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GENOTOXIC AND ANTIPROLIFERATIVE EFFECTS OF THREE NEWLY SYNTHESIZED AMINOPHOSPHONIC ACIDS

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ABSTRACT. Aminophosphonic acids are analogs of amino acids where the carboxylic group is substituted with a phosphonic group $P(O)(OH)_2$. A large part of the present research of aminophosphonic acids and their derivatives is focused on their herbicide, antiviral and antitumor activity. This report refers to the investigation of genotoxic and antiproliferative effect of newly synthesized aminophosphonic acids.
For the cytogenetical investigation male and female C57Bl mice were administered i.p. with **KT-2, KT-3, KT-4** at doses of 10mg/kg and 100mg/kg. Mitomycin C (Kyowa) 3.5 mg/kg was used as a positive control. Animals injected with 0.9% NaCl were used as a negative control.
The clastogenic and antiproliferative effects of newly synthesized original aminophosphonic acids were investigated for the first time. The studied compounds do not possess clearly expressed relationship “dose-effect” in their clastogenic effects, as this property is specific for the alkylating agents. Comparatively low percentage of bone marrow metaphases with chromosome aberrations and the lack of aberrant metaphase plates with disintegrated chromosomes and dispersed chromatin is an evidence of the moderate clastogenic effects of the studied compounds. Metaphase analysis showed that the changes of the chromosome structure were predominantly centromer/cenromeric fusions, rarely breaks and fragments. These results suggest that the aminophosphonic acids affect the centromeric chromosome regions. The obtained centric fusions involved middle size chromosomes of the laboratory mice karyotype considered as the “hot” chromosomes. The correlation between the moderate clastogenic effect and the low values of MI, obtained after the treatment with KT-2 100mg/kg, allows furthermore detailed investigations on the experimental tumor models *in vivo* and *in vitro*.

KEY WORDS. Aminophosphonic acids, chromosome aberrations, clastogenic effects, cell proliferation, mitotic index.

INTRODUCTION

Aminophosphonic acids are considered to be structural analogues of amino acids in which $P(O)(OH)_2$ replaces the carboxylic group (Kukhar and Hudson, 2000). Aminophosphonic acids are closely related to the amino carboxylic acids and are very interesting because of their diverse biological activity and insignificant toxicity in mammalian cells. N-phosphonomethyl glycine (glyphosate) is the most completely studied one. This chemical and its salts are effective herbicides (Franz et al., 1997).

Except their herbicide activity, many derivatives of N-phosphonomethyl glycine have antiviral activity too, suppressing herpes viruses, grippe viruses, rhinoviruses, etc. (Camden, 1997, 2000). These compounds are effective in suppressing the tumour growth. It was detected that they penetrate the cancer cell membranes 4-5 times easier than the membranes of the normal cells (Bandurina et al., 1978).

In the previous investigations, the cytotoxicity of some derivates of N-phosphonomethyl glycine in cell cultures *in vitro* has been investigated. It was found that α-ethyl-α-N-(hydroxyethylamino) methylphosphonic acid has dose dependent cytotoxic effect on chicken (DEC99) and beef (EBTr) cell lines. The cell sensitivity was evaluated according to CD_{50} values (Ivanov et al., 2001).

For the total characterization of these newly synthesized aminophosphonic derivates of N-phosphonomethyl glycine it is very important and appropriate to investigate their genotoxic and antiproliferative effects. The present study is the first proof of genotoxic and antiproliferative effect of some original aminophosphonates. Chromosome aberration test was applied to solve the main task. This test allows detecting the possible genotoxic effect, the type of the chromosome aberrations and the specific chromosomes of the mouse karyotype which are subjected to the effects.

The change of the proliferative activity of the bone marrow cells of the treated laboratory mice was calculated applying the statmokinetick approach.

MATERIAL AND METHODS

Chemicals

KT-2 α-ethyl-α-N-(hydroxyethylamino)methylphosphonic acid

KT-3 α-N-(2-hydroxypropilamino)methylphosphonic acid

KT-4 α-ethyl-α-N-phosphonomethyl glycine

These chemicals were synthesized by Troev et al. (1999) and Naidenova et al (2003).

Cytogenetical method. The cytogenetical investigation was conducted as described by Preston et al., (1987). Male and female C57Bl mice, weighing 20g ±1.5g were kept at standard conditions - 20° C, 12 h light/dark cycle, having free access to food and water. **KT-2, KT-3, KT-4** were administered i.p. at doses of

10mg/kg and 100mg/kg . Mitomycin C (Kyowa) 3.5 mg/kg was used as a positive control. Animals injected with 0.9% NaCl were used as a negative control.

Bone marrow chromosome aberration assay was performed on groups of animals. Each one consists of 3 males and 3 females treated with the studied compound and 5 control animals. The animals were injected i.p. with colchicine at a dose of 0.4 mg/kg, 24, 48 h after the administration of the applied chemicals or 0,9% NaCl solution and 1h before isolation of the bone marrow cells. Bone marrow cells were flushed from femur and hypotonized in a 0.075 M KCl at 37°C during 20 min. Thereafter the cells were fixed in methanol - acetic acid (3:1), dropped on cold slides and air dried. To examine the chromosome aberrations the slides were stained with 5% of Giemsa solution (Sigma Diagnostic). At least 50 well-spread metaphases were analysed per experimental animal at random.

Mitotic indices were determined by counting the number of dividing cells among 1500 cells per animal in the bone marrow slides to score aberrations.

The frequencies of abnormalities and the mitotic index were determined for each animal and then the mean±standard error for each group was calculated.

Statistical analysis. Three-way analysis of variance (ANOVA) with fixed effects, followed by two-group Student's t-test and post hoc pairwise comparison test of Dunnett with a control was performed using BMDP4V, BMDP3D and BMDP7D programs (Dixon at al., 1990). Statistical significance is expressed as ***p<0.001; **p<0.01; *p<0.05; p>0.05 - (not significant). Unless otherwise stated eight animals were used per group.

RESULTS

The analyses of the obtained results of the induced chromosome aberrations frequency by KT-2, KT-3 and KT-4 in mice cells are presented consequently.

KT-2 α-ethyl-α-N-(hydroxyethylamino)methylphosphonic acid

After the KT-2 administration the percentages of cells with aberration for the two applied concentrations and for the samples from 24th and 48th hours were close (Fig. 1) and the differences in the calculated values were not significant (p>0,05).

A more detailed analysis shows that KT-2 (10mg/kg, 24th hour) induces centromeric fusion (4,33 ± 0,61%). On the contrary, in the same group (48th hour) almost equal number of centromer/centromeric fusions (2 ± 0,73%), fragments (2 ± 1,03%) and breaks (2,33 ± 0,95%) from the whole number of cells with aberrations were obtained.

In the mice treated with 100mg/kg KT-2 the number of metaphases with centromer/centromeric exchanges increased at the 48th hour after the chemical administration (5,67 ± 0,61%) and chromosomes with telomer/telomeric fusions could be seen.

KT-3 α-N-(2-hydroxypropilamino)methylphosphonic acid

The chromosome aberrations values in the experimental animals after the treatment of KT-3 are presented on Figure 1. KT-3 at concentration 10mg/kg caused the presence of 6 ± 0,73% (24th hour) and 5,67 ± 0,8% (48th hour) bone marrow cells

with chromosome aberrations. Significant differences between treated groups injected with 10-100mg/kg at the two time-intervals were not detected ($p>0,05$). The values of centromer/centromeric fusions were close to those obtained for KT-2. The number of cells with breaks and fragments was significantly lower. Metaphases with more than two aberrant chromosomes were also observed.

KT-4 α-ethyl-α-N-phosphonomethyl glycine

KT-4 at 10mg/kg (24th hour) induced 7,76 ± 0,56% chromosome aberrations. A tendency to higher incidences of chromosome changes – cenromer/centromeric fusions, specific for KT-2 and KT-3 was also followed. In the 48th hour samples the number of cells with centromer/centromeric fusions decreased twice and the total percentage of metaphases with aberrations is surprisingly diminished to 4,67 ± 0,38%, whereas in KT-2 and KT-3 (48th hour) groups the c/c values were kept in the statistical error limits. This significant decrease of the chromosome aberrations percentage at the 48th hour ($p<0,05$) could be explained by the elimination of the cells with these damages later after KT-4 application or by abatement of the effect of these chemicals to the 48th hour.

In the experimental mice groups after KT-4 injections at 100mg/kg the percentages of chromosome aberrations are comparable to the 24th and 48th hour intervals - 5,6 ± 0,63% and 6,67 ± 0,77%.

Generally, in all treated groups the percentage of aberrant metaphases differs significantly from the control groups. It was significantly higher to the negative control and significantly lower to the positive control – Mitomycin C ($p<0,01$). These results are evidence for some clastogenic effect of the studied aminophosphonic acids. Furthermore, none dispersed metaphases were observed on the slides from the aminophosphonic acids experimental groups in comparison with those of the Mitomycin C positive control.

Some other conclusions about the genotoxicity of the studied aminophosphonic acids could be drawn using the data of the correlation between the different types of aberrations. The calculation showed that more than 41,25% of the damaged metaphases after KT-2 treatment have breaks and fragments (2,95% of the total number of analyzed cells). For a comparison, in the negative control group cells with breaks and fragments were not found, but in the positive Mitomycin C control the percentage was 71,3% (or 29,33% of the total number of analyzed cells).

In KT-3 mice groups these percentages were considerably lower – 23,08 or 1,5% from the total. After KT-4 administration 41,09% of the aberrant bone marrow metaphases possessed broken chromosomes and fragments, and according to these affects KT-4 was closely related in its effect to KT-2.

In the treated animals (24th hour) the highest percentage of chromosome aberrations provoked KT-4 (7,76 ± 0,56%), KT-2 – lower (7,2 ± 0,92%) and KT-3 – the lowest (6 ± 0,73%). These differences are not statistically significant, but some tendency exists. The described diminition of the chromosome aberration yielded in the KT-4 10mg/kg (48th hour) group distinguishes it from the experimental group treated with KT-2 and KT-3. The faster metabolic pathway of KT-4 and the higher stability of the other two compounds could explain this.

It can be summarized that KT-2, KT-3 and KT-4 possess moderate clastogenic effects. The analysis of the type of chromosome aberrations obtained after the treatment of the investigated compounds showed that in all experimental groups centromeric fusions predominate with the exception of KT-4 100mg/kg (48th hours) group, where the values of chromosome breaks are significantly higher in all variants. These results allowed a hypothesis that newly synthesized aminophosphonic acids damage predominantly the centromeric chromosome regions.

2. Effect of newly synthesized aminophosphonic acids on the proliferative activity of C57Bl mice bone marrow cells

The effect of the newly synthesized aminophosphonic acids on the proliferative activity of bone marrow cells was evaluated by the mitotic index parameter. The obtained results are presented in Figure 2.

The mitotic index values (MI) of bone marrow cells in the groups, treated with KT-2 10mg/kg, on the 24th hour (6,26 ± 0,35‰) were close to these on the 48th hour (6,13 ± 0,61‰).

The data about the effect of KT-2 100mg/kg are especially interesting. In the experimental group (24th hour) the mitotic activity of bone marrow cells was decreased nearly five fold (2,67 ± 0,55‰) in comparison to the untreated control (17,3 ± 2,49‰) ($p<0,001$). The value of the mitotic index are slightly increased on the 48th hour group (4,18 ± 0,36‰), but remain significantly lower to the untreated control as well as to the groups treated with 10mg/kg ($p < 0,001$).

Significant differences between the mitotic indexes values obtained for the investigated bone marrow cell populations at the 24th and 48th hour (treated with the two applied KT-3 concentrations) were not found. MI varied from 5,21 ± 0,49 ‰ to 7,10 ± 0,47 ‰.

In the samples scored after KT-4 injection on the 24th hour after the treatment (10mg/kg KT-4) MI was 8,36 ± 0,59‰ and kept almost unchanged till 48th hour. The higher applied concentration (100mg/kg) induced slightly decreased cell division, when compared to 10mg/kg - 6,06 ± 0,73 ‰ for 24h and 7,29 ± 0,34 ‰ for 48h.

DISCUSSION

This study was undertaken to evaluate the possible genotoxic and antiproliferative activity of three newly synthesized aminophosphonic acids *in vivo*. Our knowledge is not yet employed in the safety assessment of these chemicals.

The analysis of the all treated C57Bl samples provided evidences for relatively low genotoxicity of the investigated compounds. Compared to the data obtained for Mitomycin C – KT-2, KT-3 and KT-4 provoke about 8 fold fewer chromosome aberrations. If we emphasize on the relative part of breaks and fragments, the differences will be more significant. Concerning mitotic activity of treated bone marrow cells, the calculated values in the most experimental groups were close to these obtained after Mitomycin C treatment.

A tendency toward appearance of more metaphases with centromeric fusion in comparison with breaks and fragments was calculated. These results could be explained with damages of some chromosomes centromeric and telomeric regions

during the first mitosis after treatment. Microscopic observations demonstrated that in the obtained Robertsonian translocations middle and small size chromosomes from the C57Bl mice karyotype were involved. These data confirmed the results of Glazko and Sozinov (Глазко и Созинов, 1977) that in bone marrow cells of laboratory mice the 8th, 9th and 18th chromosomes are mostly active in interchromosome rearrangements.

In the analyzed bone marrow cells from the control animals the detected aberrations, even though in a low percentage, were from the c/c type. That is not an unexpected fact. According to Kipling et al. (1994) the rate exchanges between non-homologous chromosomes in the mice is particularly high. This might be facilitated by the telocentric nature of mice chromosomes, which allows centromer/centromeric recombinations between non-homologous chromosomes without altering the standard karyotype of mice.

There are no available data on the genotoxic and antiproliferative effects of the investigated aminophosphonic acids, but many studies concerning the genotoxic effects of N-phosphonomethyl glycine (glyphosate) are carried out. The obtained results are rather contradictory.

According to Li and Long (1988) no genotoxic activity was observed in a variety of well-established *in vitro* and *in vivo* assays, e.g. Chinese hamster ovary cell gene mutation assay and *in vivo* cytogenetic assay in rat bone marrow. Cytogenetical effects were not found in the mouse bone marrow micronucleus test, but *Allium* anaphase-telophase test showed that a significant increase in chromosome aberrations appeared after treatment of 1,44 and 2,88mg/l glyphosate isopropylamine (Rank et al., 1993). Later Lioi et al. (1998) provided new evidences for the genotoxicity and oxidative stress induced by glyphosate in bovine lymphocyte cultures *in vitro.* These results indicated a statistically significant increase of the structural aberration sister chromatide exchanges, suggesting that the glyphosate induces a mutagenic effect. Their results showed that the decrease of the mitotic index and cell viability after exposure demonstrates a high cytotoxic effect, which is always associated with the observed genotoxic effect.

Concerning the influence of the cell mitotic activity Marc et al. (2004) showed that 10mM glyphosate impeded G_2/M transition as judged from analysis of the first cell cycle of sea urchin development. Glyphosate inhibited the synthesis of DNA occurring in S phase of the cell cycle. The extent of the inhibition of DNA synthesis by glyphosate was correlated to the effect on the cell cycle.

Their aassumptions allow us to suggest that the effect of the newly synthesized aminophosphonic acids, investigated in the present study, is exerted at the level of DNA-response checkpoint of S-phase in mouse bone marrow cells too.

The data obtained by the current study on the biological effects of the newly synthesized aminophosphonic acids are generally similar to those for the data on the more intensive investigated relative compound – N-phosphonomethyl glycine.

CONCLUSION

● The clastogenic and antiproliferative effects of newly synthesized original aminophosphonic acids were investigated for the first time.

● The studied compounds KT-2, KT-3 and KT-4 did not possess clear expressed relationship "dose-effect" (high percentage of aberrations after higher dose applied) in their clastogenic effects as this relationship is specific for the alkylating agent Mitomycin C.

● Comparatively low percentage of bone marrow metaphases with chromosome aberrations and the lack of aberrant metaphase plates with disintegrated chromosomes and dispersed chromatin is an evidence of the moderate clastogenic effect of newly synthesized aminophosphonic acids.

● Metaphase analysis showed that the changes of the chromosome structure in the bone marrow cells of the treated animals were predominantly centromer/cenromeric fusions and rarely breaks and fragments. These results suggest that the investigated compounds affect the centromeric chromosome regions. The obtained centric fusions involved middle size chromosomes of the laboratory mice karyotype considered as the "hot" chromosomes.

● The correlation between the moderate clastogenic effect and the low values of MI, obtained after the treatment with KT-2 100mg/kg, is an interesting fact. We consider further detailed investigations on the experimental tumor models *in vivo* and *in vitro* as appropriate.

REFERENCES

ГЛАЗКО, Г. Б., А.А. СОЗИНОВ. 1997. Некоторые характеристики хромозомного уровня изменчивости у мышей. Цитол. и генет. **31**, 70-76.

BANDURINA, T. A., KONYUKHOV, V. N., PONOMAREVA, O. A., BARYBIN, A. S. & PUSHKAREVA, Z. V.1978. Khim-Farm Zh. **12:**35.

DIXON, J., BROWN, M., ENGELMAN, L. and JENNRICH R. 1990. BMDP Statistical Software Manual. UCLA Press, Berkely.

FRANZ, J. E., MAO, M. K. & SHIKORSKI, J. A. 1997. Glyphosate: A Unique Global Herbicide (monagraph), American Chemical Society: Washington, DC. 233-257.

CAMDEN, J. B. 1997. US Patent 5665713.

CAMDEN, J. B. 2000. US Patent 6090 796.

IVANOV, I., I. SAINOVA, E. SHIKOVA, K.TROEV. 2001. „Cytotoxicity induced by 3-ethyl-2-hydroxy-2-oxa-1,4,2-oxazaphosphorinane and -ethyl- -N-(hydroxyethylamino) methilphosphonic acid *in vitro*." Experimental Pathology and Parasitology **4**: 26-30.

JIRAUNGKOORSKUL, W., E. S. UPATHAM, M. KRUATRACHUE, S. SAHAPHONG, S. VICHASRI-GRAMS, P. POKETHITIYOOK. 2003. „Biochemical and histopathplogical effects of glyphosate herbicide on Nile tilapia (Oreochromis niliticus)." Environ Toxicol. **18(4)**: 260-7.

KIPLING, D., H. WILSON, A. MITCHELL et al. 1994. Mouse centromeric mapping using oligonucleotide probe that detect variants of the minor satellite. Chromosoma. **103**, 46-55.

KUKHAR, V. & HUDSON, H. 2000. Aminophosphonates and Aminophosphinic Acids, John Wiley and Sons, LTD.

LI, AP, LONG, TJ. 1988. An evaluation of the genotoxic potential of glyphosate. Fundam. Appl. Toxicol. **10** (3): 537-546.

LIOI, MB, SCARFI, MR, SANTORO, A., BARBIERI, R., ZENI, O., DI BERARDINO, D., URSINI, MV. 1998. Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro. Mutat. Res. **403** (1-2), 13-20.

MARC, J., BELLE R., MORALES, J., CORMIER, P., O. MULNER-LORILLON. 2004. Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. Toxicol. Sci. **82** (2), 436-442.

NAJDENOVA, E., VASSILEV, A., POPOVA, YU. & TROEV, K. 2003. Phosphonylmethylaminocyclopentane-1-carboxylic Acid, Heteroatom Chemistry **14**(3).

PRESTON, R., DEAN, B., GALLOWAY, S., HOLDEN, H., MC FEE, A.F. and SHELDY, M. 1987. Mammalian *in vivo* cytogenetic assay analysis of chromosome aberrations in bone marrow cells. Mutation Res. **189**, 157-165.

RANK, J., JENSEN, AG, SKOV, B., PEDRESEN, LH, JENSEN, K. 1993. Genotoxicity testing of the herbicide Roungup and its active ingredientglyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test. Mutat. Res. **300** (1): 29-36.

TROEV, K., KREMER, S. & HAEGELE, G. 1999. A New Synthetic Approach to α-Aminophosphonic Acids: Synthesis and NMR Characterization, Heteroatom Chemistry. **10** (7)**:** 627.

Frequences of chromosome aberrations in bone marrow cells after i.p. treatment with aminophosphonic acids

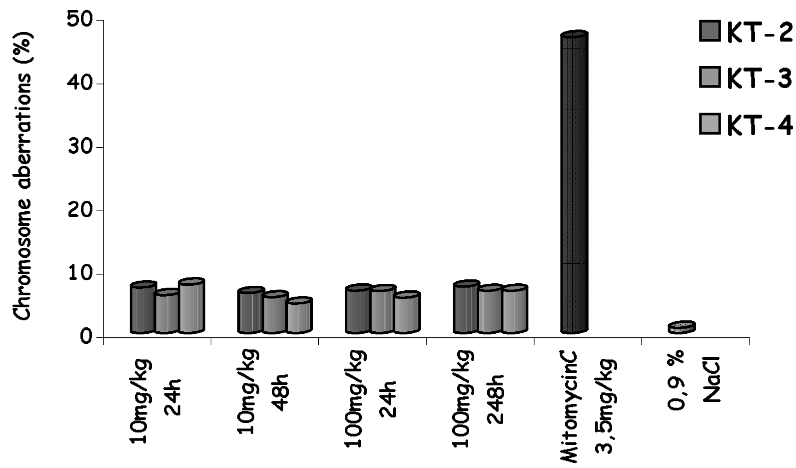

Figure 1.

Mitotic indices in bone marrow cells after i.p. treatment with aminophosphonic acids

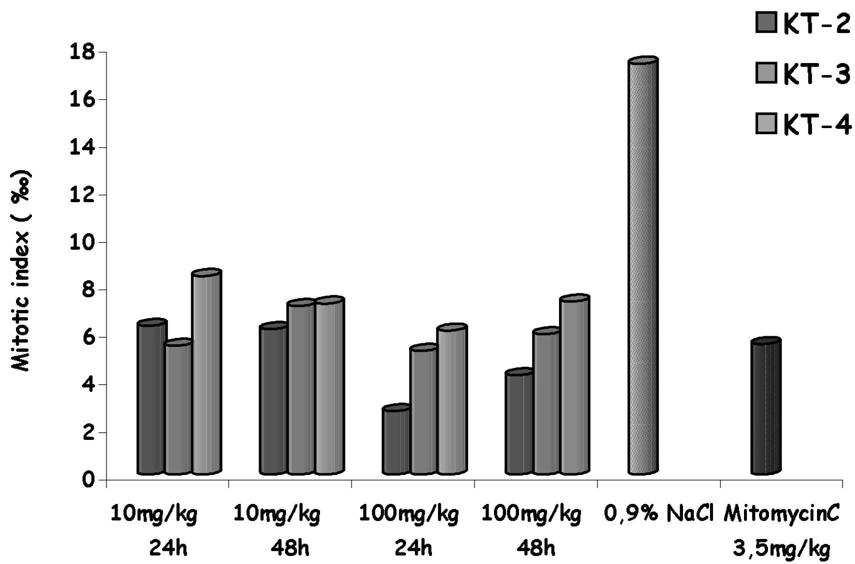

Figure 2.