Lehan, Tina and Ronan Murphy for reading the manuscript of the thesis.

Thanks are due to all the members of the toxicology team at IfADo for the warm working atmosphere, the cooperative help, and for their open ears, especially of Brigitte Begher-Tibbe, Dr. Claudia Behm, Doris Dannappel, Beate Graf, and Silke Hankinson.

I thank my best friend Nadine Lehan for being my best friend!

Mein lieber Henning, Dir danke ich von Herzen für Deine ununterbrochene, verständnisvolle Geduld, die tägliche Motivation, Deine liebevolle Unterstützung in tausend Kleinigkeiten – und besonders für „the rainbow“!

Curriculum Vitae

Personal information

	Susanne Dorn
	Born December 5th, 1976, in Rottweil/Germany
	Diploma in human biology

Education

1997 – 2002	Studies in human biology, University of Greifswald Diploma thesis: „In-vitro Biotests zur Abschätzung der toxischen Potenz wässriger Systeme“
September 2002	Diploma in human biology, University of Greifswald
1983- 1996	Joseph-von-Eichendorff-Grundschule Rottweil, Albertus-Magnus-Gymnasium Rottweil, General qualification for university entrance

Work experience

Since October 2003	Collegiate at the International Graduate College „Molecular Mechanisms of Food Toxicology“ at the Heinrich-Heine-University Düsseldorf PhD candidate at the Institut für Arbeitsphysiologie an der Universität Dortmund, Projectgroup “Chemical toxicity”
Since March 2002	Science journalist (freelancer), *Laborjournal* and *Lab Times* (01/06) Merzhausen
February – April 2003	Internship at the German Cancer Research Centre, Heidelberg, Department of press and public relations
November 2002 – January 2003	Internship at *Laborjournal*, Merzhausen

October 2000 – September 2002	Student assistant at the Institute of Hygiene and Environmental Medicine, University Greifswald, Prof. Axel Kramer; data processing, toxicological profiling of antiseptics
March – May 2001	Internship at the German Cancer Research Center Heidelberg, Division of Immunogenetics, Prof. Peter H. Krammer; Molecular investigations for the characterization of caspase-8 splice variants
August – October 2000	Internship at the Finnish Environment Institute, Helsinki, Finnland, Division of Ecotoxicology Ecotoxicological investigations of hydrocarbon-contaminated soil
March 2000	Internship at Lysoform GmbH, Berlin Determination of formaldehyde residues in indoor air
August – September 1998	Nursing internship, Pulmonary Clinic, Hemer
August 1996 – September 1997	Work with children and animals, Hofgut Aryatara, breed of Spanish arabian horses, alpacas and llamas, Hardt

Further occupational training

Since 2004	Participation in the postgraduate education program "Fachtoxikologe" of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT)

Bochum, October 24th, 2007

I prepared the present dissertation independently and without any impermissible help. The thesis has not been submitted in the present form to any other institution. Hitherto, I have not performed unsuccessful efforts to get a doctoral degree.