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An investigation of micronucleus and mutation induction by oxazepam in mammalian cells

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The benzodiazepines are a class of drugs that are widely used in the treatment of various psychiatric disorders. One member of this class, oxazepam, is also a common metabolite of several other benzodiazepines. Since the evidence for the genetic toxicity and carcinogenic properties of these compounds is inconsistent, we investigated the oxazepam-induced formation of micronuclei in Syrian Hamster embryo fibroblast (SHE) cells, human amniotic fluid fibroblast-like (AFFL) cells and L5178Y mouse cells. A dose-dependent increase in micronucleus fractions was found in all three cell lines. The time course of micronucleus induction in L5178Y cells showed a maximum at 5 h after treatment, suggesting that the micronuclei were formed in the first mitosis after treatment. Kinetochore staining (CREST-antiserum) revealed the presence of kinetochores in ~50% of the micronuclei in all three cell types. This result was further confirmed by *in situ* hybridization in L5178Y cells and indicates the presence of whole chromosomes or centric fragments as well as acentric fragments in the oxazepam-induced micronuclei. The L5178Y cells did not show a mutagenic response to oxazepam at any of the doses or expression times used.

Introduction

Benzodiazepines make up a class of drugs that are widely used in the treatment of various psychiatric disorders. One member of this class, oxazepam (Figure 1), is a common metabolite of several benzodiazepines. An epidemiological study suggested an association between increased breast cancer and use of benzodiazepines in women (Stoll, 1976). However, this association was later discounted (Kleinerman *et al.*, 1984). Although there are many reports of fetotoxic and teratogenic effects in animals (for review, see Tucker, 1985), benzodiazepines have been used to treat psychiatric complications of pregnancy (Shannon *et al.*, 1972; Kanto, 1982). There have also been reports of increases in severe congenital abnormalities in infants whose mothers took such benzodiazepines as oxazepam during pregnancy (Milkovich and van den Berg, 1974; Laegreid *et al.*, 1989); however, reports to the contrary have also been published (Hartz *et al.*, 1975).

A number of rodent studies on the carcinogenicity of benzodiazepines have been performed. Induction of tumors was reported by Fox and Lahcen (1974; Swiss Webster mice), de la Iglesia (1981; CF1 mice and Wistar rats) and Robinson *et al.* (1984; CRCD rats and CRCD-1 mice). In a study on promotion, Remandet *et al.* (1984) found no evidence of increases in liver neoplasms or enzyme altered foci with benzodiazepines in F344 rats. Preat *et al.* (1987) reported that oxazepam possessed promotional activity in Wistar rats in two different assays for hepatocarcinogenesis. Diwan *et al.* (1986) found diazepam and oxazepam to be promotors of initiated liver tumors in mice. Recently, the National Toxicology Program reported clear evidence for increases in hepatocellular adenomas and carcinomas in both sexes of the B6C3F1 and Swiss Webster mouse, and increased hepatoblastomas in B6C3F1 mice (NTP, 1993).

After *in vivo* application, the most extensively studied benzodiazepine, diazepam, induced micronuclei in erythrocytes (and nucleated cells) from mouse bone marrow (Das and Kar, 1986). However, in other studies, it did not induce chromosomal aberrations in bone marrow cells from the mouse (Miller and Adler, 1989; Xu and Adler, 1990), the Chinese hamster (Schmid and Staiger, 1969) or the Sprague-Dawley rat (Ishimura *et al.*, 1975). Adler *et al.* (1991) could not find any evidence that diazepam induced micronuclei in erythrocytes from mouse bone marrow *in vivo*. In addition, no increased sister chromatid exchanges or chromosomal aberrations were observed in the lymphocytes of treated patients (Torigoe, 1979; Husum *et al.*, 1985). Diazepam also induced micronuclei in several cell systems *in vitro*. The compound produced a significant increase in frequency of CREST positive micronuclei in CI-1 Chinese hamster cells (Antoccia *et al.*, 1991) as well as in human fibroblasts and V79 Chinese hamster cells (Bonatti *et al.*, 1992). Lafi and Parry (1988) showed that diazepam induced chromosomal aberrations in cultured Chinese hamster cells. This result was not supported in three other studies (Ishidate *et al.*, 1978; Matsuoka *et al.*, 1979; NTP, 1993) in which no evidence of diazepam-induced chromosomal aberrations in Chinese hamster cells was found. Other *in vitro* effects include mitotic arrest in human fibroblast which were induced not by affecting the microtubules but by inhibiting the separation of centrioles (Andersson *et al.*, 1981), as well as mitotic arrest, multipolar spindles, lagging chromosomes and aneuploidy (Hsu *et al.*, 1983), and disruption of mitosis with concomitant chromosome loss in Chinese hamster cells (Parry *et al.*, 1986; Lafi *et al.*, 1987). Results of tests for induction of chromosomal aberrations and sister chromatid exchange in human fibroblasts (Staiger, 1969; Kawachi *et al.*, 1980; Sasaki *et al.*, 1980) or lymphocytes (Staiger, 1970; Zhurkov, 1975) *in vitro* were negative.

There are contradictory reports on the mutagenic activity of

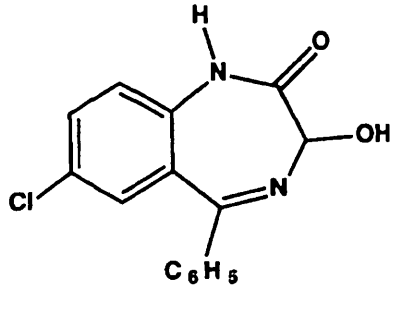

Fig. 1. Chemical structure of oxazepam.

oxazepam. The compound was found mutagenic in Salmonella strains TA100 and TA98 with metabolic activation (Batzinger *et al.*, 1978). Balbi *et al.* (1980) found no evidence for mutagenic activity of oxazepam in these strains as well as in TA1535 and 37, with and without metabolic activation.

The clear neoplastic response of B6C3F1 and Swiss Webster mouse livers to oxazepam (NTP, 1993) led us to investigate genetic toxicity endpoints that could perhaps elucidate possible mechanisms leading to this response. B6C3F1 mice typically develop a relatively high incidence of hepatic neoplasia and the application of this endpoint to predict carcinogenicity in humans is controversial.

In view of the widespread use of benzodiazepine medication, especially in children and during pregnancy, and the inconsistent results on the genetic toxicity of these compounds *in vitro* and *in vivo*, we investigated the ability of oxazepam to induce mutations at the *tk* locus of L5178Y mouse lymphoma cells and to induce micronuclei in L5178Y cells, Syrian hamster embryo fibroblast (SHE) cells and human amniotic fluid fibroblast-like (AFFL) cells. We further investigated the contents of the induced micronuclei by anti-kinetochore immunofluorescence staining as well as *in situ* hybridization.

Materials and methods

Chemicals

Bisbenzimide 33258, diethylstilbestrol (DES), ethyl methanesulfonate (EMS), hypoxanthine, methotrexate, oxazepam, thymidine, trifluorothymidine (TFT) and FITC-conjugated goat anti-human antibody were purchased from Sigma Chemie GmbH (Deisenhofen, FRG). Dimethyl-sulfoxide (DMSO) and formamide were acquired from Aldrich Company Europe (Steinheim, FRG) and CREST-Serum (anti-kinetochore antibody) was purchased from Antibodies Inc. (Davis, CA). Digoxigenin and the anti-digoxigenin detection system were acquired from Boehringer (Mannheim, Germany).

Cell culture

Mouse L5178Y cells, clone 3.7.2c (Clive, 1972), were used for micronucleus assays (including kinetochore analysis and *in situ* hybridization) and mutation assays. They were cultured in suspension in RPMI 1640 supplemented with 95 U/ml penicillin, 95 μg/ml streptomycin, 0.25 mg/ml L-glutamine, 107 μg/ml sodium pyruvate and 10% heat-inactivated horse serum (Sigma). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C. SHE cells were used for micronucleus assays (including kinetochore analysis). They were established as described previously (Schiffmann *et al.*, 1984). All experiments were performed with tertiary or quarternary cultures derived from 13 day old Syrian hamster embryos. The culture medium used was IBR-modified Dulbecco's reinforced medium (Gibco, Grand Island, NY), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 g/l $NaHCO_3$ and 15% fetal calf serum (Gibco, Eggenstein, FRG). Cell cultures were grown in a humidified atmosphere with 12% CO_2 in air at 37°C. AFFL cells (Hoehn and Salk, 1982) were used for micronucleus assays (including kinetochore analysis). They were taken from a mid-trimester pregnancy and cultured in DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 3.7 g/l $NaHCO_3$ and 10% FCS (Gibco, Eggenstein, FRG). Cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37°C.

In vitro micronucleus assay and kinetochore analysis

Treatment time was varied according to the different cell cycle durations of the cell lines used. For recovery in dose–response experiments, a time was chosen that only allowed one mitosis between treatment and fixation. Exponentially growing mouse L5178Y cells (cell cycle duration 10 h) were treated with oxazepam for 4 h. The vehicle control was 1% DMSO. After removing the chemicals by centrifugation and medium replacement, the cells were incubated for 5 h for dose–response experiments or for 0–25 h for time course experiments. The cells were then brought onto glass slides by cytospin centrifugation. Fixation was performed with methanol (−20°C, 1 h). SHE cells (cell cycle duration 16 h) were grown in culture flasks to medium density and treated for 5 h with oxazepam (dissolved in DMSO) or DMSO for 5 h. The final concentration of DMSO did not exceed 1%. After removing the chemicals by medium replacement the cells were incubated for 12 h. At that time the cells were trypsinized and brought onto glass slides by cytospin centrifugation. Fixation was performed with methanol (−20°C, 1 h). AFFL cells (cell cycle duration 24 h) were plated on glass coverslips in 35 mm Petri dishes and grown to medium density. Then the cell culture medium was replaced by culture medium containing oxazepam (diluted in DMSO) or DMSO (1%). Following an incubation period of 10 h the compound was removed by changing the medium. At 12 h later cells were fixed with methanol (−20°C, 1 h). For staining of nuclei and micronuclei, the slides or coverslips were washed with distilled water, incubated with bisbenzimide 33258 (1 μg/ml, 5 min), washed three times with distilled water and mounted for microscopy. Kinetochore staining was achieved by incubating the fixed cell preparations [after rinsing with phosphate buffered saline (PBS)] with CREST serum (60 min) in a humidified chamber at 37°C. After rinsing with PBS again, the cells were incubated as before with FITC-conjugated goat anti-human antibody (diluted 1:100 in PBS), rinsed again and counterstained with bisbenzimide 33258 (1 μg/ml, 5 min). Using a magnification of 1250× the number of nuclei and micronuclei were scored. Each data point represents the mean of three slides with 2000 nuclei evaluated per slide. The synthetic estrogen DES was used to illustrate the effects of an aneugen.

In situ hybridization

Mouse L5178Y cells were centrifuged onto glass slides (cytospin) and dehydrated in an ethanol series (70, 80, 95, 100%, 2 min each) after fixation in cold methanol:acetic acid (3:1, −20°C, 30 min). Prior to hybridization slides were immersed in 70% formamide/2×SSC (pH 7.0, 2 min, 70°C) to denature the DNA. Subsequently, they were again dehydrated as described above. For hybridization, cells were incubated in 50% formamide/2×SSC/10% dextran sulfate, containing 20 ng of digoxigenated DNA probe. A mouse minor satellite probe that binds to or near to the kinetochore domain of every mouse chromosome (Wong and Rattner, 1988) was used for detection of centromeric DNA regions in these cells. Hybridization was performed overnight in a humidified (2×SSC) chamber at 37°C. Immunochemical detection was carried out using a fluorescent FITC–anti-digoxigenin conjugate (Boehringer). Whole DNA was counterstained with bisbenzimide 33258 (1 μg/ml, 5 min) and the slides were evaluated for the presence of centromeric DNA signals in micronuclei with fluorescence microscopy.

Mutation assay

Cultures of mouse L5178Y cells were treated with methotrexate before each experiment to kill pre-existing TFT-resistant cells. To accomplish this, cells were incubated for 24 h in culture medium plus methotrexate (0.3 μg/ml), thymidine (9 μg/ml), hypoxanthine (15 μg/ml) and glycine (22.5 μg/ml). The cells were then incubated for at least 48 h in the same medium without methotrexate. To measure chemically induced mutations using the *in situ* procedure (Rudd *et al.*, 1990; Spencer *et al.*, 1993), duplicate cultures containing 6×10^6 cells in 10 ml medium were treated with DMSO (control) or oxazepam (dissolved in DMSO). EMS (300 μg/ml) was used as a positive control. Incubation was performed for 4 h in a rotating drum, then the cells were washed twice with fresh medium. From each tested culture. 0.5×10^6 cells were added to 50 ml of semisolid culture medium (containing 0.25% granulated agar, Baltimore Biological Laboratories, Baltimore, MD) and plated into two plastic 100 mm culture dishes and allowed to solidify at room temperature. TFT-resistant cells were selected by adding an overlay of TFT (final concentration 8 μg/ml) in semisolid medium after an appropriate expression time. Cloning efficiency was determined by adding 600 cells to 100 ml of semisolid medium in three 100 mm culture dishes. All plates were incubated for a total of 11–12 days at 37°C in 5% CO_2 for colony growth. An automatic colony counter was used to count the number of TFT-resistant colonies. The counter detected colonies larger than 0.25–0.3 mm diameter (Model 982B; Artek Systems, Farmingdale, NY).

Results

Oxazepam (Figure 1) induced the formation of micronuclei in a dose dependent manner in three different mammalian cell types (Figures 2–5). In mouse L5178Y cells, the micronucleus fraction increased up to a concentration of 200 μg/ml oxazepam (Figure 2). At the next higher dose tested (250 μg/ml), we observed fragmentation of nuclei which made evaluation of cells for micronucleus induction impossible. Oxazepam did not affect Trypan blue exclusion under micronucleus assay conditions up to a concentration of 200 μg/ml (data not shown). In SHE cells, the micronucleus fraction reached a maximum at 100 μg/ml oxazepam and decreased at higher concentrations (Figure 3). In AFFL cells, the micronucleus fraction increased up to a concentration of 250 μg/ml oxazepam (Figure 4). Higher concentrations were not applied, since microscopically visible crystals due to limited solubility of oxazepam appeared in the culture medium at concentration of 200 μg/ml and higher. When we examined the time course of micronucleus induction in

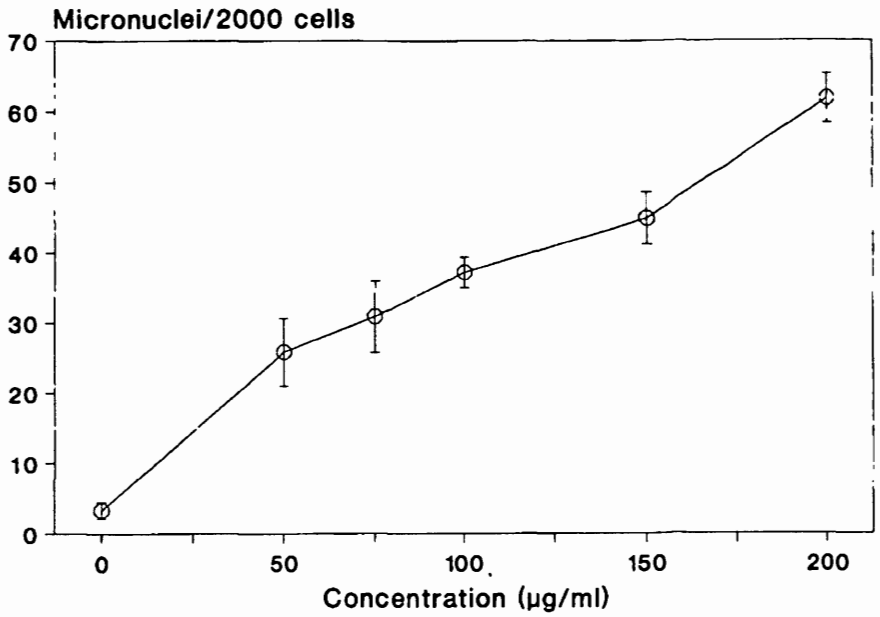

Fig. 2. Induction of micronucleus formation in L5178Y cells. Cells were treated with oxazepam for 4 h and evaluated 5 h later.

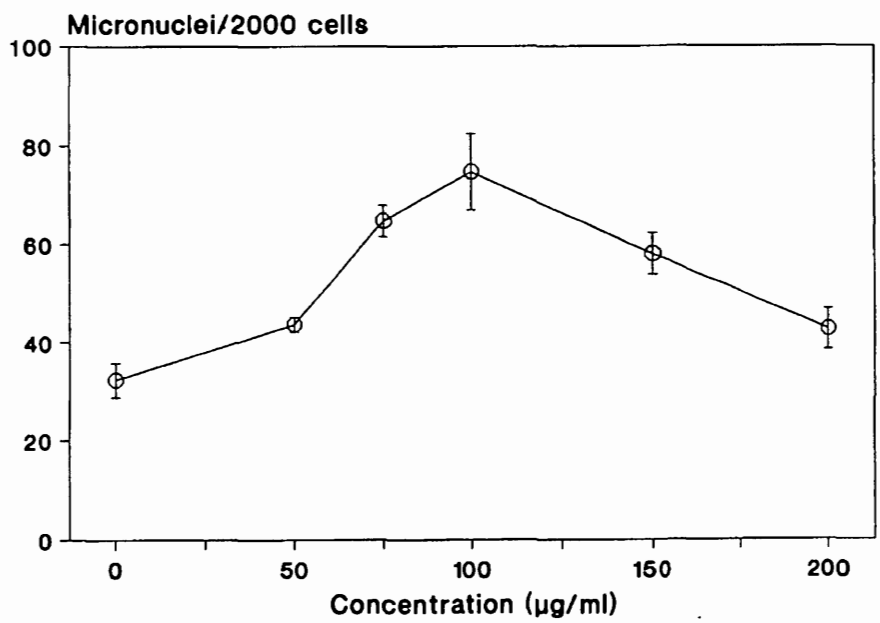

Fig. 3. Induction of micronucleus formation in SHE cells. Cells were treated with oxazepam for 5 h and evaluated 12 h later.

L5178Y cells, the fraction of micronuclei was maximal 5 h after the oxazepam treatment (Figure 5).

Anti-kinetochore staining of micronuclei (Table I) revealed that 41–61% of the oxazepam induced micronuclei in all three cell types contained a signal, compared with 22–42% in the vehicle controls. The aneugenic compound DES also induced micronuclei and 77–85% were stainable for kinetochore protein in mouse L5178Y and SHE cells. Kinetochore staining was carried out under optimal conditions which were determined for each cell type and were derived from the dose–response and time course experiments of micronucleus induction and the known cell cycle times (10 h for mouse L5178Y cells, 16 h for SHE cells and 24 h for AFFL cells).

The staining of centromeric DNA was achieved by *in situ* hybridization of mouse L5178Y cells with mouse minor satellite DNA. This DNA binds to the kinetochore region of the chromosomes. After a 4 h treatment and fixation 5 h later (Table II), 47% of the 200 µg/ml oxazepam-induced micronuclei showed a signal, whereas 28% of the spontaneous micronuclei and 82% of the 10 µg/ml DES-induced micronuclei contained stainable centromeric DNA.

We examined mouse L5178Y cells for their mutagenic response to oxazepam. This cell line is heterozygous for the thymidine kinase gene and the active allele can be inactivated by mutational events and render the cell resistant to TFT. In the assay used here (Rudd *et al.*, 1990; Spencer *et al.*, 1993) every surviving mutant cell forms a single colony due to cloning in semisolid agar immediately after exposure to the chemical. The

selecting agent, TFT, is then added after appropriate expression times. The positive control EMS at 300 μg/ml yielded a 9-fold increase in mutation fraction (Tables III and IV) compared to the vehicle control. However, oxazepam did not show a significant increase in TFT resistance over the vehicle control at any of the doses (50–200 μg/ml) examined (Table III). After different expression times, the positive control EMS (300 μg/ml) showed a time dependent increase in mutation fraction (Table IV), but oxazepam at 200 μg/ml did not show any significant increase.

Discussion

The benzodiazepine oxazepam induced the formation of micronuclei in the three cell types derived from three different mammalian species: Mouse (L5178Y), Syrian hamster (SHE) and human (AFFL). In mouse L5178Y and AFFL cells, the micronucleus fraction increased linearly with increasing dose up to 200 or 250 μg/ml, depending on the cell line used. In SHE cells, a maximum was reached at 100 μg/ml and higher doses resulted in decreased values. This could be due to an increased sensitivity of these cells to irreversibility of cell cycle arrest which would render the cells countable but unable to undergo mitosis and produce micronuclei.

Since the peak in the oxazepam-induced micronucleus fraction occurred 5 h after treatment, the induction of micronuclei by oxazepam is likely to occur during the course of the first mitosis after treatment. The kinetics of micronucleus induction by oxazepam was similar to the one observed after treatment with

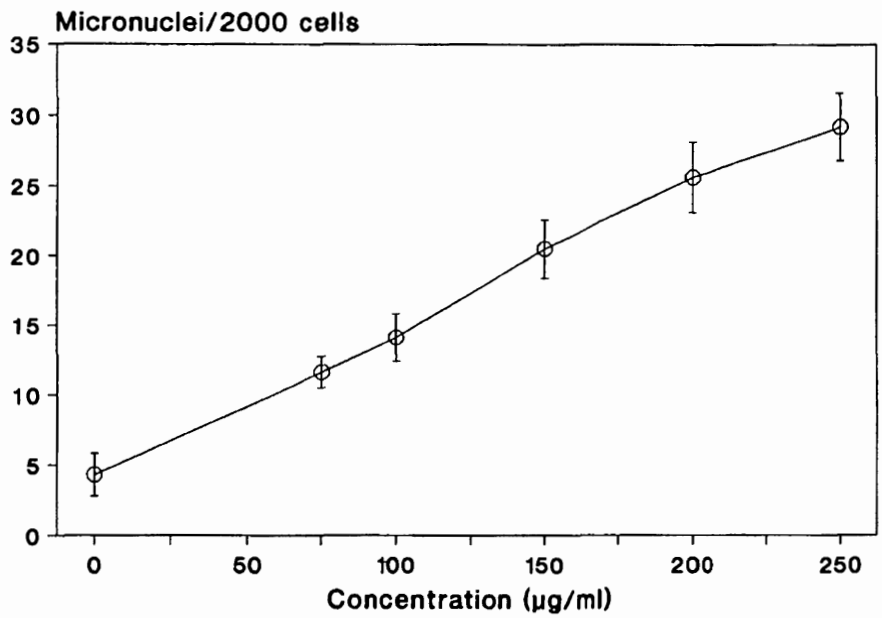

Fig. 4. Induction of micronucleus formation in AFFL cells. Cells were treated with oxazepam for 10 h and evaluated 12 h later.

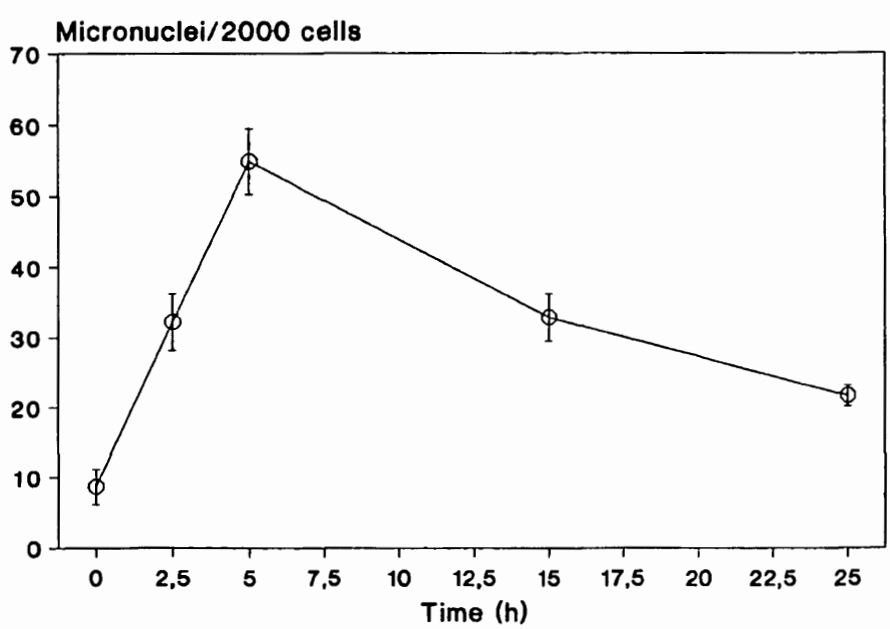

Fig. 5. Time course of micronucleus induction in L5178Y cells. Cells were treated for 4 h with 200 μg/ml oxazepam and evaluated at the indicated times after that. The spontaneous (background) micronucleus fraction was 3.32 ± 1.14 micronuclei/2000 cells in this experiment.

the aneuploidy-inducing estrogen DES (H.Stopper *et al.*, manuscript in preparation). The mechanism of micronucleus induction by DES is known to operate during the course of mitosis (Schiffmann and DeBoni, 1991). Hsu *et al.* (1983) also showed that diazepam induced mitotic disturbances (metaphase arrest during treatment, lagging chromosomes in anaphase and multipolar spindles after treatment) in Chinese hamster cells. These authors also showed moderate aneugenic properties of diazepam. Mitotic disturbances with concomitant chromosome loss were also reported by Parry *et al.* (1986) and Lafi *et al.* (1987) in Chinese hamster cells. When we applied supravital UV microscopy according to the technique of Schiffmann and DeBoni (1991), we found an increased number of metaphases during the treatment of SHE cells with 100 μg/ml oxazepam. Shortly after the treatment, the cells went through mitosis, indicating the reversibility of the arrest. There was also an increased number of cells in telophase showing narrow chromatin bridges (unpublished observations).

Substances that interfere with the course of mitosis can lead to the loss of whole chromosomes (aneuploidy). To investigate whether oxazepam-induced micronuclei contained whole chromosomes, we applied CREST-staining for detection of kinetochores. About half of the oxazepam induced micronuclei stained positive in all three cell types, whereas DES yielded ~80% kinetochore-positive micronuclei. Therefore, in addition to its aneugenic properties, oxazepam exerts some clastogenic activity. Because of the early appearance of these micronuclei, chromosomal breakage must occur during the first mitosis after treatment. Since it is possible that kinetochore staining may give erroneous results when the kinetochore protein is damaged (Miller *et al.*, 1991), we hybridized mouse minor satellite DNA to mouse L5178Y cells *in situ*. The results verified our kinetochore data. Bonatti *et al.* (1992) found 65% of diazepam induced micronuclei kinetochore-positive in three human and Chinese hamster cell types and Antoccia *et al.* (1991) showed the presence of kinetochores in 75% of diazepam induced micronuclei in Chinese hamster cells.

Even though diazepam also seems to have some clastogenic effect, the clastogenic effect of oxazepam accounts for half of the induced micronuclei. It should be noted that, even though the induction of micronuclei by diazepam in cultured cells *in vitro* had been demonstrated by two groups (Antoccia *et al.*, 1991; Bonatti *et al.*, 1992), the induction of micronuclei *in vivo* could not be demonstrated in erythrocytes of the mouse bone marrow (Adler *et al.*, 1991). However, according to findings by Das and Kar (1986) micronuclei were elevated in the mouse bone marrow assay.

Oxazepam shows no consistent mutagenic activity. The mutagenicity of oxazepam has been investigated in Salmonella (Ames test) with variable results. Batzinger *et al.* (1978) found it to be mutagenic, whereas Balbi *et al.* (1980) concluded that it was non-mutagenic. We therefore investigated the mutagenic potential of this compound in mouse L5178Y cells at the *tk* locus. Oxazepam did not show a significant increase of mutants at any of the doses or expression times tested. In this context, an

Table I. Presence of kinetochores in micronuclei

Cell type	Oxazepam concentration (μg/ml)	No. of evaluated micronuclei	% K^+
L5178Y	0	100	22
	200	300	53 ± 4.6
	DES (10 μg/ml)	300	77 ± 4.2
AFFL	0	120	42
	200	300	41 ± 4.4
SHE	0	300[a]	23 ± 7
	100	120	47
	DES (13.4 μg/ml)	300[a]	85 ± 3

Presence of kinetochores in micronuclei (K^+) of three different cell lines. Experimental conditions: 4 h treatment and evaluation 5 h after that for L5178Y cells, 10 h treatment and evaluation 12 h after that for human amniotic fluid cells, 5 h treatment and evaluation 12 h after that for SHE cells.
[a]Data taken from Stopper *et al.* (1992).

Table II. Presence of centromeric DNA in micronuclei

Compound	Concentration (μg/ml)	No. of evaluated micronuclei	% C^+ (no. C^+)
Control (DMSO)	(1%)	75	28 (21)
Oxazepam	200	100	47
DES	10	100	82

Presence of centromeric DNA (*in situ* hybridization signal % C^+) in micronuclei of L5178Y cells. Cells were treated with the chemicals for 4 h and evaluated 5 h after that.

Table III. Dependence of mutation fractions on substance concentration

Substance	Concentration (μg/ml)	CE	MnF	Relative MnF
Control (1% DMSO)	0	0.72	230	1.0
EMS	300	0.66	2120	9.2
Oxazepam	50	0.73	232	1.0
	100	0.54	332	1.4
	200	0.49	362	1.6
	250	0.47	344	1.5

Mutation experiments were performed with mouse L5178Y cells. Each number given is the average from two independent cultures treated in one experiment. Expression time is the time between the end of substance treatment and TFT addition; expression time was 47.5 h. A dose–response curve was also generated at an expression time of 41.5 h and no mutagenic response was observed. CE is cloning efficiency, MnF is mutation fraction and RMnF is the mutation fraction relative to the control.

Table IV. Dependence of mutation fractions on expression time

Expression time (h)	Control (1% DMSO)		EMS (300 μg/ml)		Oxazepam (200 μg/ml)	
	MnF	RMnF	MnF	RMnF	MnF	RMnF
4	12	1.0	11	0.9	8	0.7
23.5	23	1.0	83	3.7	23	1.0
28	30	1.0	198	6.6	28	0.9
45	105	1.0	1130	10.8	116	1.1
52	168	1.0	1154	6.9	199	1.2
69.5	478	1.0	2049	4.3	451	0.9

See Table III for explanation.

observation by Seredenin *et al.* (1986) is interesting. They reported an antimutagenic effect of benzodiazepines and attributed it to their possible radical scavenger properties.

In our studies oxazepam was found to induce genetic damage as shown by the micronucleus assay. It is not known whether the loss of chromatin material enclosed in micronuclei can result in the expression of a mutant phenotype. However, since the mouse L5178Y mutation assay could not detect this type of damage, it may be advisable to combine both assays for future genotoxicity testing.

The doses necessary for micronucleus induction are rather high for oxazepam and would be equivalent to several grams of intake per average person. The induction of mutation in mammalian cells could not be demonstrated here. However, the use of low but chronic doses of benzodiazepines may also present a risk to human health. Therefore, further investigations seem appropriate to further clarify the effect of benzodiazepines on the human organism.

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