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ORIGINAL ARTICLE

Modulation of ionising radiation generated oxidative stress by HI-6 (asoxime) in a laboratory rat model

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Abstract

OBJECTIVES: HI-6 is an antidotum suitable for treatment of intoxication by nerve agents. The recent investigation appointed its modulation of inflammatory response as well as vegetative nervous system activity. However, the present experiments were carried out in order to assess the antioxidant effect of HI-6 in irradiated animals.
METHODS: male Wistar rats were irradiated by ionizing radiation (7.5 Gy, $LD_{50/30}$). Animals were divided into four groups: i.e. controls (A), irradiated (B), treated with HI-6 (C), and both irradiated and treated with HI-6 (D). Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and glutathione reductase activity were assayed in liver, spleen, plasma, and whole blood. Clinical biochemistry markers were determined in plasma samples.
RESULTS: We found significantly increased FRAP levels in liver, while its levels decreased in the spleen of B group animals. Ionising radiation (B group) also significantly elevated TBARS values in spleen. HI-6 reversed FRAP and TBARS values to control levels. Glutathione reductase activity was significantly elevated in spleen and liver of animals exposed to HI-6 (C and D groups). Clinical biochemistry markers were shifted only slightly. The in vitro test confirmed the inhibitory effect of HI-6 towards acetylcholinesterase.
CONCLUSIONS: In conclusion, HI-6 is potent in suppressing oxidative stress and might be a promising drug in the field of radiation protection.

INTRODUCTION

Although deleterious effects, including acute radiation sickness or genomic instability associated with higher cancer risks (Donnelly *et al.* 2010; Liu 2010), are well known, highly effective and broad antidotes protecting from ionizing radiation impact have not been found despite extensive research efforts (Zhorova *et al.* 2010). The ionizing radiation is a source of oxidative stress, i.e. generation of reactive oxygen and nitrogen species, which leads not only to DNA damage but also affects the function of proteins regulating basic cellular mechanisms including DNA repair (Shuryak & Brenner 2009; 2010). To prevent ionizing radiation effects, antioxidants such as polyphenols and flavonoids extracts have been investigated as drugs reducing oxidative stress and its impact on the irradiated organisms (Verma *et al.* 2010). Nevertheless, the protective index of alimentary administered antioxidants is not usually higher than 1.3 (Okunieff *et al.* 2008; Calabro-Jones *et al.* 1998).

Another promising way to reduce oxidative stress is based on the up-regulation of antioxidant enzymes. The enzymes cannot be administered directly due to poor distribution and possible alteration of the immune system. Therefore, experiments aimed at supplementation of the metal cofactors of enzymatic antioxidants such as selenium have been carried out (Micke *et al.* 2009; Mucke *et al.* 2010). However, metal toxicity seems to be a limiting factor of this approach (Wallenberg *et al.* 2010; Jamier *et al.* 2010).

Asoxime chloride (CAS 344433-31-3; 1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride), known as HI-6, is an oxime reactivator used for restoration of enzyme acetylcholinesterase (AChE) acitivity after its previous inhibition by organophosphate compounds such as nerve agents (Bajgar 2004). Beside the reactivation process, HI-6 probably plays a more complex role when administered into the body. For instance, HI-6 seems to be able to counteract the antineoplastic drug irrinotecan toxicity (Radic *et al.* 2007). In the consequent experiment, the authors observed HI-6 antioxidant effect (Vrdoljak *et al.* 2009), which might be involved in its protective action. In our previous research, we found a significant role of HI-6 in modulation of oxidative stress associated with toxicity of nerve agents (Pohanka *et al.* 2009a; Pohanka *et al.* 2010a) and strong modulation of tularemia progression (Pohanka *et al.* 2010b). In this study, we evaluate the HI-6 modulatory effect on ionising radiation generated oxidative stress *in vivo*. Moreover, *in vitro* characterizations and *in silico* characterization of HI-6 is irreplaceable part of experiments.

MATERIAL AND METHODS

Animal exposure

A total of 32 two-month-old male Wistar rats (Anlab, Prague, Czech Republic) weighing 180 to 200 g were used in our experiments. During the whole experiment, rats were kept under steady controlled conditions (temperature 22±2 °C, humidity 50±10%, light period 7 a.m to 7 p.m., food and water provided *ad libitum*). The ethic Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic permitted and supervised the whole experiment as well as manipulation with animals. The experiment started after obligatory adaptation period. Animals were divided into four groups of 8 rats. The first group (A) was exposed to saline only; the second (B) was irradiated by a single dose of 7.5 Gy ($LD_{50/30}$) using ^{60}Co unit (Chirana, Prague, Czech Republic) at a dose rate of 0.9 Gy min^{-1} with a target distance of 1 m and administered with saline 30 min before irradiation; the third group (C) was administered with 5% of HI-6 LD_{50} – 39 mg/kg; the last group (D) received 7.5 Gy and was administered with 5% of HI-6 30 min before irradiation. Saline and HI-6 were administered intramuscular into posterial femoral muscles. Animals were sacrificed 6.5 hours after saline or HI-6 administration (6 hours after irradiation) in CO_2 narcosis. Blood was collected from the carotid artery into heparinized tubes and spun immediately (3000×g for 15 minutes, 15 °C). Plasma, blood mass, liver and spleen were stored in a refrigerator adjusted up to –75 °C.

Ferric reducing antioxidant power assay

Low molecular weight antioxidants were estimated using the ferric reducing antioxidant power (FRAP) assay. The previous protocol was slightly adapted (Pohanka *et al.* 2009). 2.5 ml of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/l hydrochloric acid was poured together with 2.5 ml of 20 mmol/l ferric chloride and 25 ml of 0.1 M pH 3.6 acetate buffer. 200 µl of mixture, 30 µl of sample (or saline solution as blank)

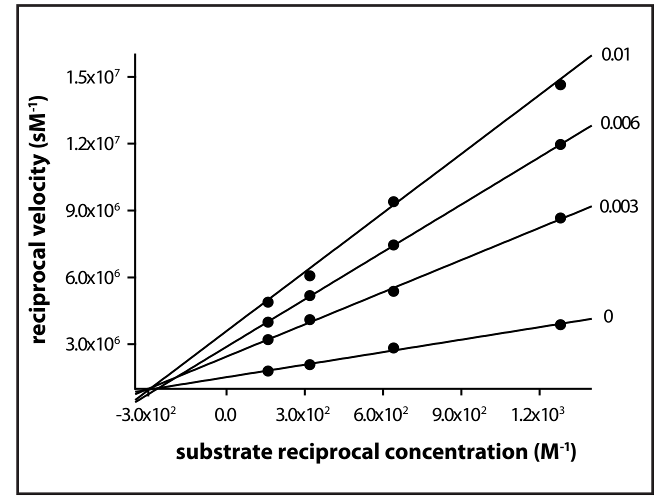

Fig. 1. Double reciprocal plot for human erythrocytes AChE, acetylthiocholine as substrate and HI-6 as an inhibitor. Molar level of HI-6 is indicated on the right edge for each plot.

and 770 μl of deionized water were injected into plastic tube after the mixture incubation at 37 °C for 10 minutes. After other 10 minutes of incubation, the mixture was spun at 10,000×*g* for 10 minutes. Absorbance of the supernatant was measured at 593 nm against the blank.

Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were assayed in a way as mentioned in the reference (Pohanka *et al.* 2010c). First, 67 mg of thiobarbituric acid were mixed with 1 ml of dimethylsulfoxide and then with 9 ml of deionized water. Samples (200l) were purified from proteins by precipitation with 400 μl of 10% trichloroacetic acid. After centrifugation at 3000×g for 15 minutes, 400 μl of supernatant or 400 μl of saline solution (blank) were injected into a plastic tube together with the same volume of the above-prepared mixture and heated at 100 °C for 10 minutes. Absorbance was measured at 532 nm against the blank.

Glutathione reductase assay

A cuvette was consequently filled with 100 μl of 10 mmol/l oxidized glutathione, the same volume of 1 mmol/l NADPH, and 650 μl of PBS. The reaction was started by addition of 50 μl of the sample. Absorbance at 340 nm was measured in the selected interval 120 seconds. Glutathione reductase (GR) activity was calculated considering molar consumption of NADPH (extinction coefficient $\varepsilon = 6\,220\ M^{-1}cm^{-1}$).

In vitro HI-6 affinity to AChE

The adapted photometrical Ellman´s method was used for AChE activity evaluation (Pohanka *et al.* 2008). A polystyrene cuvette was filled with 0.4 ml of 0.4 mg/ml of 5,5´-dithiobis(2-nitrobenzoic acid), further DTNB. Consequently, 25 μl of AChE (from human erythrocytes; Sigma-Aldrich, St. Louis, MO, USA) solution in phosphate buffered saline with the overall activity of 0.5 μkat, 25 μl of tested HI-6 concentration and 450 μl of PBS were injected into the cuvette. The reaction was started by addition of acetylthiocholine chloride in concentration of 10 μmol/l–10 mmol/l. Absorbance was measured at 412 nm against mixture with AChE replaced by saline only. AChE activity was calculated using extinction coefficient for thionitrobenzorate: $\varepsilon = 14\,150\ M^{-1}cm^{-1}$. Every assay was repeated three times. The enzymology assessment was carried out using the Lineweaver-Burk plot. Inhibitory constants K_{i1} (HI-6 affinity to AChE alone) and K_{i2} (HI-6 affinity to complex AChE-substrate) were calculated.

In silico estimation of the HI-6 impact

The EPI Suite (Office of Pollution Toxics and Syracuse Research Corporation; US Environmental Protection Agency) software was used throughout. Octanol water partition coefficient was estimated using the module Kowwin and biodegradability was calculated using the module Biowin.

Biochemical assays

Plasma samples were analysed using an automated analyzer SPOTCHEM TM EZ SP-4430 (Arkray, Japan) for ALP (μkat/l) – alkaline phosphatase; ALT (μkat/l) – alanine aminotransferase; AST (μkat/l) – aspartate aminotransferase; CPK (μkat/l) – creatine phosphokinase, CRE (μmol/l) – creatinine; GLU (mmol/l) – glucose; LDH (μmol/l) – lactate dehydrogenase; IP – (mmol/l) – inorganic phosphate, T-Pro (mmo/l) – total plasma protein; T-bil (μmol/l) – total bilirubin; TG (mmol/l) – triglyceride.

Statistics

The software Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA) was used for data processing and statistical evaluation of results. Significance of markers between individual groups was estimated by one-way ANOVA with Tukey test. Both $p \leq 0.05$ and $p \leq 0.01$ probability levels were calculated for the examined groups of 8 specimens.

RESULTS

HI-6 adjusted at concentration from 10^{-6} to 10^{-2} M was assayed in the same way as biological samples. Trolox, a water soluble derivate of tocopherol, was used as a positive control for the assay. We did not recognize any significant antioxidant effect of HI-6. Contrary to this, the standard antioxidant Trolox was able to significantly reduce the ferric ion to the ferrous form. *In vitro* assay of affinity toward AChE proved a non-competitive inhibitory mechanism, as can be seen in double reciprocal plot (Figure 1). The individual plots were crossed on the axis representing reciprocal concentration of substrate. That fact confirms idea of non-competitive mechanism of inhibition. The affinity of HI-6 to the substrate free AChE (K_{i1}) was $(2.4 \pm 0.3) \times 10^{-4}$ mol/l. The affinity to the AChE with acetylthiocholine in its active site was $(1.0 \pm 0.2) \times 10^{-4}$ mol/l. EPI Suite provided selected toxicological and chemical data about HI-6. The logarithm of octanol-water partition coefficient was –3.11. The seven available models of Biowin module did not prove oxidative as well as anaerobic degradability.

The FRAP value was altered differently in the used matrices. The complete experimental data are attached as Table 1. Ionizing radiation significantly decreased antioxidants in spleen and increased in liver ($p \leq 0.01$). HI-6 in combination with ionizing radiation was significantly increased in spleen and decreased in liver ($p \leq 0.01$), i.e. the level of antioxidants in the group D (both HI-6 and radiation exposed) was turned into the level of the controls (A). HI-6 alone had only a limited impact on antioxidants and it was not significantly shifted to the controls.

The TBARS value was the second parameter assessed *in vivo*. The values found are shown in Table 2. There were no significant changes in liver, plasma, and packed blood cells due to ionizing radiation and/or HI-6. One excep-

Tab 1. Ferric reducing antioxidant power (FRAP) value ± standard error of mean. Columns: A - control, B - irradiated, C - HI-6 exposure, D - HI-6 exposure and irradiation. Significance testing - probability level: * 0.05; ** ≤0.01 by Fisher test. Numerator: significance against the controls (column A), denominator: significance against the group D.

	A	B	C	D
liver (µmol/g)	2.71±0.04	2.93±0.05 (**/**)	2.61±0.07 (–/–)	2.59±0.04 (–)
spleen (µmol/g)	2.72±0.10	2.24±0.07 (**/**)	2.61±0.07 (–/*)	2.88±0.07 (–)
plasma (mmol/l)	0.321±0.015	0.305±0.013 (–/–)	0.343±0.038 (–/–)	0.378±0.043 (–)
packed blood cells (mmol/l)	1.69±0.12	1.63±0.11 (–/–)	1.64±0.04 (–/–)	1.73±0.011 (–)

Tab. 2. Thiobarbituric acid reactive substances (TBARS). The description is same as in Table 1.

	A	B	C	D
liver (µmol/g)	0.647±0.031	0.634±0.073 (–/–)	0.567±0.054 (–/–)	0.510±0.021 (–)
spleen (µmol/g)	0.608±0.025	0.779±0.065 (*/*)	0.668±0.028 (–/–)	0.659±0.0.031 (–)
plasma (µmol/l)	53.2±2.0	55.8±2.1 (–/–)	47.8±1.8 (–/–)	51.2±2.0 (–)
packed blood cells (mmol/l)	56.9±2.5	53.0±1.0 (–/–)	54.1±1.1 (–/–)	58.9±2.3 (–)

Tab 3. Glutathione reductase activity. The description is same as in Table 1.

	A	B	C	D
liver (µkat/g)	2.95±0.20	2.54±0.15 (*/–)	3.18±0.10 (–/*)	2.78±0.6 (–)
spleen (µkat/g)	2.32±0.21	2.86±0.06 (**/–)	3.80 ±0.25 (**/–)	3.32 ±0.12 (**)

tion concerned the spleen. HI-6 alone slightly increased TBARS against the controls. However, the increase was insignificant. Ionizing radiation significantly ($0.01<p\leq 0.05$) increased TBARS in the spleen. Surprisingly, HI-6 administration caused a decrease in spleen TBARS down to the level insignificant to the controls.

Glutathione reductase was assayed in the spleen and liver samples where it indicates the rise in oxidative stress (see table 3). The shifts of glutathione reductase activities in the liver samples were lower than in the spleen samples. Radiation caused a significant ($0.01<p\leq 0.05$) decrease of glutathione reductase in liver. On the other hand, HI-6 caused a significant increase ($0.01<p\leq 0.05$). HI-6 administered to irradiated animals caused equilibration of the glutathione reductase activity to the equal level with the activity of controls. Glutathione reductase activity was significantly ($p\leq 0.01$) elevated in spleen of all animals exposed to radiation and/or HI-6.

Assay of standard biochemistry markers is summarized as Table 4. Most markers were not shifted or the shifts were insignificant. Aspartate aminotransferase and blood urea nitrogen in the irradiated animals (groups B and D) were significantly elevated ($0.01<p\leq 0.05$). Animals exposed to radiation and HI-6 had a significantly decreased triglyceride level.

DISCUSSION

The compound HI-6 contains two pyridinium rings. Lundy *et al.* proved that HI-6 has not well distributed in the central nervous system. Contrary, the central nervous system contains the lowest level from the followed organs (Lundy *et al.* 1990). HI-6 is mainly accumulated in kidney and excreted without significant modification (Lundy *et al.* 1990). The parameters described by Lundy *et al.* are in compliance with our results. The *in silico* calculation of partition coefficient predicts poor

Tab. 4. Plasma biochemistry. The description is same as in Table 1. Abbreviations used in the graph: ALP - alkaline phosphatase; ALT - alanine aminotransferase; AST - aspartate aminotransferase; BUN - blood urea nitrogen; CPK (µkat/l) - creatine phosphokinase; CRE - creatinine; GLU -glucose; LDH - lactate dehydrogenase; IP - inorganic phosphate; T-Pro - total plasma protein; T-Bil - total bilirubin; TG – triglycerides.

	A	B	C	D
ALP (µkat/l)	3.49±0.38	3.67±0.38 (–/–)	3.26±0.43 (–/–)	2.79±0.23 (–)
ALT (µkat/l)	0.383±0.053	0.375±0.049 (–/–)	0.415±0.059 (–/–)	0.338±0.035 (–)
AST (µkat/l)	1.24±0.14	1.64±0.09 (*/–)	1.25±0.14 (–/*)	1.60±0.07 (*)
BUN (mmol/l)	5.45±0.29	6.97±0.48 (*/–)	5.73±0.25 (–/–)	6.87±0.54 (*)
Ca (mmol/l)	2.39±0.08	2.25±0.06 (–/–)	2.39±0.07 (–/–)	2.34±0.06 (–)
CPK (µkat/l)	7.36±0.32	5.61±0.41 (–/–)	6.63±1.04 (–/–)	6.34±1.56 (–)
CRE (µmol/l)	64.2±3.7	59.8±3.8 (–/–)	62.0±2.2 (–/–)	55.8±4.1 (–)
GLU (mmol/l)	8.37±0.58	9.07±0.55 (–/–)	9.68±0.94 (–/–)	9.03±0.30 (–)
IP (mmol/l)	1.67±0.09	1.65±0.12 (–/–)	1.59±0.04 (–/–)	1.56±0.07 (–)
LDH (µkat/l)	18.7±5.0	19.3±2.0 (–/–)	24.0±7.9 (–/–)	18.0±3.0 (–)
Mg (mmol/l)	0.895±0.032	0.795±0.039 (–/–)	0.877±0.041 (–/–)	0.832±0.072 (–)
T-Bil (µmol/l)	10.8±1.2	9.67±2.11 (–/–)	11.2±2.1 (–/–)	12.8±2.7 (–)
T-Pro (g/l)	54.2±1.6	51.8±1.1 (–/–)	53.7±1.7 (–/–)	52.7±2.2 (–)
TG (mmol/l)	1.34±0.12	0.953±0.071 (–/–)	1.25±0.22 (–/*)	0.785±0.106 (*)

penetration of HI-6 through lipidic membranes. The indicated poor distribution through membranes well correlates with the experiments describing low availability of oxime reactivators in brain due to persistency of the blood brain barrier (Okuno *et al.* 2008; Sakurada *et al.* 2003). In compliance with the mentioned facts, HI-6 effect should be expected in the peripheral nervous system and organs rather than the central one.

As presented in the results, FRAP assay for HI-6 *in vitro* and the affinity toward AChE *in vitro* enabled to estimate the mechanism of antioxidant action observed after HI-6 administration *in vivo*. We proved that HI-6 has no antioxidant ability, therefore the HI-6 impact on oxidative stress could not be based on direct scavenging of reactive oxygen or nitrogen species. This fact is opposite to the experiments carried out by Vrdoljak *et al.* (2009). From the chemical point of view, no significant antioxidant activities of HI-6 can be expected since there is no simply oxidizable group in its chemical structure. Pyridiniums in HI-6 structure allow interaction with AChE active site. Moreover, there is also affine interaction with the peripheral anionic site (Hornberg *et al.* 2010). AChE inhibition is the result of HI-6 - AChE interaction. We recognized and described strong inhibition of different cholinesterases in one of the previous papers (Pohanka *et al.* 2007). Here, we proved a non-competitive inhibitory mechanism. *In vitro*, HI-6 steadily binds AChE as well as AChE-substrate complexes. Though the affinity of HI-6 to AChE is lower than typical for myastenia gravis or Alzheimer disease drugs (Musilek eta l., 2010; Ahmed *et al.* 2006), biological effects can be expected. The HI-6 effect on cholinergic system is probably more complicated as the interaction with acetylcholine receptors is literary documented (Soukup *et al.* 2008) and similar situation can be expected for other oxime compounds (Loke *et al.* 2002). Vegetative nervous system is probably implicated in the HI-6 effect here observed.

In our study, we proved that HI-6 can modulate oxidative stress following exposure to ionizing radiation. We expected oxidative stress in liver (Gencel *et al.* 2010). On the contrary, it seems that liver is extensively synthesizing low molecular weight antioxidants for distribution throughout the body. It can be deduced from the fact that antioxidants were exhausted in spleen and presented in liver. Opposite to it, malondialdehyde, a lipid peroxidation marker assayed as TBARS, was not elevated in liver but it was present in spleen. HI-6 turned the level of low molecular weight antioxidants as well as malondialdehdyde into values found in controls. The beneficial effect of HI-6 on oxidative stress is probably associated with the enzymatic antioxidants defence system. HI-6 seems to force cells to express glutathione reductase in spleen over the controls and keep glutathione reductase in liver in the same level as controls.

Biochemistry examination did not show extensive pathological findings in laboratory animals due to ionizing radiation and/or HI-6 administration. Aspartate aminotransferase was significantly elevated in groups B and D. It can be deduced that it was caused by ionizing radiation as HI-6 alone had no significant impact and there was no significant difference between animals irradiated and irradiated in combination with exposure to HI-6. The meaning of elevated aspartate aminotransferase is unclear as the other markers (alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, creatine phosphokinase, total bilirubin) were steady, therefore, liver, heart and muscle damage cannot be related to the change. Blood sample haemolysis might be the reason of unnatural AST elevation; however, lactate dehydrogenase excludes it. The increase of aspartate aminotransferase is unprecedented since injury to organs should be accompanied by an increase in the alanine aminotransferase plasma level (Zhu *et al.* 2010) or level of the other markers. Blood urea nitrogen appoints at either slight damage of kidney or triggered proteosynthesis due to ionizing radiation in groups B and D. There was found neither synergic effect nor potentiation by HI-6. Triglycerides were slightly decreased in the B group. HI-6 potentiated ionizing radiation regards to triglycerides. Unfortunately, we did not assess whether the animals had lower appetite nor whether the consumption of feed could be related to decreasing triglycerides. It should be emphasized that the changes of biochemical markers presented in Table 4 are quite low and did not appoint at serious organs failure or metabolic disorders.

CONCLUSIONS

HI-6 can influence oxidative stress generation in animals exposed to ionizing radiation. The effect is probably based on the vegetative nervous system modulation. The compound up-regulates glutathione reductase (antioxidant enzyme) expression and provides protection from ionizing radiation impact. We conclude that HI-6 is a promising drug applicable as a radioprotectant enhancing oxidative stress suppression even despite of no direct HI-6 antioxidant impact.

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