In vitro genotoxicity of two widely used benzodiazepines: alprazolam and lorazepam

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ABSTRACT: Alprazolam (AZ) and Lorazepam (LZ) belong to benzodiazepines, a multi-membered group of biologically active substances. Even though they are widely used as drugs for the relief of anxiety, sedation and in the treatment of epilepsy, knowledge about their cytogenetic activity is limited.

Materials and Methods: In the present study the cytotoxic and cytostatic actions of AZ and LZ have been evaluated in normal human lymphocyte cultures of peripheral blood at final concentrations (0.16-3.84 μM for AZ and 0.62-3.72 μM for LZ) equivalent to oral dosage (for AZ 0.25-6 mg/day and for LZ 1-6 mg/day), employing Sister Chromatid Exchanges (SCEs), one of the most sensitive methods reflecting instability of DNA or a deficiency in DNA repair mechanisms, and Proliferation Rate Index (PRI), a valuable indicator of cytostatic activity.

Results: After 72h incubation in the cultures, both AZ and LZ caused a dose-dependent, statistically significant increase of SCE frequency (p < 0.001) followed by a statistically significant decrease of PRI (p < 0.001) of lymphocytes.

Conclusions: Our results suggest that AZ and LZ at oral doses exhibit statistically significant genotoxicity in normal human lymphocyte cultures.

Key Words: Benzodiazepines, Lorazepam, Alprazolam, Sister Chromatid Exchanges, Proliferation Rate Index, Cytogenetic effect.

INTRODUCTION

Benzodiazepines are a group of psychoactive drugs with anxiolytic (anti-anxiety), sedative, hypnotic, anticonvulsant, muscle relaxant and amnesic properties. These drugs are used in the symptomatic relief of anxiety and tension states resulting from a stressful environment or emotional factors. They are also useful in psychoneurotic states, panic attacks and alcohol withdrawal. Benzodiazepines produce their effects by binding to a specific site on the GABA receptor (gamma-aminobutyric acid, an inhibitory neurotransmitter) causing an allosteric change to it. This results in increasing the receptor’s affinity for GABA and leads to the depression of the central nervous system.

Alprazolam (AZ) [8-chloro-1-methyl-6-phenyl-4H-(1,2,4) triazolo [4,3a] [1,4] benzodiazepine] is a short-acting benzodiazepine used to treat moderate to severe anxiety disorders, panic attacks and as an adjunctive treatment for anxiety associated with moderate depression. Lorazepam (LZ) [7-chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-1,4-benzodiazepine-2-one] is a short-to-medium-acting benzodiazepine, which is principally used in the treatment of anxiety and transient situational stress.

One of the first benzodiazepines, diazepam, was found to present mutagenic and genotoxic effects in bone marrow cells of mice and aneuploidy in cultured mammalian cells, mitotic/meiotic arrest, structural aberrations and micronuclei in various in vivo and in vitro assays. However, the results from in vitro and in vivo studies of diazepam effects on human chromosomes are conflicting. In previous studies, we showed that diazepam is cytogenetically active evaluating the Sister Chromatid Exchanges (SCEs) and Proliferation Rate Index (PRI) in normal human lymphocyte cultures. Despite their extended use, cytogenetic effects

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of both AZ and LZ have not been studied as yet in humans. We decided, therefore, to investigate the cytotoxic and cytostatic activity of AZ and LZ in normal human lymphocyte cultures. In order to determine their influence on genetic stability and/or subsequent DNA repair (cytotoxic effect), we estimated the frequency of SCEs. The antiproliferative activity (cytostatic effect) was measured using the PRI values.

The consequences of the exposure to potential mutagens/carcinogens have been commonly studied in humans by cytogenetic methods and positive responses should be regarded as indicators of a genotoxic hazard. There are some sensitive biomarkers of genotoxicity such as chromosomal aberrations, comet assay, micronuclei, but SCE frequency has been proved as one of the most sensitive indices among them. SCEs can provide insight in the cytogenetic damage induced by various genotoxic agents at very low concentrations. The determination of PRI in lymphocyte cultures has been proved to be a very useful and sensitive indicator of the cytostatic action of various environmental hazards or therapeutic agents.

MATERIALS AND METHODS

In vitro SCE and PRI assays

Blood from two different healthy donors, 25-35 years old, non-smokers, not receiving any drugs, not consuming considerable quantities of alcohol or not having suffered from any kind of infection for the last 15 days, was used for two different experiments.

Human lymphocyte cultures were prepared by adding in 5ml chromosome medium (RPMI-1640, Biochrome, Berlin) at the beginning of culture life, the following:

- 11-12 drops of normal human heparinized whole blood
- 5µg/ml 5-Bromodeoxyuridine (BrdU) and
- the solutions (final concentrations per culture) of AZ (0.16, 0.32, 0.64, 1.28, 2.56, 3.84 μM) and LZ (0.62, 1.24, 1.86, 2.48, 3.1, 3.72 μM)

The concentrations of the drugs used were equivalent to the oral doses. The cultures were incubated at 37°C for 72 hours, in the dark to minimize photolysis of BrdU. Colchicine (0.3µg/ml) was added 2h before the collection of the cultures. The cells were then collected by centrifugation and exposed to 0.075 M KCl for 10 minutes. The hypotonic solution spreads the chromosomes and hemolyses the red blood cells. The pellet was fixed three times with methanol:acetic acid (3:1). Drops of concentrated suspension of cells were placed on microslides that allowed to air dry. For SCEs and PRI analysis, the slides were stained by a modification of the Fluorescence Plus Giemsa procedure to obtain harlequin chromosomes.

Statistical analysis

For SCEs more than 20 suitably spread 2nd division cells from each culture were blindly scored. For PRI at least 100 cells in the 1st, 2nd, 3rd and higher divisions from each culture were blindly scored. PRI=M1+2M2+3M3+/100, where M1, M2, and M3 are the percent values of cells in the 1st, 2nd, 3rd and higher divisions respectively. For the statistical evaluation of the experimental data, Student’s t-test was performed to determine whether any SCE values differed significantly from the controls and the X2 test was used for the cell kinetic comparison (PRI). Simple linear correlation between SCEs and PRI frequencies were also calculated using Pearson’s product moment correlation coefficient r. Then a criterion for testing whether r differs significantly from zero was applied, whose sampling distribution is Student’s t test with n-2 d.f.

RESULTS

The oral dosage is for AZ 0.25-6 mg/day and for LZ 1-6 mg/day. Final concentrations per culture (0.16-3.84 μM for AZ and 0.62-3.72 μM for LZ) were equivalent to the oral doses. The results show that both AZ and LZ cause a dose-dependent increase of SCE frequency. AZ induced statistically significant (p<0.001) increase at all concentrations tested in the two experi-
Table 1. Cytogenetic activity of AZ in normal human lymphocyte cultures from two different healthy donors.

<table>
<thead>
<tr>
<th>Equivalent oral dosage (mg/day)</th>
<th>Concentration (µM)</th>
<th>Concentration (µg/ml culture)</th>
<th>1st donor SCEs±SE/cell</th>
<th>2nd donor SCEs±SE/cell</th>
<th>1st donor PRI</th>
<th>2nd donor PRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>0</td>
<td>7,08±0,503</td>
<td>7,10±0,38</td>
<td>2,19</td>
<td>2,166</td>
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<tr>
<td>0.25</td>
<td>0,16</td>
<td>0,049</td>
<td>10,070±0,923*</td>
<td>10,767±0,532*</td>
<td>1,975</td>
<td>2,111</td>
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<tr>
<td>0,5</td>
<td>0,32</td>
<td>0,098</td>
<td>11,201±0,905*</td>
<td>12,604±0,530*</td>
<td>1,927</td>
<td>2,064</td>
</tr>
<tr>
<td>1</td>
<td>0,64</td>
<td>0,196</td>
<td>12,322±0,719*</td>
<td>13,266±0,487*</td>
<td>1,91</td>
<td>1,973</td>
</tr>
<tr>
<td>2</td>
<td>1,28</td>
<td>0,392</td>
<td>12,565±0,808*</td>
<td>15,063±0,587*</td>
<td>1,801**</td>
<td>1,946**</td>
</tr>
<tr>
<td>4</td>
<td>2,56</td>
<td>0,784</td>
<td>12,998±0,854*</td>
<td>16,241±0,879*</td>
<td>1,79**</td>
<td>1,95**</td>
</tr>
<tr>
<td>6</td>
<td>3,84</td>
<td>1,176</td>
<td>13,323±0,714*</td>
<td>15,788±0,686*</td>
<td>1,77**</td>
<td>1,798**</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.001) increase over the corresponding control (t-test)
**Statistically significant (p<0.001) decrease over the corresponding control (X² test)
A minimum of 20 cells was scored for SCEs from each culture
SE = standard error of the mean

Table 2. Cytogenetic activity of LZ in normal human lymphocyte cultures from two different healthy donors.

<table>
<thead>
<tr>
<th>Equivalent oral dosage (mg/day)</th>
<th>Concentration (µM)</th>
<th>Concentration (µg/ml culture)</th>
<th>1st donor SCEs±SE/cell</th>
<th>2nd donor SCEs±SE/cell</th>
<th>1st donor PRI</th>
<th>2nd donor PRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Control</td>
<td>0</td>
<td>9,272±0,458</td>
<td>6,55±0,716</td>
<td>2,517</td>
<td>2,127</td>
</tr>
<tr>
<td>1</td>
<td>0,62</td>
<td>0,199</td>
<td>10,497±0,45</td>
<td>8,12±0,582</td>
<td>2,44</td>
<td>2,06</td>
</tr>
<tr>
<td>2</td>
<td>1,24</td>
<td>0,398</td>
<td>12,433±0,75</td>
<td>9,08±0,568</td>
<td>2,441</td>
<td>1,98</td>
</tr>
<tr>
<td>3</td>
<td>1,86</td>
<td>0,597</td>
<td>13,738±0,675*</td>
<td>11,902±0,579*</td>
<td>2,366</td>
<td>1,984</td>
</tr>
<tr>
<td>4</td>
<td>2,48</td>
<td>0,796</td>
<td>16,759±0,653*</td>
<td>12,277±0,553*</td>
<td>2,397</td>
<td>1,966</td>
</tr>
<tr>
<td>5</td>
<td>3,1</td>
<td>0,995</td>
<td>16,445±0,727*</td>
<td>13,468±0,567*</td>
<td>2,324</td>
<td>1,955</td>
</tr>
<tr>
<td>6</td>
<td>3,72</td>
<td>1,194</td>
<td>18,562±0,676*</td>
<td>14,602±0,633*</td>
<td>2,240**</td>
<td>1,923**</td>
</tr>
</tbody>
</table>
ments, though LZ had this effect at the concentrations of 1.86 to 3.72 μM in the two experiments. AZ and LZ induced a decrease of PRI levels at all concentrations tested, which became statistically significant at 1.28-3.84 μM for AZ and at 3.72 μM for LZ in the two experiments. In addition, a correlation was observed (p < 0.01) between the magnitude of the SCE induction and the PRI depression (r = -0.94 and t = 6.06 p < 0.01 for AZ and r = -0.91 and t = 4.81 p < 0.01 for LZ).

The results are given at Table 1 for AZ and Table 2 for LZ.

**DISCUSSION**

Among the most interesting adverse reactions of drugs are genotoxic and carcinogenic effects. Current guidelines for genotoxicity testing of pharmaceuticals indicate a standard test battery that consists of: (i) a test for gene mutation in bacteria, (ii) an *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma tk assay, (iii) an *in vivo* test for chromosomal damage using rodent haematopoietic cells. Benzodiazepines have been extensively used since 1960 for the treatment of anxiety, as hypnotics, sedatives and for other conditions. For many of them, more than 50, lack of studies do not allow the evaluation of their genotoxic-carcinogenic potential risk to humans. Diazepam, the most extensively tested, gave both positive and negative results in genotoxicity assays and was carcinogenic to mice but not to rats, hamsters and gerbils.

In a previous series of experiments, we estimated the SCEs and PRI in normal human lymphocyte cultures, in order to study the cytotoxic and cytostatic activity of diazepam in vitro. The results showed that it causes a dose-dependent, statistically significant increase of SCE frequency, followed by an equally significant decrease of PRI in all four experiments performed.

AZ and LZ are two widely used benzodiazepines, which gave negative or equivocal responses in both genotoxicity and carcinogenicity assays in S. typhimurium, animal cells in vitro, rat bone-marrow cells and rat liver in vivo. Their effect on DNA strand breaks in human cells in vitro hasn’t been tested as yet. Carlo P. et al found absence of liver DNA fragmentation (DNA damage in vivo is considered a sensitive index of potential mutagenic-carcinogenic activity) in rats treated with high oral doses of 32 benzodiazepines, among which were AZ and LZ. Despite that, they suggest that studies performed in different laboratories or in different biological systems and consequently the use of several assays with different genetic endpoints may allow a better approach to the estimation of mutagenic-carcinogenic hazards.

In our study, both AZ and LZ exhibited dose-dependent cytogenetic activity in vitro, increasing SCE frequencies and diminishing PRI levels in normal human lymphocyte cultures. The presence of the –NHCO- group in LZ’s molecule might be responsible for its cytotoxic and cytostatic effect. The –NHCO- group is thought to interact with similar groups or with structure-specific domains in DNA and proteins. AZ has a triazolo-ring, which seems to be even more cytotoxic than the lactamic one of LZ and a -CH₃- group that could be an alkylating group. AZ, in oral doses, imposed a stronger cytogenetically effect than LZ. We found that AZ is cytotoxic at all doses and cytostatic at half of them, whereas LZ exhibited cytotoxic activity at the higher doses and cytostatic at the highest one.

Benzodiazepines are considered as safe and effective drugs in short term use, with low toxicity in humans, but for many of them published or otherwise publicly accessible data do not allow the evaluation of genotoxic-carcinogenic risk to humans. Our results suggesting that both benzodiazepines are cytogenetically active (with AZ more effective than LZ on a molar basis) are based on in vitro studies. The final concentrations of AZ and LZ in the cultures were equivalent to oral doses, but they were higher than the therapeutic doses because of the pharmacokinetic behaviour of the compounds (high protein binding) after oral administration. So we intend to extend this very interesting research in other cell lines and in vivo experiments too.

There are findings indicating that the effectiveness of SCE induction by potential antitumor agents in cancer cells in vitro and in vivo is positively correlated with in vivo tumour response to these agents. The incidence of SCEs on peripheral human lymphocytes in vivo might be an appropriate method for evaluating exposure to various agents including chemotherapeutics: peripheral lymphocytes are the dosimeter of such
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In exposure, since they circulate throughout all tissues of the body and a proportion of them are long-lived. The SCE assay has predictive value as a clinical assay for drugs for which a strong correlation between cell death and induction of SCEs has been established. Other studies investigating a relationship between SCE induction and other expressions of genotoxicity have also shown a positive relationship between SCEs, reduced cell survival and alteration in cell cycle kinetics. Since in the present study a strong correlation \( p < 0.01 \) between SCE enhancement and PRI depression was established in lymphocyte cultures (in vitro), further studies in experimental tumours (in vivo) for evaluating possible antineoplastic potential by these chemicals are warranted.

REFERENCES

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