Evaluation of HI-6 oxime: potential use in protection of human acetylcholinesterase inhibited by antineoplastic drug irinotecan and its cyto/genotoxicity in vitro

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The function of acetylcholinesterase (AChE) is the rapid hydrolysis of the neurotransmitter acetylcholine (ACh), which is involved in the numerous cholinergic pathways in both the central and the peripheral nervous system. Therefore, AChE measurement is of high value for therapy management, especially during the course of intoxication with different chemicals or drugs that inhibit the enzyme. Pyridinium or bispyridinium aldoximes (oximes) are able to recover the activity of the inhibited enzyme. Since their adverse effects are not well elucidated, in this study the efficiency of HI-6 oxime in protection and/or reactivation of human erythrocyte AChE inhibited by the antineoplastic drug irinotecan as well as its cyto/genotoxicity in vitro were investigated. HI-6 was effective in protection of AChE and increased its activity up to 30%; the residual activity after irinotecan inhibition was 7%. Also, it reactivated the enzyme previously inhibited by 50% irinotecan (4.6 µg/ml) applied at ¼ of the IC50 value. The tested concentrations of HI-6 exhibited acceptable genotoxicity towards white blood cells, as estimated by the alkaline comet assay, DNA diffusion assay and cytogenetic endpoints (structural chromosome aberrations and cytokinesis-block micronucleus assay). The results obtained warrant the further investigation of HI-6 in vivo, as well as its development for possible application in chemotherapy.

Keywords: HI-6, acetylcholinesterase, protection, reactivation, irinotecan, comet assay, apoptosis, chromosome aberrations, micronuclei

INTRODUCTION

The best-characterised function of acetylcholinesterase (AChE; EC 3.1.1.7) is the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Taylor & Radić, 1994; Massoulié et al., 1999), which is involved in the numerous cholinergic pathways in both the central and peripheral nervous system. Consequently, inhibition of AChE results in severe cholinergic toxic signs caused by increased concentration of ACh at cholinergic nerve-nerve and nerve-muscle synapses (Somani & Romano, 2001). Muscular and nerve AChE are only present in the synaptic cleft and cannot be measured directly. Since erythrocyte AChE has a similar structure as the synaptic enzyme, it appears to be a...
suitable model to reflect the various reactions at the synaptic site (Mortensen et al., 1998). Therefore, its measurement is of high value for therapy management, especially during the course of intoxication with different chemicals or drugs that inhibit the activity of the enzyme.

Clinical studies indicate the possibility of acute cholinergic side effects after intravenous administration of a new broad-spectrum anticancer drug, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin; CPT-11) (Grisaru et al., 1999; Morton et al., 1999), which, on the basis of in vitro experimental findings, has been ascribed to a direct blockade of AChE (Dodds & Rivory, 1999; Hyatt et al., 2005). This drug has shown remarkable antitumor activity in the treatment of a variety of solid tumors. However, as with all cytotoxic chemotherapeutic agents, severe toxicity is frequently seen with irinotecan administration. The principal dose-limiting toxicity of irinotecan is diarrhoea. It can cause either acute diarrhoea related to a cholinergic surge from inhibition of AChE (Gandia et al., 1993; Abigerges et al., 1994; Bugat et al., 1994; Petit et al., 1997; Hyatt et al., 2005), or a delayed diarrhoea syndrome, which is possibly related to the accumulation of an active metabolite of irinotecan in the bowel (Hecht, 1998). While the cholinergic syndrome is not life-threatening, it is very unpleasant for the individual and the patient requires constant monitoring during drug infusion (Gandia et al., 1993). Since this syndrome is associated with high dose irinotecan administration, measures to prevent and reduce the side effects associated with irinotecan are currently being explored (Furman et al., 2003; Waadkins et al., 2004).

Current evidence indicates that atropine is favorable in the management of acute cholinergic syndrome (Rothenberg et al., 1993). Namely, muscarinic symptoms as nausea, vomiting, diarrhoea and bowel movements can be stopped by its administration. Unfortunately, the available literature indicates that systematic adverse reactions to atropine are possible as well. Therefore, development of better protective compounds is needed. There are two types of adverse reactions to atropine: toxic (atropine toxicity) and allergic (O’Connor & Mummma, 1985; Aguilera et al., 1988). The toxic reaction to atropine results from its anti-cholinergic action and includes a variety of peripheral and central manifestations. This toxic effect may occur at the usual therapeutic doses (Economacos & Kanakis, 1981). Although cases of severe systemic reaction to atropine are very rare, it is important to identify patients allergic to atropine and to provide treatment regimens with alternative drugs.

Derivatives from the group of bis-pyridinium aldoximes (called oximes), such as HI-6 [(1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxopropane dichloride)] are employed for the protection/reactivation of inhibited AChE. The mechanism of action of HI-6 as a therapeutic component is clear now. It reactivates AChE inhibited by organophosphorus agents and may also have beneficial antinicotinic effects (Tattersal, 1983; Sheridan et al., 2000). Also, HI-6 can be highly effective in restoring some active AChE where the nicotinic effects of excessive ACh are not antagonized by atropine (Jonson et al., 2000).

According to the exceptional properties of HI-6, it seems reasonable to investigate the potential use of this oxime in protection of AChE inhibited by some antineoplastic drugs (US patent 5916903) too. Taking into account the assumption by Gandia et al. (1993), that the mechanism of cholinergic syndrome induced by irinotecan might be ascribed to a direct stimulation of nicotinic autonomic ganglia, we attempted here to investigate the use of HI-6 oxime to protect or reactivate AChE inhibited by irinotecan in vitro. Since in drug research it is desirable to find not only the most active, but also the least toxic substances, we wanted to assess the toxicity of HI-6 in the dose range used for effective protection/reactivation of inhibited AChE. In order to estimate the potential cytogenic and genotoxic effects of HI-6 in human peripheral blood leukocytes, a variety of assays were used: alkaline comet assay, DNA diffusion assay, analysis of structural chromosome aberrations and cytochalasin-block micronucleus assay.

MATERIAL AND METHODS

Blood sampling. Blood sample was obtained from a healthy female donor (age 37 years, non-smoker) who gave an informed consent for participation in the study. The donor had not been exposed to diagnostic or therapeutic irradiations or to known genotoxic chemicals for a year before blood sampling. Venous blood (40 ml) was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson) containing lithium heparin as anticoagulant.

Enzyme assay and preparation. The activity of AChE was measured spectrophotometrically according to the Ellman procedure with the thiol reagent DTNB (final concentration 0.30 mmol/l) (Ellman et al., 1961). All experiments were done in 0.1 mol/l phosphate buffer, pH 7.4, and enzyme activities were measured in the presence of 1.0 mmol/l acetylthiocholine iodide (ATCh) as substrate at 37°C. Intact native human erythrocytes were used as the source of AChE; the final dilution during enzyme assay was 600-fold. The reaction was performed in
the total volume of 3.0 ml. The increase in absorbance was read at 412 nm at 15 s intervals against a blank that contained the erythrocytes suspended in buffer and DTNB. All spectrophotometric measurements were performed using a CECIL 1000 spectrophotometer with temperature controller.

**Chemicals and reagents.** The structure of the studied oxime HI-6 and of irinotecan is given in Fig. 1. HI-6 was synthesized in Bosnalijek, Sarajevo (Bosnia and Herzegovina). It was kept at room temperature and was dissolved in sterile injection water immediately before use. Concentrations of HI-6 tested for the cyto/genotoxicity corresponded to ¼ LD$_{50}$, IC$_{50}$, P$_{50}$ and a concentration that yielded 50% enzyme reactivation (R$_{50}$). Irinotecan (CAMPTO$^\text{®}$) was purchased from Aventis Pharma Ltd. It was used in the form of irinotecan hydrochloride trihydrate. Before use, it was dissolved in 5 ml of provided sterile solution to a final concentration of 20 mg/ml. The concentrations of the antineoplastic drug tested in our in vitro experiment were 9.0 µg/ml and 4.6 µg/ml, corresponding, respectively, to doses of 350 mg/m$^2$, recommended in monotherapy, and 180 mg/m$^2$ recommended in combination chemotherapy in adult patients. Other chemicals and reagents used, if not specified, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Inhibition of AChE by HI-6 and irinotecan.**

a) The concentration of the compound yielding a 50% enzyme inhibition (IC$_{50}$) was determined by incubating erythrocytes with four or more different concentrations of both compounds at 37°C and assaying for AChE activity after 15 min. The changes in the enzyme activity are presented as the percent-age of activity of the respective of control. Only values between 10% and 90% inhibition were used for calculation. b) For the purpose of biochemical experiments, we also determined the catalytic activity of AChE previously inhibited with two different concentrations of irinotecan (9.0 and 4.6 µg/ml) or HI-6 (37.7 µg/ml; ¼ of its IC$_{50}$). The reaction mixture was incubated for 10, 30, 60, 120, 150 and 180 min at 37°C after addition of each drug and the activity of AChE was measured. Each sample was assayed in triplicate, and a mean value was then calculated. The effects of irinotecan and HI-6 were expressed as percentages of the AChE activity of the respective of control.

**Protective potency of HI-6.** The protective potency of HI-6 with respect to irinotecan-inhibited AChE was tested by measurement of enzyme inhibition in the presence and absence of HI-6. Native human erythrocytes were diluted with phosphate buffer, pH 7.4; HI-6 (37.7 µg/ml) and irinotecan (4.6 and 9.0 µg/ml) were added in succession. Irinotecan was added 1, 5 or 15 min after HI-6, the reaction mixture was then incubated at 37°C for further 30 min and activity of AChE was measured. The in vitro protective efficiency of HI-6 against irinotecan inhibition of human erythrocyte AChE was expressed as protective potency (Pp).

**Reactivating potency of HI-6.** Native human erythrocytes were incubated with two different concentrations of irinotecan (4.6 or 9.0 µg/ml) for 15 min at 4°C to obtain approx. 90–95% inhibition of AChE activity. The reaction mixture was diluted (1:600) with phosphate buffer and the activity of AChE was measured in the presence of irinotecan and HI-6 (37.7 µg/ml). Simultaneously, the activity of AChE from native erythrocytes diluted identically was measured in the presence of HI-6. Prior to AChE assay, the reaction mixture was incubated at 37°C for 0.5, 1 or 24 h. The percentage of reactivation (%R) was calculated according to De Jong et al. (1989).

**Assessment of cyto/genotoxicity of HI-6.**

**Experimental design:** Two independent experiments were conducted on the same blood sample and the data were pooled. Aliquots of whole blood (V = 0.5 ml) were placed in sterile plastic tubes containing RPMI culture medium (Gibco; V = 6 ml) without serum or mitotic activator. Duplicate cultures per sample were set up, treated with HI-6 and were incubated for 30 min at 37±1°C in humified atmosphere with 5.0% CO$_2$ (Heraeus Hera Cell 240 incubator). The final concentrations of HI-6 in culture medium with human blood cells in this in vitro experiment were 2.5×10$^{-1}$ mM (¼ LD$_{50}$); 4.2×10$^{-1}$ mM (IC$_{50}$); 2.2×10$^{-1}$ mM (P$_{50}$) and 2.0×10$^{-2}$ mM (R$_{50}$). Negative and positive controls were also included and handled in the same manner. Following treatment, cultures were centrifuged for 10 min at 600 r.p.m.,
and the culture medium containing HI-6 was carefully removed. A part of the treated samples was immediately used for the preparation of slides for the alkaline comet assay and DNA diffusion assay. Other blood samples were used for the analysis of structural chromosome aberrations and cytochalasin-blocked micronucleus assay (CBMN assay). These blood cells after centrifugation were washed twice in fresh RPMI medium and cultivated for further 48 h in vitro according to the standard procedure.

The alkaline comet assay. The comet assay was carried out under alkaline conditions, basically as described by Singh et al. (1991). Fully frosted slides were covered with 1% normal melting point (NMP) agarose. After solidification, the gel was scraped off from the slide. The slides were then coated with 0.6% NMP agarose. When this layer had solidified a second layer containing the blood sample (5 µl) mixed with 0.5% low melting point (LMP) agarose was placed on the slides. After 10 min of solidification on ice, slides were covered with 0.5% LMP agarose. Afterwards the slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris/HCl, 1% Na-deoxycholate, pH 10) with 1% Triton X-100 and 10% dimethyl sulfoxide added fresh to lyse cells and allow DNA unfolding. The slides were then randomly placed side by side in a horizontal gel-electrophoresis tank, facing the anode. The unit was filled with freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13.0) and the slides were left in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 min at 25 V (300 mA). After electrophoresis the slides were washed gently three times at 5-min intervals with a neutralisation buffer (0.4 M Tris/HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 µg/ml) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis. To prevent additional DNA damage, handling of blood samples and all steps in the preparation of slides for the comet analysis were conducted under yellow light or in the dark. Furthermore, to avoid possible position effects during electrophoresis two parallel replicate slides per sample were prepared. Each replicate was processed in a different electrophoretic run. Corresponding negative and positive controls were also included. As a positive control hydrogen peroxide treatment (1 mM; 5 min exposure on ice) was used.

Slides were examined at 250× magnification using a fluorescence microscope (Zeiss, Germany), equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The microscope was connected through a black and white camera to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd, UK). A total of 100 comets per sample were scored (50 from each of two replicate slides). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. To avoid the potential variability, one well-trained scorer performed all scoring of the comets. Three comet parameters: tail length, tail DNA percentage and tail moment were evaluated. Statistical analyses were carried out using Statistica 5.0 software (StatSoft, Tulsa, USA). Each sample was characterized for the extent of DNA damage by considering the mean (± standard error of the mean), median and range for the comet tail lengths measured. In order to normalize the distribution and to equalize variances, a logarithmic transformation of data was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analysis of differences was done by Scheffé test. The level of statistical significance was set at P<0.05.

DNA diffusion assay. Twelve hours after the end of each treatment, DNA diffusion assay was performed. We followed the protocol described by Singh (2003). Chemicals needed for DNA diffusion assay were provided by Sigma. Agarose-precoated slides were made by spreading 50 µl of 0.7% normal melting agarose on each slide and drying them at room temperature. Microgels were made on the agarose-precoated slides by mixing 25 µl of whole blood culture (10000 lymphocytes) with 50 µl of 0.8% high-resolution agarose and pipetting it onto the slide. The gel was immediately covered with a coverslip. The slides were coded and cooled on ice for 1 min. The cover glasses were removed, and 200 µl of 2% agarose solution was layered and again covered with the cover glass. After keeping slides for 1 min on ice, cover glasses were removed and the slides were immersed in a freshly made lysis solution (1.25 M NaCl, 1 mM EDTA, 5 mM Tris/HCl pH 10, 0.01% sodium lauroyl sarcosine, 0.2% DMSO added freshly, 300 mM NaOH added freshly) for 10 min at room temperature. After lysis, the slides were immersed in neutralizing solution (50% ethanol, 1 mg/ml spermine, 20 mM Tris/HCl pH 7.4) for 30 min at room temp., twice. The slides were air-dried and stored at room temperature. Slides were stained with YOYO-1 dye (0.25 µM YOYO-1 in 2.5% DMSO, 0.5% sucrose) for 15 min. One-thousand lymphocytes per slide were analyzed. Lymphocytes undergoing apoptosis or necrosis were distinguished from normal cells according to figures and instructions given by Singh (2003). Apoptotic cell nuclei have a hazy or undefined outline without any clear boundary due to nucleosomal-
sized DNA diffusing into agarose. Necrotic cell nuclei are bigger and are poorly defined. They have a clear, defined outer boundary of the DNA halo and a relatively homogeneous appearance of the halo. In the contrast, cell nuclei with only damaged DNA (not necrotic or apoptotic) are clearly defined and their nuclei have a larger halo with a clear outer boundary.

Chromosome aberration (CA) test. The chromosome aberration test was performed according to current IAEA guidelines (2001) with some modifications. Blood samples were cultivated in RPMI-1640 medium (Gibco) containing inorganic salts, all amino acids, vitamins and other components. The medium was supplemented with 20% fetal calf serum (Gibco), 10% autologous serum, penicillin (100 IU/ml; Sigma), and streptomycin (100 µg/ml; Sigma). For mitotic stimulation phytohaemagglutinin (Apogent) was added. Duplicate cultures per sample were set up and incubated at 37 ± 0.5°C for 48 h in humified atmosphere with 5.0% CO₂. Corresponding negative and positive controls (ethyl methanesulfonate, 30 µg/ml) were also included. Preparations were made according to standard procedure. Slides were stained with 5% Giemsa solution (Sigma). All slides were coded and scored blindly. Five-hundred metaphases per sample were analysed for chromosomal aberrations. Structural chromosome aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 45–47 centromeres were analysed. Percentages and types of aberrations per each sample were determined. Statistical significance of the results obtained was evaluated by χ² test. The level of statistical significance was set at P<0.01.

Cytochalasin-blocked micronucleus assay (CBMN). Lymphocyte cultures were incubated according to a standard protocol for CBMN assay (Fenech & Morley, 1985). Corresponding negative and positive controls (bleomycin, 10 µg/ml) were also included. Cytochalasin B at a final concentration of 6 µg/ml was added to the culture at 44 h. For MN identification the criteria of Fenech et al. (2003) were used. The number of micronuclei (MN) in 2000 binucleated (BN) cells was scored for each treatment and the number of BN cells with MN was also recorded. On the same slides the proliferation index was also evaluated by classifying cells according to the number of nuclei (cytokinesis-block proliferation index, CBPI), as suggested by Surralles and co-workers (1995) until 1000 binuclear lymphocytes were scored. We used their formula: CBPI=[M1+2M2+3(M3+M4)]/N where M1–M4 indicate the number of cells with 1–4 nuclei, respectively, and N the total number of cells scored.

RESULTS

Inhibition of AChE by HI-6 and irinotecan

To further address the question of whether irinotecan acts as specific AChE blocker, we checked its action in vitro on human erythrocyte AChE. Results obtained in previous in vitro studies suggest that this drug interacts directly with various molecular forms of AChE, leading to a non-competitive inhibition of this enzyme (Morton et al., 1999; Dodds & Rivory, 1999). In our experiments, the inhibitory power (IC₅₀) of irinotecan on AChE of human erythrocytes was determined and compared with the HI-6 oxime a known reversible inhibitor of AChE and antidotal drug in organophosphorus poisoning. The IC₅₀ values obtained were 5.0×10⁻⁴ mmol/l for irinotecan and 4.2×10⁻¹ mmol/l for HI-6 (Fig. 2). The values indicate that HI-6 is an approx. 840-fold less potent inhibitor than irinotecan, which was found to be a strong inhibitor of ACh hydrolysis by AChE. Also, a decrease of the catalytic activity of a human erythrocye AChE with prolonged incubation of cells with either dose of irinotecan. There was a slight reduction in the inhibition of AChE by both of doses of irinotecan with increased incubation times. The higher dose of irinotecan inhibited the AChE activity with a potency similar to that estimated for physostigmine in our previous study (Kopjar et al., 2007). HI-6 inhibited the AChE activity by up to 50% 150 min after its addition (Fig. 3).

Protective potency

The protective potency of HI-6 towards human erythrocyte AChE inhibited by two different doses of irinotecan is given in Table 1. The residual activities after inhibition with irinotecan (4.6 and 9.0 µg/ml) were 6.7 and 6.0%, respectively. HI-6 demonstrated a good protective potency against inhibition

![Figure 2. In vitro inhibition of AChE by HI-6.](image-url)
of AChE with both concentrations of irinotecan. The AChE activity in the presence of HI-6 was increased up to 30% of the irinotecan-inhibited level. The best result was obtained when the oxime was used 5 min before irinotecan (4.6 µg/ml). Also, under same experimental conditions a higher dose of irinotecan did not induce an expected decrease in AChE activity that was decreased only by 8–10%. We obtained identical or similar results when irinotecan was added 1 or 15 min after HI-6. All in all, these results indicated that HI-6 is able to protect AChE against inhibition by irinotecan.

Reactivating potency

The ability of HI-6 (applied at a final concentration of ¼ of its IC\textsubscript{50} value) to reactivate irinotecan-inhibited human erythrocyte AChE is shown in Table 1, too. Reactivation was quantitated using the De Jong’s equation for the calculation of percentage of reactivation. The range of reactivation was from 36 to 50%. The highest percentage of AChE activity (50%) previously inhibited by irinotecan (4.6 µg/ml) was obtained 24 h after addition of HI-6. According to the obtained results, the HI-6 oxime seems to be a potent AChE reactivator at the concentration of ¼ of its IC\textsubscript{50}. This concentration level could be relevant for human use (Tattersall, 1993).

The alkaline comet assay

Results of the alkaline comet assay indicate that the exposure of human blood to HI-6 in concentrations tested did not induce any significant increase of primary DNA damage in white blood cells as compared to negative control. On the other hand, the values of all comet parameters (tail length, tail DNA percentage and tail moment) recorded in the positive control were significantly increased (\(P<0.01\), ANOVA with post-hoc Schêffe test) as compared both to HI-6-treated samples and untreated cells (Table 2). This is also clearly visible in Fig. 4, where the results of the evaluation of DNA damage in individual samples are displayed in the form of Box and Whisker diagrams.

DNA diffusion assay

Results of the DNA diffusion assay indicate that the exposure of human blood to HI-6 in concentrations tested induced only a slight, non-significant increase in the percentage of apoptotic cells as compared to negative control. On the other hand, the percentage of apoptotic cells observed in the positive control were significantly higher (\(P<0.01\), \(\chi^2\) test) as compared both to HI-6-treated samples and untreated cells. Necrotic cells were observed only in blood sample treated with HI-6 in concentration corresponding to R\textsubscript{50} (but this was not statistically significant) as well as in the positive control (Table 2).

Analysis of structural chromosome aberrations in lymphocytes

\textit{In vitro} treatment with HI-6 did not induce a significant increase of chromosomal damage in peripheral blood lymphocytes as compared to negative control. It mainly induced one to four additional chromatid breaks, while a single acentric fragment was recorded only in the sample treated with the concentration corresponding to IC\textsubscript{50}. On the other

<table>
<thead>
<tr>
<th>Concentration of irinotecan (µg/ml)</th>
<th>Pp (%)</th>
<th>R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>9.0</td>
<td>20</td>
<td>17</td>
</tr>
</tbody>
</table>

\(a\)Pp is the protective potency of HI-6 expressed as percentage of preserved activity of AChE in the presence of two concentrations of irinotecan; R is percentage of reactivation of human erythrocyte AChE with HI-6, previously inhibited by irinotecan.
HI-6 protection/reactivation of AChE inhibited by irinotecan in vitro

hand, ethyl methanesulfonate (positive control) caused a clear positive response both in the frequency and types of chromosomal aberrations recorded (Table 3).

Table 2. Results of alkaline comet assay and DNA diffusion assay on human white blood cells treated in vitro with HI-6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alkaline comet assay</th>
<th>DNA diffusion assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tail length (µm)</td>
<td>Tail intensity (DNA %)</td>
</tr>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Min</td>
</tr>
<tr>
<td>HI-6 ¼ LD₅₀</td>
<td>13.61 ± 0.13</td>
<td>11.54</td>
</tr>
<tr>
<td>HI-6 IC₅₀</td>
<td>12.87 ± 0.13</td>
<td>10.26</td>
</tr>
<tr>
<td>HI-6 P₅₀</td>
<td>13.08 ± 0.12</td>
<td>10.26</td>
</tr>
<tr>
<td>HI-6 R₅₀</td>
<td>13.27 ± 0.16</td>
<td>10.26</td>
</tr>
<tr>
<td>Positive control</td>
<td>18.62 ± 0.84*</td>
<td>10.26</td>
</tr>
<tr>
<td>Negative control</td>
<td>13.24 ± 0.10</td>
<td>10.26</td>
</tr>
</tbody>
</table>

* Treatments lasted for 30 min. Negative control and positive control (1 mM hydrogen peroxide) were also included. Primary DNA damage was evaluated in 100 comets per sample and statistical evaluation (ANOVA with post-hoc Schéffe test) was done on logarithmically transformed data. For DNA diffusion assay 1000 cells per slide were analyzed and statistical evaluation was performed by χ² test.

Table 3. Structural chromosome aberrations in human peripheral blood lymphocytes treated in vitro with HI-6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells with CA (%)</th>
<th>No. of structural chromosome aberrations/500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Σ</td>
<td>Chromatid break</td>
</tr>
<tr>
<td>HI-6 ¼ LD₅₀</td>
<td>0.8</td>
<td>4</td>
</tr>
<tr>
<td>HI-6 IC₅₀</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>HI-6 P₅₀</td>
<td>0.6</td>
<td>3</td>
</tr>
<tr>
<td>HI-6 R₅₀</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>Positive control</td>
<td>4.4*</td>
<td>25*</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.4</td>
<td>2</td>
</tr>
</tbody>
</table>

* Treatments lasted for 30 min. Negative control and positive control (ethyl methanesulfonate, 30 µg/ml) were also included. Statistic evaluation was done using χ² test; *P<0.01 as compared to all other samples.

Table 4. Results of CBMN assay in human peripheral blood lymphocytes treated in vitro HI-6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Σ MNed BN</th>
<th>No. of BN with</th>
<th>Σ MN</th>
<th>Total No. of</th>
<th>Lymphocyte proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 MN</td>
<td>2 MN</td>
<td>3 MN</td>
<td>Total</td>
</tr>
<tr>
<td>HI-6 ¼ LD₅₀</td>
<td>13</td>
<td>13</td>
<td>–</td>
<td>–</td>
<td>13</td>
</tr>
<tr>
<td>HI-6 IC₅₀</td>
<td>16</td>
<td>15</td>
<td>1</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>HI-6 P₅₀</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>–</td>
<td>14</td>
</tr>
<tr>
<td>HI-6 R₅₀</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>–</td>
<td>15</td>
</tr>
<tr>
<td>Positive control</td>
<td>60</td>
<td>45</td>
<td>12</td>
<td>3</td>
<td>78*</td>
</tr>
<tr>
<td>Negative control</td>
<td>13</td>
<td>12</td>
<td>1</td>
<td>–</td>
<td>14</td>
</tr>
</tbody>
</table>

* Treatments lasted for 30 min. Negative control and positive control (bleomycin, 10 µg/ml) were also included. Binucleate cells, 2000, (i.e. 4000 nuclei) were scored to determine total number of micronuclei (MN) for each experimental point. MNed, micronucleated; BN, binucleate cells; NPB, nucleoplasmic bridges; APO, apoptosis; NC, necrosis; CBPI, cytokinesis-block proliferation index is computed by the formula CBPI = [M1 + 2M2 + 3(M3 + M4)]/N where M1–M4 indicate the number of cells with 1–4 nuclei, respectively, and N the total number of cells scored. Statistic evaluation was done using χ² test; *significantly different (P<0.05) as compared with all other samples; #significantly different (P<0.05) as compared with all samples except positive control.
concentration of the oxime tested, the MN frequency was not significantly different compared to negative control. On the other hand, bleomycin (used as a positive control) increased the number of micronuclei over 4-fold. The majority of BN in HI-6-treated samples contained only one MN with only a small proportion with 2 MN, while BN with 3 MN were recorded only in the positive control (Table 4). Besides the induction of MN, in vitro treatment with HI-6 also induced formation of nuclear buds, nucleoplasmic bridges and apoptotic cells, but their frequencies were not significantly higher than in the negative control. In HI-6-treated samples and negative control no necrotic cells were observed, while their frequency in the positive control was 8 necrotic cells per 2000 binuclear cells (Table 4). The values of cytokinesis-block proliferation index indicate that HI-6 retarded lymphocyte proliferation in vitro only at the concentration corresponding to IC50 (Table 4).

**DISCUSSION**

The treatment of cancer disease by chemotherapy is presently common. Chemotherapy may be used either alone or in conjunction with surgical removal of a tumour and/or radiation therapy. Although the goal of chemotherapy is to eliminate cancer cells while sparing the normal ones, some adverse effects in normal cells are almost always observed. The two major obstacles in an effective cancer therapy are drug resistance and toxicity to normal organs that prevent the use of optimal doses and schedules. The development of non-toxic selective and effective cytoprotective compounds that preferentially protect normal tissues from chemotherapy toxicity, without protecting the malignant tissue, is a major challenge in cancer chemotherapy research. Such compounds could potentially protect against the genetic damage, mutation, immune system alterations, as well as teratogenic effects.

The most susceptible cells during chemotherapy are those with a high rate of cell division, e.g., bone marrow, hair bulb and the gastrointestinal tract. Besides, the nervous system is frequently a site of symptomatic toxicity of antineoplastic agents. Chemotherapy induces stress in the nervous system (both central and peripheral) by inhibiting its ability to efficiently transmit nerve impulses along the synapse. Neurotoxicity, as a common unwanted side-effect of antineoplastic drugs, can cause significant long-standing disability because regeneration of the nervous system is poor. For example, it has been found that administration of high levels of the therapeutic agent taxol may result in severe neurotoxicity in the form of peripheral neuropathy (US Patent 5916903). Furthermore, most of the chemotherapy regimens currently used for treatment of the colorectal carcinoma are neurotoxic. It is also well documented that oxaliplatin, the newest drug used in treatment of colorectal carcinoma, induces peripheral neuropathy (Goetz et al., 2003). However, it is the only treatment modality for patients who cannot be treated with irinotecan due to a preexisting liver dysfunction or other reasons. Irinotecan, on the other hand, is a potent AChE inhibitor, which activity is likely responsible, at least in part, for the cholinergic syndrome that is observed following its intravenous infusion (Tobin et al., 2004; Hyatt et al., 2005).

Until today the cholinergic syndrome induced with irinotecan has been treated mainly with atro-
pine (ACh receptor antagonist), which reduces or alleviates the effects of antineoplastic disease treatment. Unfortunately, after its administration, a toxic reaction to atropine resulting from its anti-cholinergic action and including a variety of peripheral and central manifestations cannot be excluded. This toxic effect may occur at the usual therapeutic doses (Economacos & Kanakis, 1981).

AChE reactivators (oxime), such as HI-6, have been used in conjunction with the muscarinic cholinergic receptor antagonist atropine to provide in vitro protection against nerve agents and other organophosphorus compounds (Somani & Romano, 2001). However, AChE reactivators have not been previously employed to alleviate the side effects of antineoplastic disease treatment (US Patent 5916903). In this in vitro study an attempt was made to establish whether the HI-6 oxime, being known AChE reactivator, has a potency to enhance the activity of red blood cell AChE in human whole blood samples previously treated with irinotecan. All our initial results seem very promising, although they cannot provide evidence for therapeutic effectiveness of HI-6 in clinical settings. Irinotecan and HI-6 showed different in vitro toxic effects on human erythrocyte AChE, which corresponds well to the results of their toxicity previously obtained by other authors (Mesić et al., 1991; Dodds & Rivory, 1999; Tobin et al., 2004). In contrast to irinotecan, HI-6 is a poor inhibitor of human erythrocyte AChE in vitro. The IC_{50} value of HI-6 indicates low affinity of this compound for AChE, although it contains a quaternary pyridinium moiety in the molecule, which is known to have a certain affinity for human erythrocyte AChE (Bevandić et al., 1985; Galoši et al., 1988). The enzymatic activity decreased by up to 50% when the enzyme was exposed to irinotecan at the concentration of 5.0 × 10^{-4} mmol/l, or to HI-6 at 4.2 × 10^{-3} mmol/L. Continuous decrease of activity of AChE up to 180 min after addition of HI-6 was by 40–50%. Under the same experimental conditions the activity of AChE was inhibited by irinotecan in doses comparable to those recommended in mono- or combination therapy, by between 85 and 95%, and correlates with the activity of the same enzyme after inhibition with the well-known AChE irreversible blocker physostigmine (Kopjar et al., 2007). In our experiments we also demonstrated that HI-6 had a protective effect and reduced the degree of inhibition of AChE by irinotecan at least two-fold. Its chemical structure (the pyridinium rings, the bridge between pyridinium rings and the nucleophilic activity of the oxime anion that is bound to the pyridinium ring) is known to be important for the protective potency and rate of the reactivation of inhibited AChE. The much better reactivation than protection potency obtained with a concentration of HI-6 relevant for humans corresponded to its antidotal efficacy in organophosphorus poisoning (Kassa et al., 1997).

Usually, the reactivating efficacy of an oxime in vitro corresponds to its therapeutic efficacy in vivo. For this reason we hope that this in vitro study will contribute to the progress in delineating new research strategies in the treatment of the cholinergic syndrome in patients with tumors. It is known that HI-6 crosses the blood-brain barrier (Clement, 1982) and we expect that it might protect or reanimate irinotecan-inhibited AChE in vivo, in the peripheral as well as in the central compartments of the nervous system.

Based on the results of the first part of this study concerning human erythrocyte AChE activity and its interactions with HI-6 oxide and the antineoplastic drug irinotecan, the relevance of this enzyme as surrogate marker of neuronal AChE activity is confirmed. Moreover, the data obtained in vitro indicate the potential of irinotecan to induce toxicity in the central nervous system which needs to be taken into account in future studies.

The present study reports for the first time results concerning possible genotoxic and cytoprotective effects of HI-6. Although the spectrum of cytoprotective agents available is relatively broad, most of them are either toxic or lack selective cytoprotective activity. Thus, HI-6, a member of the oxime family is certainly one of the promising candidates for further studies (US Patent 5916903). According to its exceptional properties, confirmed earlier in the treatment of organophosphate poisoning, it seems reasonable to investigate its potential use in protection of AChE inhibited by antineoplastic drugs. Since the drug is still experimental, extensive studies on its toxicity are necessary before it may be approved for the use in human subjects. Therefore, to elucidate the toxicity profile of HI-6, in the present study the cytogenotoxicity of the compound to peripheral blood leukocytes was studied in vitro using a variety of biomarkers.

Many techniques for detecting DNA damage could be used to identify potentially genotoxic substances. During recent years the comet assay or single cell gel electrophoresis has been established as a sensitive method for detecting DNA damage (Møller et al., 2000; Singh, 2000; Tice, 2000, Faust et al., 2004). One of the successful modifications of the method is the DNA diffusion assay (Singh, 2003) also employed in the present study in parallel with conventional cytogenetic techniques: chromosome aberration analysis and cytokinesis-block micronucleus assay.

Despite cytoprotective effects observed in non-proliferating cells, a substance could harm actively proliferating cells. To check this assumption
we used three techniques that substantially differ in the levels of the damage detection. Whereas the comet assay detects DNA damage in single cells and independently of cell proliferation, chromosome aberration analysis and the cytokinesis-block micronucleus assay are applicable to proliferating cells only. Combination of such methods is extremely important when studying the effects of compounds with potential use in human and animal medicine.

The present study confirmed that the alkaline comet assay is a useful complement to the standard cytogenetic analyses. The results obtained using the alkaline comet assay suggest that HI-6 did not induce significant DNA damage in human leukocytes. The results obtained using DNA diffusion assay also indicate a low cytotoxicity of HI-6 in concentrations tested. In vitro treatment with HI-6 did not induce a significant increase of chromosomal damage in peripheral blood lymphocytes (a few additional chromatid breaks only). Moreover, the frequency of MN even at the highest concentration of the oxime tested was not significantly increased. However, the values of cytokinesis-block proliferation index indicate that HI-6 retarded lymphocyte proliferation in vitro in the high concentration corresponding to IC$_{50}$. Trying to explain this observation, we also tested a higher concentration of the compound (corresponding to LD$_{50}$) and confirmed the same growth inhibitory effect. Since the effects of HI-6 in our study were investigated in human peripheral blood lymphocytes, a cell type that is predominantly in a DNA presynthetic stage of the cell cycle (i.e. the G$_0$ phase), additional analyses using other cell types and metabolic activation are necessary. Namely, dormant cells often differ in their response to the treatment and usually have a lower DNA repair capacity as compared to actively proliferating cells.

Based on the results of all experiments performed in the present study, we can conclude that HI-6 possesses acceptably low genotoxicity in vitro and therefore might be also useful as a potential cytoprotector. Nevertheless, its activity should be tested on other cell types to elucidate whether it is applicable for extensive use in vivo.

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