MMSL
Military Medical
Science Letters

ORIGINAL ARTICLE

COMPARISON OF POTENTIAL CYTOTOXICITY AND GENOTOXICITY OF SELECTED ANTIDOTES AGAINST ORGANOPHOSPHATES INHIBITING ACETYLCHOLINESTERASE

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Summary

Organophosphorous compounds cause fatal intoxication based on inhibition of acetylcholinesterase, an essential enzyme of neurosynapses and neuromuscular junctions. There is an obvious need to develop appropriate treatment against them due to their application in agriculture and chemical industry or their misuse in terrorist or war attack.

In the Czech army some medicaments have been established to be used against this poisoning; Obidoxime, Methoxime, Atropine and Benactyzine. In present *in vitro* study we focused on potential cytotoxic and genotoxic effect evaluation of these drugs by the clonogenic and comet assay, respectively.

Obtained results show that oximes exhibit pronounced toxic effect, namely obidoxime in term of genotoxicity and methoxime in term of cytotoxicity.

Key words: genotoxicity; cytotoxicity; antidotes; organophosphates; DNA damages, oximes; anticholinergic; comet assay; clonogenic assay

INTRODUCTION

In terms of recent global political situation it is clear that readiness against intoxications caused by nerve agents, such as tabun, sarin, soman, VX or cyclosarin is still genuie. Concerning their properties i.e., high toxicity, the immediate efficiency, and easy ava-

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ilability, nerve agents could be abused in a terrorist or war attack (1, 2).

These organophosphorous compounds act as acetylcholinesterase (AChE) irreversible inhibitors. It leads to accumulation of the acetylcholine in neurosynapses of the nervous system or neuromuscular junctions. This results in overstimulation, excitation, and life threatening events such as convulsions and depression of cardiovascular and breath centres (3, 4, 5, 6).

The standard therapy consists of the administration of anticholinergics, reactivators of AChE, and anticonvulsants (7). In the Czech army, atropine, benactyzine and trihexyphenidile as anticholinergics, the obidoxime, the methoxime, and oxime HI-6 as reactivators, and diazepam as an anticonvulsive drug

are established. The commercial preparates are an autoinjector Combopen (obidoxime and atropine), solutions for injection for intramuscular administration Chonol I (atropine) and Chonol II (benactyzine), Renol (methoxime) and tablets Panpal (trihexyphenidyle, benactyzine, pyridostigmine). Their pharmacological profile is well known but there is a lack of information about their possible genotoxic and cytotoxic effects.

In the present study, we have focused on the *in vitro* cytotoxic and genotoxic potency, assayed as an extent of DNA damage, of above-mentioned substances.

To analyse the DNA damage level, we have employed comet assay (single cell gel electrophoresis, SCGE) (8, 9) modified for the estimation of oxidative DNA damage by use of the specific enzyme endonuclease III. This enzyme cleaves specifically the abasic sites and oxopyrimidines. This leads to the formation of alkalilabile sites (Endo III sites) in the DNA, which are in the comet assay analysed as additional DNA breaks (10).

The cell line A549 (human lung carcinoma cell line) is widely used for toxicological studies. In our case, they were chosen because they are used for studies of the influence of chemicals on the activity of AChE (11), which is important from the point of view of the mechanism of action of compounds tested in this study. They were used for the measuring of cytotoxicity by clonogenic assay and for the DNA estimation by SCGE.

Because metabolites of tested compounds could be genotoxic, also perpetual cell line HepG2 (hepatocellular carcinoma cells) was chosen as a suitable cell culture for SCGE. This cell line was shown to maintain most of the functions of various phase I and phase II enzymes and their inducibility (12). The presence of enzymes relevant for the metabolism of xenobiotics gives preference to HepG2 cells for genotoxicity assays, because they reflect more adequately the toxification and detoxification process that occurs *in vivo*. These cells grow as apical formations with high morphological and functional differentiation, which is the reason why they cannot be not used for clonogenic assay.

MATERIALS AND METHODS

Chemicals:

All oximes studied were prepared in our department according to the synthesis described by Kuča

et al. (13). The purity (98.5%) of this reactivator was detected using the TLC technique, the HPLC technique and NMR (14, 15).

They were kept at room temperature and freshly dissolved in PBS saline shortly before the experiment. Anticholinergics were obtained commercially (BioTech, Praha).

Cell lines:

HepG2 cells were cultivated in specific Williams medium E supplemented with 10% of bovine foetal serum (FBS), (PAA, (BioTech, Praha). Cells were grown under controlled conditions (37°C, 5%CO_2).

The A549 cell line (obtained from the European collection of cell culture), were cultured in medium DMEM supplemented with 10% of FBS, (PAA, (BioTech, Praha). Cells were grown under controlled conditions (37°C, 5%CO_2).

Clonogenic assay:

Two hundred cells were seeded per each 40 mm petri dish (TPP) in 1.5 ml of culture medium specific for this type of cell line. Next day cells were treated by anticholinergics and oximes in concentrations mentioned above. Two parallel dishes were used per experimental point. After 24 hours, medium was changed and cells were allowed to grow up and form colonies (groups at least of 50 cells) for 7 days. Then the colonies were fixed by methanol acetic - acid (3:1), stained by Giemsa and counted. e. Results are presented as percentage of negative control, without treatment to treated samples. Each experiment was repeated 2 – 4 times.

Comet assay:

Cultivated cells were treated for 2 and 24 hours by above-mentioned chemicals with concentrations 10mM, 5mM, 2.5mM, 1.25mM, 0.6mM, in the case of benactyzine also with 0.3mM, 0.15mM. In parallel, negative controls without any treatment were also included into the experimental design. The concentrations were chosen on the bases of results of clonogenic assay respecting current OECD (2004) guidelines for genotoxicity testing in mammalian cells which require that the top concentration with soluble and non-toxic substances should be 10 mM (16).

In indicated intervals, single cell suspension was prepared for processing for DNA damage de-

termination by the comet assay (10). Briefly, cells were embedded in agarose on a microscope slide and cell membranes were degraded in lysing buffer (2.5M NaCl, 0.1 M Na2EDTA, 10 mM Tris-HCl, pH 10.1, 1% triton) for 1 hour. After this step, 30 µl of endonuclease III was added to each second gel for 45 min/37°C. After the alkaline unwinding in alkaline electrophoresis buffer (0.3 M NaOH, 10 mM EDTA, 40 min/4°C, pH > 13 (17)) and electrophoresis (25 V, 30 min), the comets were viewed by fluorescence microscopy and the parameter "% tail DNA" was measured (25 cells per experimental point) by image analysis (Lucia G, Laboratory Imaging, Prague).

Statistics:

For the testing of significance of the antidotes effect the Kruskal-Walis test was used, the significance of the difference between DNA breaks (%tail DNA) in controls and treated group was retested by Mann-Whitney test using the Sigma Stat 2.03 software.

RESULTS AND DISCUSSION

Clonogenic assay:

The ability of cells to form colonies under presence of various compounds in different concentrations was tested. Cells were treated with the chemicals for 24 hours and the formed colonies were counted after 7 days. The colony forming ability of cells decrease in a dose-dependent manner. Figure 1 shows benactyzine as a compound with the least pronounced cytotoxic effect, nevertheless, at concentration 1.25 mM viability declines rapidly.

Contrary to benactyzine, obidoxime, and atropine dramatically decrease the survival of cells already at 0.3 mM concentration. It seems that methoxime acts as the most toxic compound, because its cytotoxic effect manifests already at 0.15mM. Both atropine and methoxime halted cell proliferation earlier, at concentration 0.625mM. Additionally, it seems plausible that used anticholinergics possess lower cytotoxic effect, because its colony forming ability is completely inhibited completely at 2.5 mM concentration.
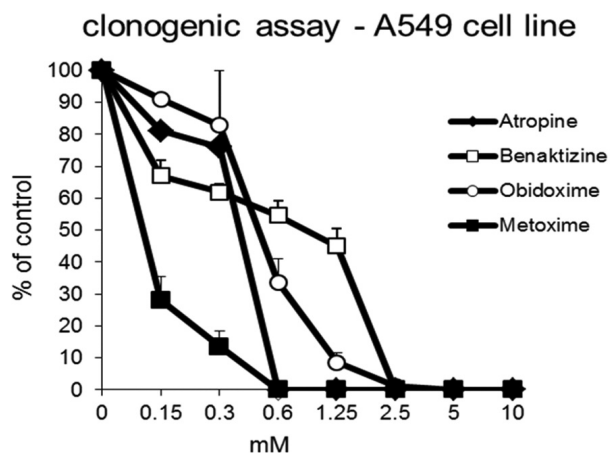

Figure 1. The ability of A549 cells to form colonies under influence of various compounds in different concentrations was followed for 24 hours. At a negative control, 100% viability of cells can be observed. It was measured by clonogenic assay.

Comet assay:

Briefly, analysed cells are split to two samples, cells of each sample embedded in agarose on a microscope slide and incubated in lysing solution (1 hour, 4°C). After this step, one of two parallel slides is left in the lysing solution (analysis of DNA breaks), the gel with cells on the other slide was incubated with 40 µl of endonuclease III

(45 min, 37 °C) (analysis of endo III sites (see methods for details). The DNA damage (single strand breaks, SSBs) is expressed as the percentage of DNA in tail (% tail DNA). According to our calibration of the method by X ray irradiation published previously, the amount of $SSB/10^9$ daltons of single stranded DNA can be calculated as % tail DNA x 0.042 under conditions used in our modification of the method.

Results (Figure 2) show the influence of chosen chemicals on DNA after 2 hours (Figure 2a) and 24 hours (Figure 2b). Figure 2a shows the DNA damage caused by atropine. The number of DNA breaks (SSB) is not increased with increasing concentration. The similar effect is observed in the presence of endonuclease III, except for a concentration of 2.5 mM. where the amount of endo III sites increased up to 30% DNA in tail.
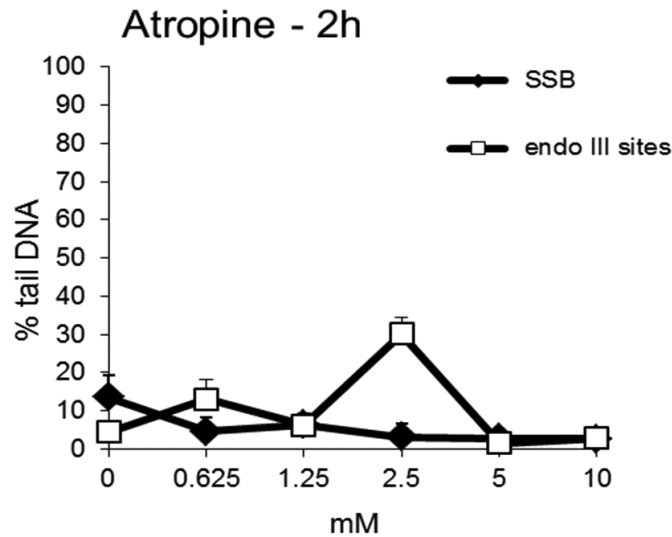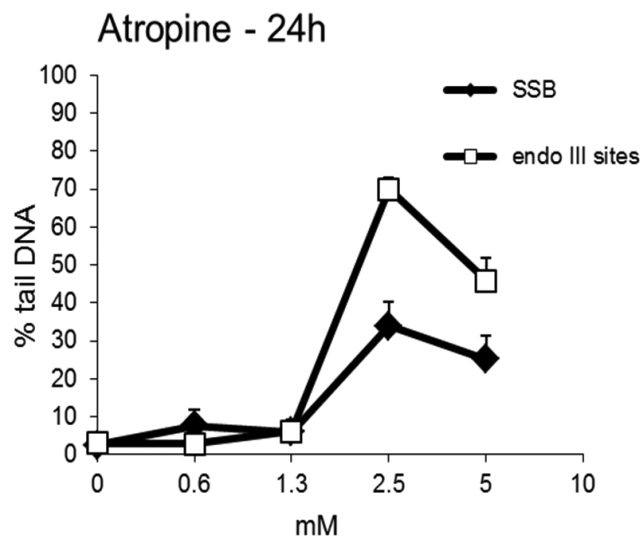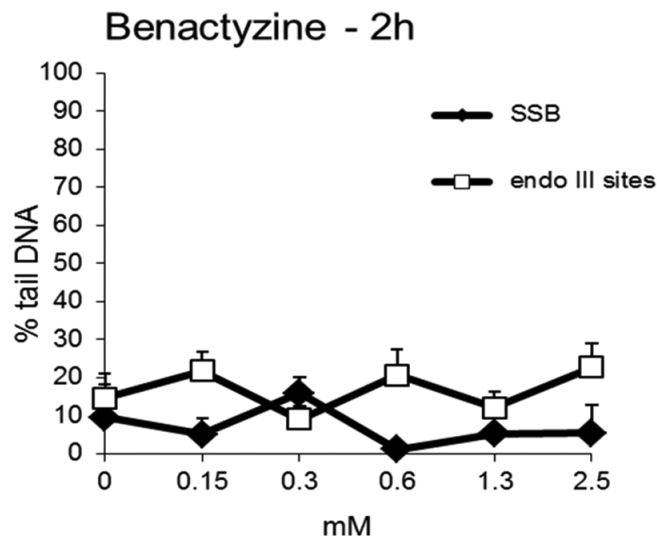

Figure 2a. DNA damage induced in HepG2 cells by atropine for 2 hours

Figure 2b. DNA damage induced in HepG2 cells by atropine for 24 hours

We did not observe any effect up to concentration 1.5mM after 24 hours (see Figure 2b). At higher concentrations, the level of the DNA damage increases, both SSB (up to 34 % tail DNA) and oxidative DNA damage (up to 70% tail DNA). The difference between the DNA breaks (34% tail DNA) and endo III sites (70% tail DNA) may express the amount of oxidative DNA damage

induced with Paradoxically, compared to these values, a lower DNA damage was found in cultures treated with 5 mM (28 % and 46 % respectively). This may be a result of the damaging effect of the higher dose, after which the DNA in the majority of cells is highly damaged and these cells may disappear during the electrophoresis. Only cells which were more resistant to the treatment remain, showing relatively lower DNA damage.

Benactyzine (see Figure 3a and 3b) does not seem to influence significantly DNA damage at either time interval.

Figure 3a. DNA damage induced in HepG2 cells by benactyzine for 2 hours
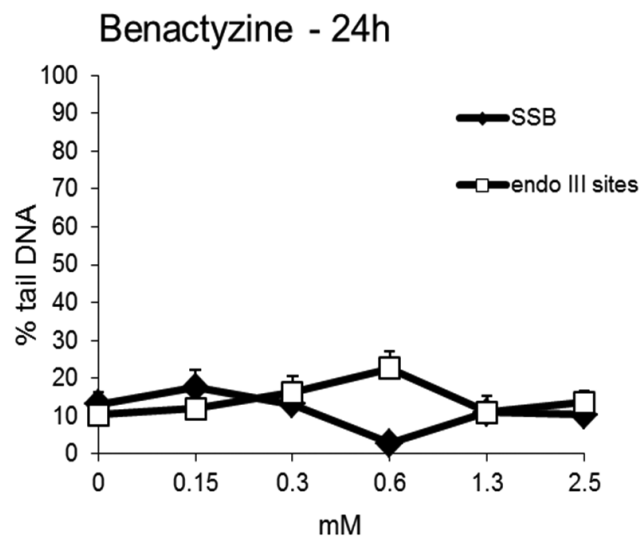

Figure 3b. DNA damage induced in HepG2 cells by benactyzine for 24 hours

Obidoxime (see Figure 4a) showed modest decrease of SSB and endo III sites in comparison with control cells (16% tail DNA) up to concentration 2.5 mM for SSB and 1.25 mM for oxidative damage. From these points, measured values increase up to 26% for DNA breaks and up to 24% for endo III sites with increasing concentrations. But this increase of percentage of DNA damage in tail is not significant as

in the case of obidoxime after 24 hours. At the treatment interval of 24 hours there is substantial increase of both SSB and endo III sites (see Figure 4b) from values close to 20 % of tail DNA up to 51% of

SSB and up to 70%, of endo III sites with increasing concentrations. However, this damage may reflect the DNA degradation thanks to the cytotoxic effect, rather than true induction of DNA breaks or endo III sites.
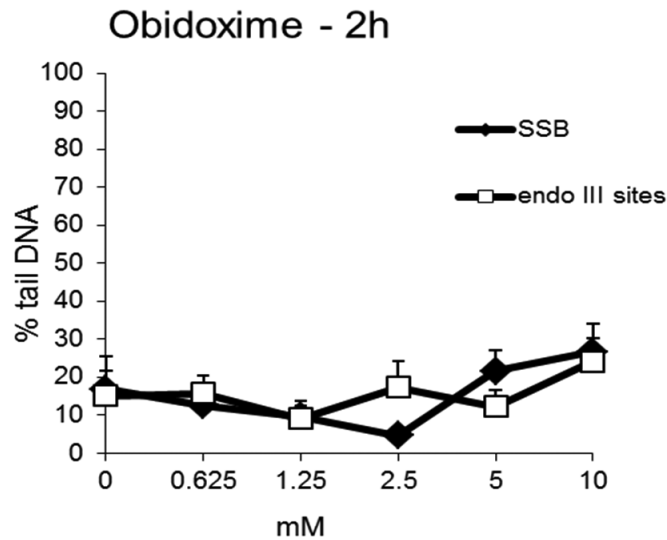

Figure 4a. DNA damage induced in HepG2 cells by obidoxime for 2 hours
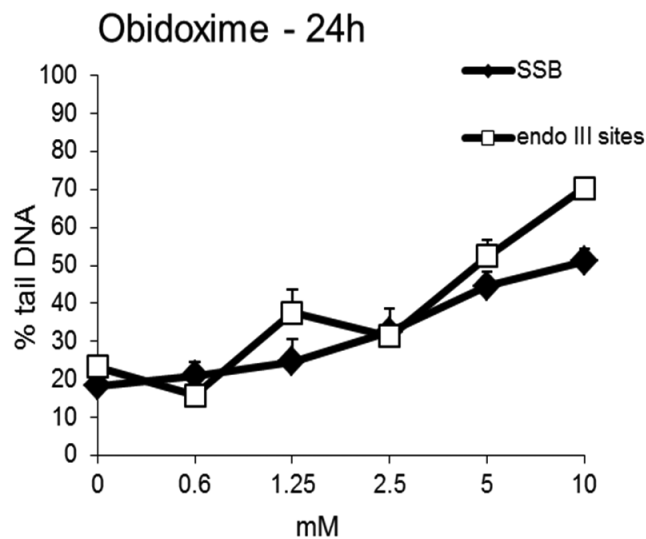

Figure 4b. DNA damage induced in HepG2 cells by obidoxime for 24 hours

Methoxime after 2 hours (see Figure 5a) did not induce any significant changes compared to negative control. On the other hand, after 24 hours (see Figure 5b) there is an increase of DNA breaks in

concentration range between 1.25 mM and 2.5 mM (40% tail DNA). From this point, results exhibit modest decrease up to 20% of SSB and 26% tail DNA in oxidative damage of DNA.
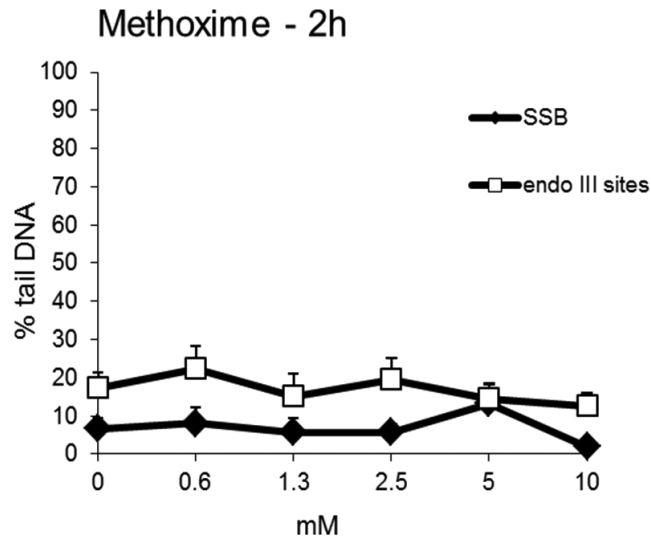
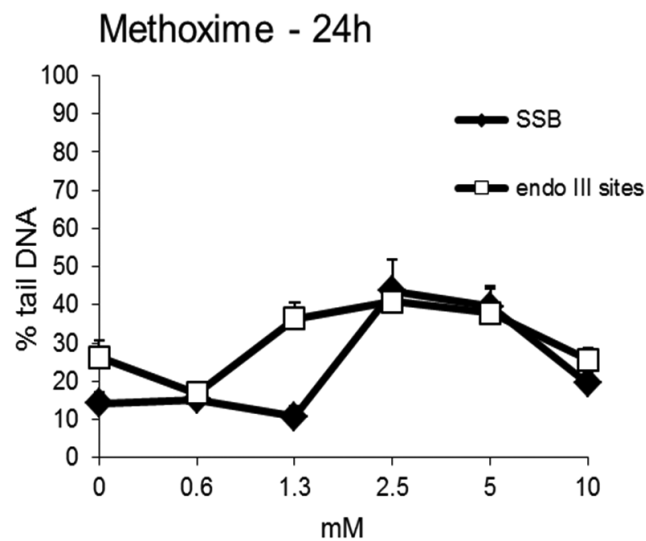**Figure 5a.** DNA damage induced in HepG2 cells by methoxime for 2 hours

Figure 5b. DNA damage induced in HepG2 cells by methoxime for 24 hours

The same measurements of the induction of DNA damage carried out on A549 cells treated with tested chemicals did not show any significant induction of DNA damage (results not shown). These results support our use of HepG2 cells in this study, indicating a higher sensitivity of HepG2 cells compared to A549.

In conclusion, we can say that none of the tested compounds showed significantly pronounced genotoxicity. Practically, none of the tested compounds exert the induction of DNA damage after the treatment of cells for 2 h with the highly cytotoxic concentration 2.5 mM. On the other hand, after 24 hours of the treatment with this high concentration the degradation of DNA takes place (except of obidoxime), most probably as a consequence of cell death by the apoptosis or necrosis.

These presented measurements, facilitate comparison and evaluation of cytotoxic and genotoxic effect of established medicaments on cells, which might contribute to our understanding of possible interactions in organism.

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