



## Toxic effects of diazinon and its photodegradation products

Mirjana Čolović<sup>a</sup>, Danijela Krstić<sup>b</sup>, Sandra Petrović<sup>a</sup>, Andreja Leskovac<sup>a</sup>, Gordana Joksić<sup>a</sup>, Jasmina Savić<sup>a</sup>, Mladen Franko<sup>c</sup>, Polonca Trebše<sup>c</sup>, Vesna Vasić<sup>a,\*</sup>

<sup>a</sup> Department of Physical Chemistry, Vinča Institute of Nuclear Sciences, P.O. Box 522, 11001 Belgrade, Serbia

<sup>b</sup> University School of Medicine, Institute of Medicinal Chemistry, University of Belgrade, Belgrade, Serbia

<sup>c</sup> Laboratory for Environmental Research, University of Nova Gorica, Nova Gorica, Slovenia

### ARTICLE INFO

#### Article history:

Received 6 July 2009

Received in revised form

13 November 2009

Accepted 25 November 2009

Available online 3 December 2009

#### Keywords:

Diazinon

Photodegradation

Acetylcholinesterase

Na<sup>+</sup>/K<sup>+</sup>-ATPase

Lipid peroxidation

Cytogenetic damage

### ABSTRACT

The toxic effects of diazinon and its irradiated solutions were investigated using cultivated human blood cells (lymphocytes and erythrocytes) and skin fibroblasts. Ultra Performance Liquid Chromatography (UPLC)–UV/VIS system was used to monitor the disappearance of starting diazinon during 115-min photodegradation and formation of its by-products (diazoxon and 2-isopropyl-6-methyl-4-pyrimidinol (IMP)) as a function of time. Dose-dependent AChE and Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibition by diazinon was obtained for all investigated cells. Calculated IC<sub>50</sub> (72 h) values, in M, were:  $7.5 \times 10^{-6}/3.4 \times 10^{-5}$ ,  $8.7 \times 10^{-5}/6.6 \times 10^{-5}$ , and  $3.0 \times 10^{-5}/4.6 \times 10^{-5}$  for fibroblast, erythrocyte and lymphocyte AChE/Na<sup>+</sup>/K<sup>+</sup>-ATPase, respectively. Results obtained for reference commercially purified target enzymes indicate similar sensitivity of AChE towards diazinon (IC<sub>50</sub> (20 min)– $7.8 \times 10^{-5}$  M), while diazinon concentrations below 10 mM did not noticeably affect Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. Besides, diazinon and IMP induced increasing incidence of micronuclei (via clastogenic mode of action) in a dose-dependent manner up to  $2 \times 10^{-6}$  M and significant inhibition of cell proliferation and increased level of malondialdehyde at all investigated concentrations. Although after 15-min diazinon irradiation formed products do not affect purified commercial enzymes activities, inhibitory effect of irradiated solutions on cell enzymes increased as a function of time exposure to UV light and resulted in significant reduction of AChE (up to 28–45%) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (up to 35–40%) at the end of irradiation period. Moreover, photodegradation treatment strengthened prooxidative properties of diazinon as well as its potency to induce cytogenetic damage.

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### 1. Introduction

Diazinon (O,O-diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl phosphorothionate) is a commonly used thionophosphorous organophosphate (OP) pesticide to control a variety of insects in agriculture and household environment (Cox, 1992). Despite its low persistence in the environment, it is a nonspecific insecticide and highly toxic to animals and humans. Moreover, the toxicity of OPs is increased by their break-down products, which may be bioactivated within an organism or through exposure to the sunlight. The pathway of degradation is assumed to be substitution of sulfur by oxygen in P=S bond, cleavage of the pyrimidine ester bond, and the oxidation of isopropyl group (Kouloumbos et al., 2003; Shemer and Linden, 2006). Diazinon undergoes fast hydrolysis at acidic and basic conditions (Mansour et al., 1999; Zhang and Pehkonen, 1999) in environment, is unstable under

UV irradiation (Pehkonen and Zhang, 2002) and can be transformed to the more toxic diazoxon due to the enzymatic reaction in birds, fish, insects and mammals (Zhang and Pehkonen, 1999). Its major degradation products are hydroxydiazinon, diazoxon, hydroxydiazoxon and 2-isopropyl-6-methyl-4-pyrimidinol (IMP) (Kouloumbos et al., 2003), which may cause acute toxic effects to workers employed in the manufacture and application of this pesticide. Diazoxon is originated probably through oxidative desulfuration by OH radical attack on thiono group or through oxidative mechanism acting directly on diazinon (Kouloumbos et al., 2003). Other metabolites identified were 2-(2'-hydroxy-2'-propyl)-4-methyl-6-hydroxypyrimidine, diethyl-phosphorothioic acid and diethylphosphoric acid. IMP and its hydroxylated metabolites were reported to be much less toxic as compared to its parent compound diazinon and represent the major metabolites, which are stable and readily excreted in urine (Ku et al., 1998).

Like other OPs, the main toxic action of diazinon is inhibition of acetylcholinesterase activity (AChE, EC 3.1.1.7) by phosphorylation of the serine hydroxyl group in the substrate-binding domain of the enzyme which results in accumulation of acetylcholine and associated neurotoxicity (Fulton and Key, 2001; Oruc and Usta, 2007). It is

\* Corresponding author at: Laboratory of Physical Chemistry, P.O. Box 522, 11001 Belgrade, Serbia. Tel.: +381 11 2453 967; fax: +381 11 2447 207.

E-mail address: [evasic@vinca.rs](mailto:evasic@vinca.rs) (V. Vasić).

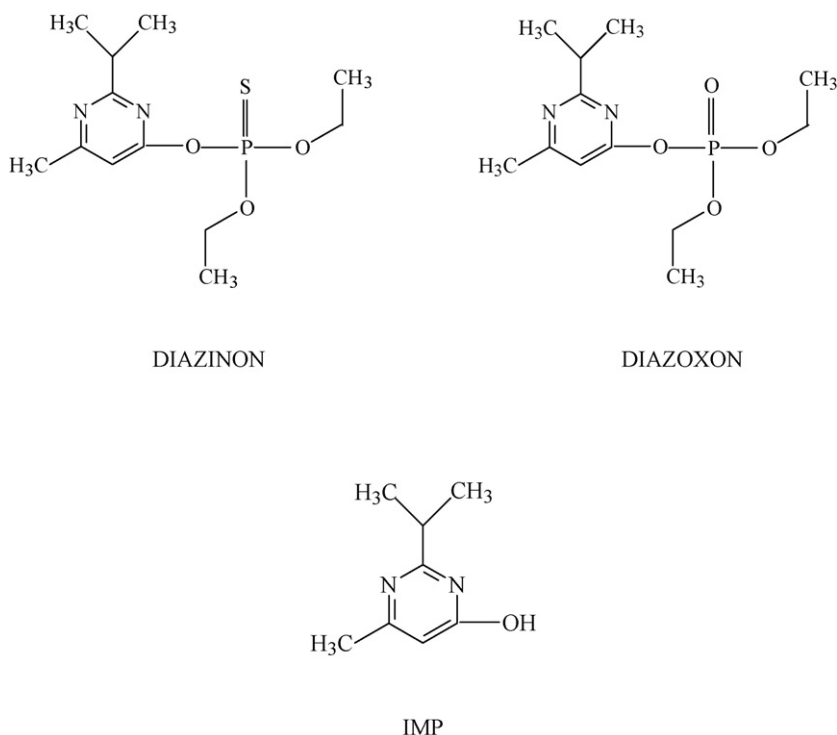


Fig. 1. The chemical structures of diazinon and the products of its photochemically induced transformation.

established also that some OPs inhibit different ATPases, the group of enzymes playing an important role in biochemical processes (Vasić et al., 2008) in various tissues (Rahman et al., 1997). Pesticides exert biological effect on the ATPase system by partitioning in the enzyme complex (Kinter et al., 1972) and it is also reported that an allosteric change results in a decrease of ATPase activity (Reddy et al., 1992). The proper functioning of these enzymes is critical for cellular viability because they control many essential cellular functions, and any inhibition of the activity may be considered as a sensitive indicator of toxicity. Both  $\text{Na}^+/\text{K}^+$ -ATPase (EC 3.6.1.37) and AChE are membrane bound enzymes and their activities depend on the phospholipids environment of the membrane. Therefore, any change in the lipid component of the membrane will directly affect the activities of these enzymes (Sahoo et al., 1999). Besides, the pesticides may also induce oxidative stress and genetic material damage leading to cell malformations, generation of free radicals and cause lipid peroxidation (Banerjee et al., 1999; Goel et al., 2005; Kehrer, 1993).

Moreover, there is a concern that the risk of genotoxicity might be appreciably greater than that predicted from standard toxicity tests (Attia, 2007). Therefore, cytogenetic damage (chromosomal aberrations—CA, micronuclei—MN and sister-chromatid exchanges—SCE) in circulating lymphocytes has been widely used as a biomarker of exposure and effects of pesticides. Measuring micronuclei frequency is an approach used extensively in molecular epidemiology and cytogenetics to evaluate the presence and the extent of the chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile. DNA damage induced by xenobiotics appears primarily in the form of alterations to the phosphate backbone, sugar or base modifications such as alkylations, crosslinks, or formation of DNA adducts that are substrates for DNA repair (Bajpayee et al., 2006) when miss repaired they are seen as increased incidence of CA, MN or SCE. Phosphorus moiety in the pesticides appears to be a good substrate for nucleophilic attack leading to phosphorylation of DNA which is an instance of DNA damage (Das et al., 2007). Micronuclei may be

induced by strand breaks in DNA due to oxidative stress (Fenech, 1993).

Although diazinon is similar in basic chemical structure and metabolite profile to other organophosphates, there is still significant gap both in data on diazinon and its metabolites toxicity and in genotoxicity studies on humans. In the present study we investigated *in vitro* the toxicity potential and oxidative stress responses of diazinon and its photo degradation products (diazoxon and IMP, formed in nature or during water purification processes using UV light) by determining the activity of AChE and ATPases in harvested human blood cells (erythrocytes, lymphocytes), human skin fibroblasts and purified enzymes, lipid peroxidation product malondaldehyde (MDA), incidence of micronuclei and cell proliferation potential (CBPI), comparatively.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of analytical grade. Acetylcholinesterase (AChE, E.C. 3.1.1.7, specific activity 500 IU/mg protein) from human erythrocytes,  $\text{Na}^+/\text{K}^+$ -ATPase from porcine cerebral cortex (specific activity 25.8  $\mu\text{mol Pi/h/mg protein}$ ), acetylthiocholine iodide (AChI), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), adenosinetriphosphate (ATP), propidium iodide and Cytochalasin B were purchased from Sigma Chemicals Co. (Germany). PB-max karyotyping medium was purchased from Invitrogen-Gibco (UK). Lymphoprep (Lymphocyte separation medium) was from PAA Laboratories (Austria). Proteinase K and RNase were from Fermentas (Canada). FITC labeled  $\alpha$ -satellite DNA probe was purchased from ICN Biomedicals (USA). Other medium assay chemicals (magnesium chloride, Tris-HCl sodium chloride, potassium chloride, stannous chloride, calcium chloride, potassiumhydrogen phosphate ( $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$ ), sodium citrate, ammonium molybdate and Giemsa were from Merck (Germany). Formamide was

purchased from AppliChem (Germany). Diazinon (O,O-diethyl-O-(2-isopropyl-6-methyl-pyrimidine-4-yl)phosphorothioate) (97.3%), O-analog diazinon (diazoxon) solution (100 ppm in acetonitrile) and 2-isopropyl-6-methyl-4-pyrimidinol (IMP) (93%) were from Pestanal (Germany). The chemical structures of studied compounds are collected in Fig. 1.

## 2.2. Photodegradation experiments

10 mL of absolute ethanol was added to 30.4 mg of diazinon and prepared  $1 \times 10^{-2}$  M solution was then diluted 10 times by deionised water. Photodegradation of  $1 \times 10^{-3}$  M (10% ethanol) diazinon solution (10 mL) was performed using a 125 W Cermax Xenon parabolic lamp emitting light with wavelengths above 200 nm, irradiating the quartz sample cell with the optical path length of 1.0 cm placed 65 cm from the light source during six different periods of time: 0, 5, 15, 30, 60 and 115 min. These irradiated solutions were diluted 50 times in blood and fibroblast cultures as well as in incubation medium for determination of AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activities.

## 2.3. UPLC analysis

Quantification of diazinon and its decomposition products (IMP and diazoxon) in aliquots of the irradiated solutions was performed on Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC)–UV/VIS detection system, using an ACQUITY UPLC™ BEH C<sub>18</sub>, 1.7 μm, 50 mm × 2.1 mm column. The mobile phase was composed of 0.1% formic acid (phase A) and acetonitrile (phase B) in the ratio 25:75 (for diazinon and diazoxon analysis), and 10:90 (for IMP analysis). Flow rate was 0.3 mL/min, injection volume 10 μL and the column temperature 25 °C. The separation of diazinon and diazoxon was monitored in a single wavelength mode at 245 nm, while IMP determination was carried out at 230 nm. The retention times for diazinon, diazoxon and IMP were 1.9 min, 1.2 min and 1.8 min, respectively. Limits of detection (LOD) for diazinon and diazoxon were  $3 \times 10^{-7}$  and  $1 \times 10^{-6}$  M for IMP. For quantification purposes calibration curves in the concentration range from  $1 \times 10^{-6}$  to  $1 \times 10^{-3}$  M for diazinon and IMP and from  $3 \times 10^{-7}$  to  $1 \times 10^{-4}$  M for diazoxon were prepared.

## 2.4. Blood cultures

Blood sample was obtained from healthy 30 years old male, non-smoking volunteer in the Medical Unit in accordance with the current Health and Ethical regulations in Serbia (Law on Health Care, 2005). Aliquots of heparinized whole blood (0.5 mL) were placed in cultures containing PB-max karyotyping medium. Two parallel sets of blood cultures were set up in the presence of increasing doses of diazinon ( $2 \times 10^{-8}$  to  $2 \times 10^{-4}$  M) and its photodegradation products yielded after irradiation of  $2 \times 10^{-5}$  M solution. Micronuclei formation and cell proliferation potential were scored in the first sets of samples and the corresponding controls, whereas the second set was used for measurement of AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activities and MDA level. For that purpose, after 72 h of incubation harvested cells were separated on Lymphoprep, lymphocytes were collected by centrifugation, washed in physiological saline, and frozen at  $-70$  °C for later analysis. The erythrocytes were collected from the bottom and RBC membranes were prepared according to the method of (Post et al., 1960).

Medium of each culture in which cells were grown during incubation (incubation medium) was also kept for measurement of MDA level.

## 2.5. Fibroblasts

Human skin fibroblasts were set up as parallel tissue. When being confluent, cells were expanded in  $15 \times 2$  flasks, and 1 h after trypsinisation, cells were treated with diazinon and its by-products as used for blood cells. The fibroblast cell density was  $1 \times 10^5$  cells in 75 cm<sup>2</sup> flasks. Micronuclei formation and cell proliferation potential were then scored in the first set of samples and the corresponding controls. In the second set of samples cells were collected by centrifugation, washed in physiological saline, and frozen at  $-70$  °C for later analysis of AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activities as well as MDA level.

## 2.6. AChE assay

The inhibition of AChE was measured in erythrocytes, lymphocytes and fibroblasts treated with desired diazinon concentrations and its irradiated solutions, while commercially available human erythrocytes enzyme, *in vitro* exposed to the same solutions was used as a reference. AChE activity was determined using Ellman's procedure (Ellman et al., 1961) and expressed as  $\Delta A/(\text{min} \times \text{mg protein})$ . The *in vitro* experiments were performed by 20 min exposure of 0.02 IU commercial enzyme to investigated compounds in final volume 0.650 mL. AChI was applied as the enzyme substrate in combination with DTNB as a chromogenic reagent. The product 5-thio-2-nitrobenzoate, formed in the reaction of thiocholineiodide (product of enzymatic reaction) and DTNB was measured spectrophotometrically (Perkin Elmer Lambda 35 UV-VIS spectrophotometer) at 412 nm (in buffer solution). All experiments were made in triplicates. Preliminary studies showed that diazinon and its by-products did not interfere with quantization of the yellow product 5-thio-2-nitrobenzoate. Total protein was quantified by the procedure of Lowry's method (Lowry et al., 1951) using Folin's reagent, and bovine serum albumin as standard.

## 2.7. ATPase assay

The specific activity of  $\text{Na}^+/\text{K}^+$ -ATPase in erythrocytes, lymphocytes and fibroblasts treated with desired diazinon concentrations and its irradiated solutions was determined in a standard incubation medium (200 μL), containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM ATP. The *in vitro* experiments were performed by exposure of 0.0125 IU commercial porcine cerebral cortex  $\text{Na}^+/\text{K}^+$ -ATPase (reference model) to investigated compounds in the same medium assay. Incubation mixtures were preincubated at 37 °C and the reaction was started by the addition of ATP, and allowed to proceed until stopped by ice cold HClO<sub>4</sub>. The activity obtained in the presence of 2 mM ouabain (without the NaCl and KCl) was attributed to Mg<sup>2+</sup>-ATPase (ecto-ATPase).  $\text{Na}^+/\text{K}^+$ -ATPase activity was calculated as a difference between the total ATPase and Mg<sup>2+</sup>-ATPase activity. The concentration of adenosinediphosphate (ADP) liberated due to the hydrolysis of ATP was measured by UPLC method (Sudo et al., 2000). The mobile phase was composed of 4 mM tetrabutylammonium hydroxide (TBAH) in 4 mM phosphate buffer (phase A) and methanol (phase B) in the ratio 75:25. Flow rate was 0.25 mL/min, injection volume 10 μL and the column temperature 40 °C. The separation was monitored in a single wavelength mode at 254 nm. In addition, the concentration of liberated orthophosphate (Pi) was measured using modified spectrophotometric procedure based on the stannous chloride method (Jorgensen et al., 2003; Vasić et al., 1999), by reading the absorbance at 690 nm and was compared to the concentration of ADP. Excellent agreement was obtained, confirming that only orthophosphates liberated from enzyme catalyzed ATP hydrolysis were detected.

## 2.8. Thiobarbituric acid (TBA) assay

Defrosted lymphocyte and fibroblast suspensions, their incubation media and RBC membranes were used for measurement of malondyaldehyde, spectrophotometrically (Aruoma et al., 1989) at the 532 nm wavelength. The values are expressed as nmol of thiobarbituric acid-reactive substance (MDA equivalent)/mg protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

## 2.9. Micronuclei analysis

For micronuclei preparation, the cytokinesis block method of Fenech et al. was followed (Fenech, 1993) Cytochalasin B at a final concentration of 4 µg/mL was added to each sample for micronucleus assay. Cell suspension was prefixed in methanol/acetic acid 3:1, washed 3 times with fixative and dropped onto clean slide. Slides were air dried and stained in alkaline Giemsa (2%). At least 1000 binucleated (BN) cells per sample were scored. All slides were analyzed with an Axiophot-2 microscope using magnification 400× or 1000× when necessary. A minimum of 1000 BN cells were scored to evaluate the percentage of cells with micronuclei.

## 2.10. In situ hybridization

Parallel unstained micronuclei slides were analyzed using Fluorescent *in situ* hybridization with FITC labeled α-satellite DNA probe (ICN Biomedicals, USA), hybridizing exclusively the centromeres of all human chromosomes. Subsequently slides were pretreated with RNase (100 µg/mL in 2xSSC (saline-sodium citrate buffer), for an hour, and proteinase K (0.5 µg/mL in 20 mM TRIS, 2 mM CaCl<sub>2</sub>, pH 7.5 for 15 min at 37 °C. The slides were dehydrated in an ethanol series (70%, 90%, 100%), for 5 min and were denatured in 70% formamide/2xSSC, pH 7.0 at 70 °C for 2 min. After denaturation the slides were dehydrated in ethanol series. Target DNA was denatured at 70 °C for 5 min, placed on ice for 10 min and than applied to slides. The slides were incubated for 16 h in moist chamber at 37 °C. After posthybridization washes (5 min in 50% formamide, 2xSSC) and two changes in 2xSSC for 2 min each, slides were dehydrated in cold ethanol series and were stained with propidium iodide (PI) antifade solution. Centromere micronuclei were analyzed on Axiomager A1 fluorescent microscope using FITC and PI filters and magnification 1450×. According to presence of centromere's signal in micronuclei, they were classified as centromere positive (MNC+) and centromere negative (MNC–) (Bolognesi, 2003).

## 2.11. Cell proliferation index

A cytokinesis-block proliferation index (CBPI) was calculated according to method of Surrales et al. (1995) as follows:  $CBPI = MI + 2MII + 3(MIII + MIV)/N$ , where MI–MIV represent the number of cells with one to four nuclei, respectively, and *N* is the number of cells scored.

**Table 2**

Effect of diazinon on Na<sup>+</sup>/K<sup>+</sup>-ATPase and AChE activity in different human cells after 3 days exposure and in purified commercial preparations. All results present mean value of at least two experiments done in triplicate.

Tissue	Na <sup>+</sup> /K <sup>+</sup> -ATPase		AChE	
	Specific activity (control), µmol P <sub>i</sub> /h/mg protein	IC <sub>50</sub> (72 h), M	Specific activity (control), ΔA <sub>412</sub> /min/mg protein	IC <sub>50</sub> (72 h), M
Fibroblasts	1.110	3.4 × 10 <sup>-5</sup>	0.109	7.5 × 10 <sup>-6</sup>
Erythrocytes	0.189	6.6 × 10 <sup>-5</sup>	0.518	8.7 × 10 <sup>-5</sup>
Lymphocytes	0.081	4.6 × 10 <sup>-5</sup>	0.929	3.0 × 10 <sup>-5</sup>
Commercial purified enzyme*	25.8	>10 <sup>-4</sup> **	2418	7.8 × 10 <sup>-5</sup> **

\* Na<sup>+</sup>/K<sup>+</sup>-ATPase from porcine cerebral cortex; AChE from human erythrocytes.

\*\* IC<sub>50</sub> (20 min).

**Table 1**

Concentration of products formed due to the exposure 1 × 10<sup>-3</sup> M diazinon to UV irradiation as the function of irradiation time.

Irradiation time (min)	Diazinon (M)	Diazoxon (M)	IMP (M)
0	1.0 × 10 <sup>-3</sup>	–	–
5	9.0 × 10 <sup>-4</sup>	2.0 × 10 <sup>-6</sup>	3.2 × 10 <sup>-5</sup>
15	7.2 × 10 <sup>-4</sup>	4.0 × 10 <sup>-7</sup>	6.4 × 10 <sup>-5</sup>
30	4.1 × 10 <sup>-4</sup>	–	1.1 × 10 <sup>-4</sup>
60	2 × 10 <sup>-4</sup>	–	2.7 × 10 <sup>-4</sup>
115	–	–	4.5 × 10 <sup>-4</sup>

Note: The irradiated solutions were diluted 50 times in blood and fibroblast cultures as well as in incubation medium for determination of AChE and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities.

## 2.12. Statistics

A statistical analysis of each of the parameters of interest was carried out using statistical software package Statistics, version 6 for Microsoft Windows. Analysis considered the incidence of micronuclei in binucleated cells, the level of produced malondyaldehyde and cytokinesis-block proliferation index of treated cells. Among parameters under consideration, Product-Moment and partial correlations and Student's *t*-test were used. The *P* value < 0.05 was considered significant.

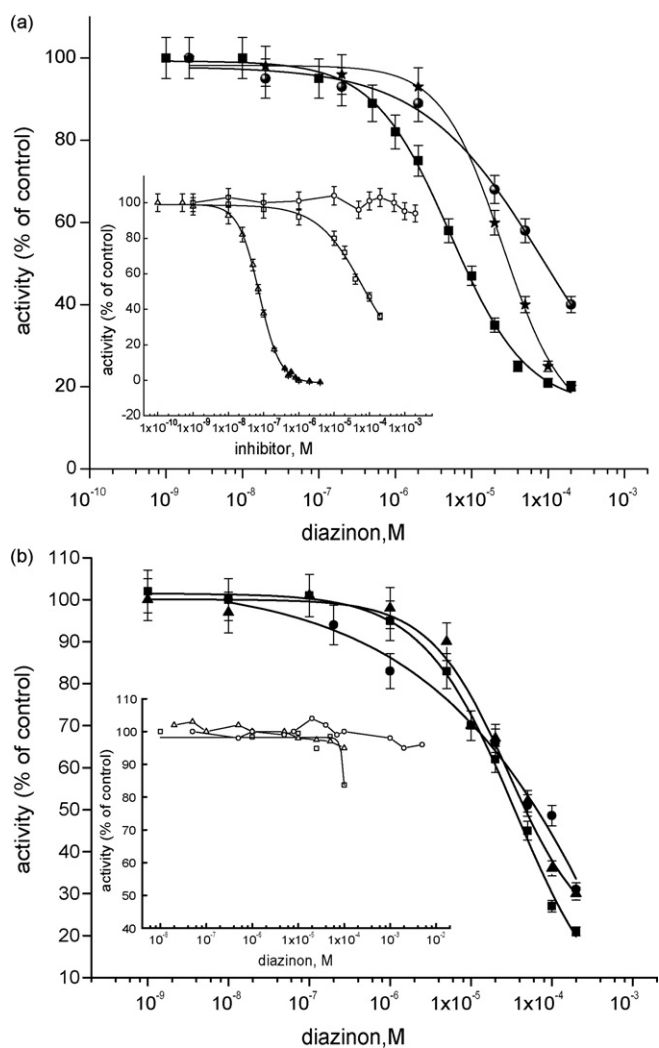
## 3. Results

### 3.1. Photochemical degradation of diazinon

The absorption spectrum of diazinon, diazoxon and IMP display strong absorption bands in the range from 200 to 280 nm. This fact implies that they have the potential to be photolyzed by any wavelengths below 280 nm. The concentrations of products (diazoxon and IMP) formed due to the exposure 1 × 10<sup>-3</sup> M diazinon to UV irradiation as the function of irradiation time are presented in Table 1. The results, obtained by UPLC analysis, indicate the complete degradation of the parent compound within 115 min of irradiation. IMP was observed already after 5 min (3.2 × 10<sup>-5</sup> M) and reached maximal concentration at the end of experiment when 45% of irradiated diazinon was converted to IMP. Diazoxon was only detected at 5 min and 15 min, while after 15 min of irradiation its concentration was under LOD. Maximal diazoxon concentration was measured at 5 min of the experiment and corresponded to 0.2% of the initial parent compound concentration. It is obvious (Table 1) that the sum of concentrations of diazinon, diazoxon and IMP in each moment of photodegradation is less than the initial concentration of diazinon suggesting the presence of other compounds (not detected) in the irradiated mixtures.

### 3.2. Effect of diazinon on AChE and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity

The effect of diazinon on AChE and Na<sup>+</sup>/K<sup>+</sup>-ATPase was investigated by determination of specific enzyme activities in human limfocytes, erythrocytes and fibroblasts which were cultivated dur-



**Fig. 2.** (a) Inhibition of AChE activity in cells (solid symbols): fibroblasts (square), lymphocytes (asterisk) and erythrocytes (circle) induced by diazinon. Inset: Inhibition of commercial human erythrocyte AChE (open symbols) induced by diazinon (square), diazoxon (triangle) and IMP (circle). (b) Inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase activity in cells (solid symbols): fibroblasts (square), lymphocytes (triangle) and erythrocytes (circle) induced by diazinon. Inset: Inhibition of commercial porcine cerebral cortex  $\text{Na}^+/\text{K}^+$ -ATPase (open symbols) induced by diazinon (square), diazoxon (triangle) and IMP (circle).

ing 72 h in the absence (control) and presence of the inhibitor (concentration range from  $1 \times 10^{-9}$  to  $2 \times 10^{-4}$  M). Fig. 2(a and b) illustrates the dose-dependent diazinon induced enzyme activities inhibition in all investigated cells. The dependence of AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activity, expressed as a percentage of the control value, on diazinon concentration fits a sigmoidal function in all cases. The concentrations of diazinon with capability to inhibit 50% of the enzyme after given exposure time ( $\text{IC}_{50}$  values) were determined by sigmoidal fitting the experimental results and summarized in Table 2. It is obvious that fibroblasts AChE shows the highest sensitivity ( $\text{IC}_{50}$  (72 h)— $7.5 \times 10^{-6}$  M), while the erythrocytes AChE is one order of magnitude less sensitive toward the investigated organophosphate ( $\text{IC}_{50}$  (72 h)— $8.7 \times 10^{-5}$  M), that could be attributed to lower capability of fibroblasts to detoxify diazinon than blood. On the other hand,  $\text{Na}^+/\text{K}^+$ -ATPase shows similar sensitivity in all examined cell types. Obtained  $\text{IC}_{50}$  (72 h) values, in M, are:  $4.56 \times 10^{-5}$ ,  $6.60 \times 10^{-5}$  and  $3.41 \times 10^{-5}$  for human lymphocytes, erythrocytes and fibroblasts, respectively.

The *in vitro* influence of diazinon on AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activities was followed exposing the reference model systems (commercial purified: AChE from human erythrocytes and cerebral cortex  $\text{Na}^+/\text{K}^+$ -ATPase) to the investigated compound in the same concentration range. In addition, the inhibitory potencies of diazoxon and IMP (detected diazinon photoinduced by-products) were tested on commercial enzymes. The obtained concentration-dependent inhibition of AChE (Fig. 2a, inset), indicates the similar response in the presence of diazinon as in the examined cell types. Moreover, similar  $\text{IC}_{50}$  values for commercial and human erythrocytes AChE were obtained, while  $\text{IC}_{50}$  value for the target enzyme from fibroblasts was ten times lower (Table 2). Obtained results show that diazoxon is about 1000 times more potent inhibitor than its parent compound ( $\text{IC}_{50}$  (20 min)— $6.8 \times 10^{-8}$  M), while the inhibitory effect of IMP was negligible at concentrations below 2 mM (Fig. 2a, inset). Unlike AChE and  $\text{Na}^+/\text{K}^+$ -ATPase in the investigated cells, activity of commercially purified reference  $\text{Na}^+/\text{K}^+$ -ATPase was not affected by diazinon (as well as diazoxon and IMP) concentrations lower than  $1 \times 10^{-4}$  M (Fig. 2b, inset, Table 2).

### 3.3. Influence of diazinon irradiated solutions on AChE and $\text{Na}^+/\text{K}^+$ -ATPase activity

The influence of diazinon photochemical treatment on its AChE and  $\text{Na}^+/\text{K}^+$ -ATPase inhibitory potency was investigated by determination of specific enzyme activities in the same model cells (erythrocytes, lymphocytes and fibroblasts). The cells were cultivated during 72 h in the presence of previously photochemically treated (during several irradiation periods) diazinon solutions under the conditions described in Section 2. The initial irradiated solutions were diluted 50 times in cell incubation medium. In addition, *in vitro* effects of these diluted irradiated solutions on the commercial purified reference enzymes were followed. Fig. 3a illustrates the effect of irradiation time of diazinon on AChE inhibition (expressed as a percentage of the control value) in all model systems. As can be seen, inhibitory efficiency of diazinon irradiated solutions increased as a function of the exposure time to the UV light in all investigated cells. Moreover, first 5 min of irradiation induced a rapid decrease of enzyme activity (about 25%) compared to inhibition caused by non-irradiated  $2 \times 10^{-5}$  M diazinon, while a gradual decrease in AChE activity was obtained during next 110 min of photochemical treatment. Obviously, obtained AChE fibroblasts inhibition in the presence of irradiated samples was higher than inhibition of blood cells AChE, as in the case of non-irradiated diazinon. In addition, in the case of commercial purified enzyme the maximum inhibition was achieved after 5 min irradiation. However, in samples with prolonged irradiation time (Fig. 3a, inset) the increasing commercial AChE activity was noticed. Moreover, after 115 min the irradiated solution did not noticeably affect enzymatic activity (96% of control activity).

Fig. 3b illustrates that inhibitory efficiency of diazinon irradiated solutions increases as a function of photodegradation time in all investigated cells. Gradual decrease in  $\text{Na}^+/\text{K}^+$ -ATPase activity was obtained during 115 min of photochemical treatment. The presence of 115-min irradiated solution resulted in diminishing  $\text{Na}^+/\text{K}^+$ -ATPase activity 35–40% in respect to the initial activity (in the presence of unirradiated  $2 \times 10^{-5}$  M diazinon) (Fig. 3b). On the other hand, diazinon photochemical treatment did not remarkably affect its influence on reference  $\text{Na}^+/\text{K}^+$ -ATPase (Fig. 3b, inset). Obtained  $\text{Na}^+/\text{K}^+$ -ATPase activity (% of control) ranged from 93 to 105 in the presence of irradiated solutions containing diazinon and/or its by-products and corresponded to the  $\text{Na}^+/\text{K}^+$ -ATPase response on unirradiated  $2 \times 10^{-5}$  M diazinon (Fig. 2b, inset).

**Table 3**  
Incidence of micronuclei (MN) and proliferation index in human lymphocytes and fibroblasts treated with diazinon and IMP.

Concentration (M)	Control	$2 \times 10^{-8}$	$2 \times 10^{-7}$	$2 \times 10^{-6}$	$2 \times 10^{-5}$	$2 \times 10^{-4}$
<b>Diazinon</b>						
<i>Lymphocytes</i>						
MN/1000 BN cells	4.61	10.18	14.07	15.04	12.9	–
CBPI	1.53	1.42	1.35	1.27	1.26	–
<i>Fibroblasts</i>						
MN/1000 cells	3.50	9.20	11.10	12.30	9.10	–
CBPI	1.32	1.31	1.26	1.26	1.31	–
<b>IMP</b>						
<i>Lymphocytes</i>						
MN/1000 cells	4.61	10.21	11.75	20.95	20.21	18.63
CBPI	1.53	1.40	1.31	1.27	1.20	1.19
<i>Fibroblasts</i>						
MN/1000 cells	3.50	8.30	9.20	16.10	15.30	15.10
CBPI	1.32	1.29	1.28	1.18	1.17	1.12

### 3.4. Effects of diazinon and its irradiated solutions on MDA level

The investigated diazinon concentrations up to  $2 \times 10^{-6}$  M did not noticeably ( $p > 0.05$ ) alter MDA level (compared to the control) in lymphocytes, as well as in incubation medium, while increasing MDA level was observed in the presence of diazinon concentrations above  $2 \times 10^{-6}$  M (Fig. 4a). The approximately 1.5- and 2.8-fold enhancement of MDA level (in respect to the control) was observed in lymphocytes and incubation medium, respectively, treated with  $2 \times 10^{-4}$  M diazinon. In addition, the effect of IMP, the diazinon hydrolysis product formed during its photodegradation, on lipid peroxidation was tested in order to compare it to the parent compound (diazinon) effect. IMP also induced the increase in MDA level, both in lymphocytes and incubation medium, in a dose-dependent manner (Fig. 4a, inset). The MDA level in lymphocytes induced by IMP at concentrations above  $2 \times 10^{-6}$  M was approximately 50–80% higher (statistically significant,  $t = 11.32$ ,  $p = 0.008$ ) than that induced by the same concentrations of diazinon. Similar results were obtained analyzing the diazinon and IMP effects in incubation medium.

Results obtained for lymphocytes and incubation medium exposed to  $2 \times 10^{-5}$  M irradiated diazinon solutions are presented in Fig. 4b. The statistically significant ( $t = -3.22$ ,  $p = 0.018$ ) gradual enhancement of MDA level was observed with the increasing irradiation time and achieved the maximum value at the end of the irradiation process. The level of MDA at the end of photochemical degradation was approximately 2-fold higher in respect to the control and level obtained in the presence of unirradiated  $2 \times 10^{-5}$  M diazinon solution.

Similar results were obtained for diazinon and its by-products effects on lipid peroxidation in erythrocytes and fibroblasts (data not shown).

### 3.5. Cytotoxic effects of diazinon and its irradiated solutions

The effects of diazinon and its by-product IMP on the incidence of micronuclei and proliferation index in cell cultures (lymphocytes and fibroblasts) are illustrated in Table 3. In lymphocyte cultures, significant increase ( $p < 0.05$ ) of the incidence of micronuclei in a dose-dependent manner was observed in all samples treated with diazinon and IMP at concentration range from  $2 \times 10^{-8}$  to  $2 \times 10^{-6}$  M, compared to the control. Even low concentration levels, below  $2 \times 10^{-6}$  M of both compounds (Table 3) display clastogenic properties seen as induction of micronuclei and lowering cell proliferation potential. IMP behaved as a more powerful inducer of MN than diazinon. The incidence of micronuclei reached

the maximum at concentration  $2 \times 10^{-6}$  M, afterwards decreased and further increase of diazinon as well as IMP concentration consequently inhibited cell proliferation potential, lowering all biochemical processes in cells. Concentration dependent inhibition of CBPI in lymphocytes treated with diazinon and IMP was statistically significant ( $t = 4.08$ ,  $p = 0.009$  and  $t = 4.61$ ,  $p = 0.004$ , respectively). The incidence of micronuclei and CBPI in cultures treated with diazinon as well as with IMP correlated inversely, statistically significant ( $r = -0.92$ ,  $p = 0.027$  and  $r = -0.93$ ,  $p = 0.008$ , respectively).

The similar trend was observed in fibroblasts treated with increasing concentrations of diazinon and IMP. The incidence of micronuclei significantly increased in a dose-dependent manner in fibroblasts treated with both, diazinon and IMP, reaching the maximum at concentration of  $2 \times 10^{-6}$  M ( $t = -7.37$ ,  $p = 0.02$  and  $t = -3.12$ ,  $p = 0.035$ , respectively).

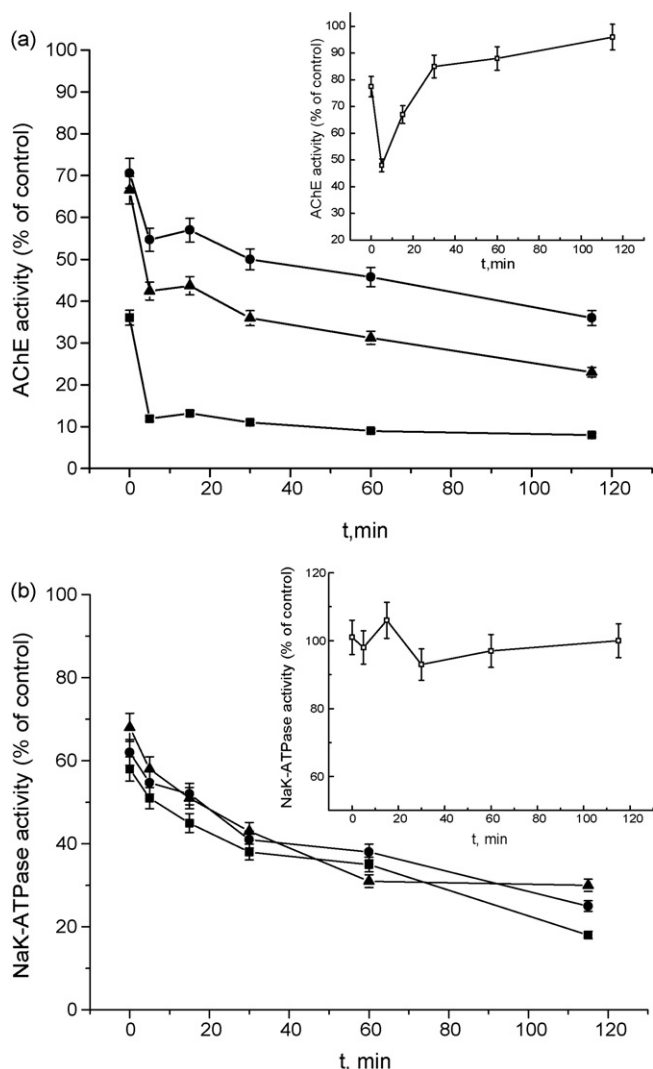
The increasing concentration of IMP significantly lowered ( $t = 2.49$ ,  $p = 0.047$ ) proliferation potential of fibroblasts. On the contrary, treatment with diazinon did not induce significant changes in CBPI at the investigated concentration range ( $p > 0.05$ ). Proliferation potential of cells was slightly reduced at concentration  $2 \times 10^{-6}$  M (compared to control) and remained almost unchanged with increasing the dose.

In lymphocyte cultures treated with IMP positive correlation between micronuclei and the level of MDA was observed ( $r = 0.91$ ,  $p = 0.03$ ), whereas the level of MDA and CBPI correlated inversely ( $r = -0.93$ ,  $p = 0.02$ ).

The data of lymphocyte and fibroblast micronuclei monitoring as well as CBPI in samples treated with irradiated diazinon (initial concentration  $2 \times 10^{-5}$  M), are presented in Table 4. Micronuclei incidence significantly increased with irradiation time in fibroblasts ( $t = -2.49$ ,  $p = 0.047$ ) and at the border of significance in lymphocytes. In both tissues, maximal incidences of micronu-

**Table 4**  
Incidence of micronuclei (MN) and proliferation index in human lymphocytes and fibroblasts treated with irradiated diazinon (initial concentration  $2 \times 10^{-5}$  M).

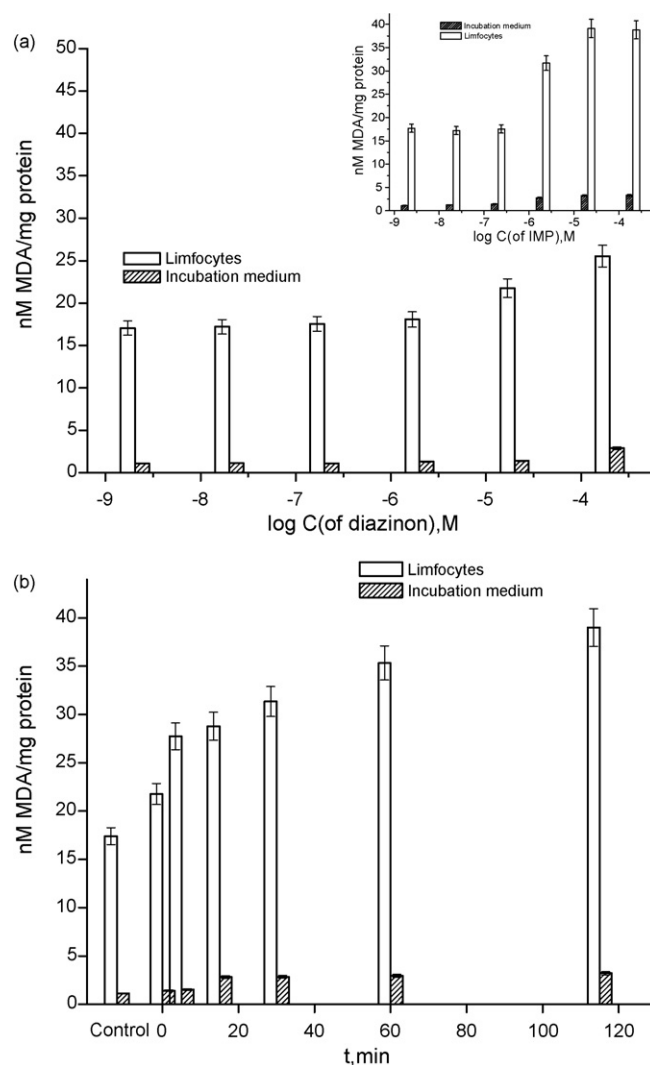
Irradiation time (min)	0	5	15	30	60	115
<b>Lymphocytes</b>						
MN/1000 cells	12.90	15.20	21.44	24.22	43.74	49.45
CBPI	1.26	1.20	1.17	1.15	1.11	1.10
MNC+	7	6	5	6	5	5
MNC–	6	8	17	18	39	45
<b>Fibroblasts</b>						
MN/1000 cells	9.10	13.00	16.00	17.00	29.00	31.00
CBPI	1.31	1.24	1.22	1.28	1.24	1.20
MNC+	7	6	5	6	5	5
MNC–	2	7	11	11	24	26



**Fig. 3.** (a) Inhibition of AChE activity in cells (solid symbols): fibroblasts (square), lymphocytes (triangle) and erythrocytes (circle) induced by  $2 \times 10^{-5}$  M diazinon irradiated solutions. Inset: Inhibition of commercial human erythrocyte AChE induced by  $2 \times 10^{-5}$  M diazinon irradiated solutions. Note: Irradiated solutions of  $1 \times 10^{-3}$  M diazinon were diluted 50 times in blood and fibroblast cultures as well as in incubation medium for determination of AChE activity. (b) Inhibition of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in cells (solid symbols): fibroblasts (square), lymphocytes (triangle) and erythrocytes (circle) induced by  $2 \times 10^{-5}$  M diazinon irradiated solutions. Inset: Inhibition of commercial porcine cerebral cortex Na<sup>+</sup>/K<sup>+</sup>-ATPase induced by  $2 \times 10^{-5}$  M diazinon irradiated solutions. Note: Irradiated solutions of  $1 \times 10^{-3}$  M diazinon were diluted 50 times in blood and fibroblast cultures as well as in incubation medium for determination of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity.

clei were observed at 115 min of irradiation. The proliferation potential of cells significantly decreased with prolonged irradiation time in both tissues ( $t=4.35$ ,  $p=0.005$  and  $t=3.78$ ,  $p=0.009$ , for lymphocytes and fibroblasts, respectively). In lymphocyte cultures, statistically significant inverse correlation between incidence of micronuclei and proliferation index was observed ( $r=-0.91$ ,  $p=0.012$ ). The level of MDA and incidence of micronuclei correlated positively ( $r=0.94$ ,  $p=0.005$ ).

Evaluation of centromere signals in induced micronuclei employing FISH method have shown the clastogenic properties of diazinon and its photodegradation products since majority of induced micronuclei in lymphocytes and fibroblast were centromere negative illustrating that they arise from chromosomal breaks and acentric fragments (Fig. 5).



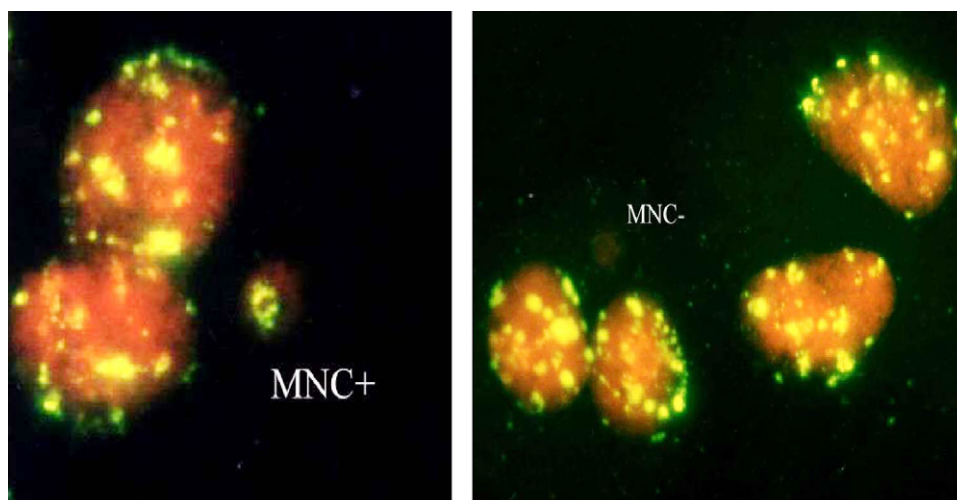
**Fig. 4.** (a) MDA level in lymphocytes and incubation medium after exposure to different diazinon and IMP (inset) concentrations. (b) MDA level in lymphocytes and incubation medium after exposure to  $2 \times 10^{-5}$  M irradiated diazinon as dependence of irradiation time.

#### 4. Discussion

Na<sup>+</sup>/K<sup>+</sup>-ATPase and AChE activity measurements, determination of MDA level as a product of lipid peroxidation and some parameters of cytogenetic damage in the present work have been used to compare the toxic properties of diazinon and some its decomposition products (diazoxon and IMP).

The results of UPLC analysis (Table 1) confirm the presence of other compounds beside these followed by-products. Specifically, the presence of IMP strongly suggests the presence of either diethyl phosphoric acid and/or diethyl thiophosphoric acid (diazinon hydrolysis product), although other not detected compounds might be also present in the irradiated mixtures (Kouloumbos et al., 2003).

The present study demonstrated mostly a dose-dependent inhibition of AChE and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in different human cells induced by diazinon suggesting that this compound might have affected both synaptic nerve transmission and nerve conduction with significant potency. Similar results were previously reported for these enzymes in rats and fishes for diazinon, malathion, parathion and other OPs (Basha and Nayeemunnisa, 1993; Oruc and Usta, 2007). The obtained higher AChE sensitivity in fibrob-



**Fig. 5.** Binucleated cells hybridized with FITC labeled  $\alpha$ -satellite DNA probe: MNC+, binucleated cell with centromere positive micronuclei, MNC-, binucleated cell with centromere negative micronuclei.

lasts and lymphocytes toward diazinon (Fig. 2a), in comparison to the *in vitro* AChE investigation, is in agreement with the fact that CYP (cytochrome P450)-mediated oxidative desulfuration to diazoxon and pyrimidinol metabolites usually leads to stronger inhibition of the enzyme (Amitai et al., 1998). On the other hand, obtained less sensitivity of blood cells AChE toward diazinon compared to fibroblasts AChE (Fig. 2a) (Table 2), could be explained by detoxification activity of carboxylesterases present in plasma. It was reported that a pool of carboxylesterases unknown physiological role, of which the inhibition does not cause apparent toxic effects, exists in mammals (especially in liver and serum). Phosphorylation of these carboxylesterases circulating in plasma is able to 'scavenge' a minimum of one molecule of diazinon before this reaches target enzyme (Sogorb and Vilanova, 2002). Additionally, phosphotriesterases typically found (in higher concentrations) in mammalian serum and liver break the bond between the phosphorous atom and the releasing group in organophosphates (Vilanova and Sogorb, 1999). The obtained less sensitivity of blood cells AChE toward diazinon compared to fibroblasts AChE (Fig. 2a) (Table 2) could be explained by this efficient detoxification route.

Our results (Fig. 3a) show that photodegradation treatment affects diazinon inhibitory efficiency. As can be seen, 5-min treatment rapidly decreases AChE activity in both the investigated cells and commercial enzyme. Obtained AChE inhibition can be attributed to diazoxon concentration  $4.0 \times 10^{-8}$  M that was present in incubation medium. Moreover, obtained inhibition of commercial AChE corresponds to the sum of separate effects of  $4.0 \times 10^{-8}$  M diazoxon and  $1.8 \times 10^{-5}$  diazinon present in incubation medium after dilution of 5-min treated diazinon solution (Table 1). Obtained lower AChE inhibition in blood cells (45–55%) than in fibroblasts (90%) induced by the 5 min diazinon irradiated sample suggests that blood are able to detoxify the mixture more efficiently than fibroblasts, that is in agreement with previously reported detoxification effects of blood carboxylesterases (Sogorb and Vilanova, 2002) hydrolyzing activities associated to serum albumin (Sogorb et al., 2008) and phosphotriesterases (Vilanova and Sogorb, 1999; Sogorb and Vilanova, 2002). Prolonged irradiation time results in gradual increasing inhibitory efficiency of diazinon irradiated solutions in the investigated cells and the presence of 115-min irradiated  $2.0 \times 10^{-5}$  M diazinon depresses AChE activity approximately 25% related to the initial unirradiated solution (Fig. 3a). On the contrary, the obtained gradual recovering reference (commercial) AChE activity between 15<sup>th</sup> and 115<sup>th</sup> minutes of irradiation is a consequence of both diazinon and diazoxon degradation and

IMP formation as a result of breakage of P–O (pyrimidine group) bond (Poet et al., 2003; Shemer and Linden, 2006) (Table 1). These findings are in agreement with previously reported studies about effects of similar organophosphates and their by-products on purified AChEs from different sources (Bavcon Kralj et al., 2007; Krstić et al., 2007, 2008) as well as the fact that IMP is much less potent AChE inhibitor compared to its parent compound diazinon (Ku et al., 1998).

Since both  $\text{Na}^+/\text{K}^+$ -ATPase and AChE are membrane bound enzymes, structural and functional derangement of phospholipids membrane bilayer induced by oxidative stress alters their activities (Sahoo et al., 1999). Therefore, cell AChE activity decreasing in the presence of 30<sup>th</sup>–115<sup>th</sup> minute irradiated solutions (Fig. 3a) could be explained by increasing MDA level under the influence of IMP that does not alter the purified enzyme activity. Lipid peroxidation, as one of the main processes induced by oxidative stress, is probably responsible for  $\text{Na}^+/\text{K}^+$ -ATPase inhibition by diazinon and its by-products in investigated cells. This assumption is supported by the obtained results about commercially purified  $\text{Na}^+/\text{K}^+$ -ATPase insensitivity to diazinon and its photodegradation products (Fig. 2b (inset), Fig. 3b (inset)) and by previously reported data that  $\text{Na}^+/\text{K}^+$ -ATPase activity is highly sensitive to oxidative stress. Two different mechanisms are thought to lead to  $\text{Na}^+/\text{K}^+$ -ATPase inhibition. These are a direct effect of reactive oxygen species and MDA level, and an indirect effect of changes in membrane fluidity (Kourie, 1998; Shattock and Matsuura, 1993).

The diazinon dose-dependent lipid peroxidation (Fig. 4a) is consistent with pro-oxidative properties of OP compounds (Banerjee et al., 1999; Franco et al., 2009) as well as oxidative stress responses in different tissues of *Cyprinus carpio* induced by diazinon (Oruc and Usta, 2007). It must also be noticed that IMP, known as the less toxic product of diazinon induced by direct photolysis (Li et al., 2002), did not exert any significant influence on the purified target enzymes, but exerted the more significant effect than diazinon on MDA formation (Fig. 4a (inset)). Moreover, MDA content induced by irradiated  $2.0 \times 10^{-5}$  M diazinon increases as a function of photodegradation time (Fig. 4b) and can be attributed to IMP formation during diazinon conversion (Table 1).

Lipid peroxidation initiated the formation of MDA, which has the capacity to cross link the amino groups of lipid and protein by the formation of Schiff-bases (Radi and Matcovic, 1988). The presence of MDA is also associated with the polymerization of specific membrane proteins (Radi and Matcovic, 1988) which usually induce cell death via apoptosis, and lower cell proliferation poten-



tial. The obtained cell  $\text{Na}^+/\text{K}^+$ -ATPase inhibition, induced probably via oxidative membrane damage by diazinon and its decomposition products, may be responsible for the observed dose-dependent decrease of CBPI. This assumption is in agreement with previously published findings that some modulators of  $\text{Na}^+/\text{K}^+$ -ATPase affect cell proliferation (Gentile et al., 1997; Vasić et al., 2008). Free radicals influence gene expression, regulate cellular responses to cytokines, as well as proliferative capability of a cell. It is possible that enhanced levels of MDA can lead to cell deregulation and result in apoptosis (Jia and Misra, 2007).

It should be pointed out that diazinon and IMP induce micronuclei in a dose-dependent manner via clastogenic mode of action, inducing single and double strand breaks on DNA molecule. The clastogenic mechanism of action of organophosphate pesticides was also observed in *in vivo* studies in mice (Cicchetti et al., 1999). These findings are consistent with a number of reports in which pesticide exposure has been associated with increases in micronuclei incidence in cultured lymphocytes isolated from peripheral blood taken from exposed individuals (Bull et al., 2006; Bolognesi, 2003). The maximum of toxic activity was observed at concentration of  $2 \times 10^{-6}$  M (Table 3), afterwards the incidence of micronuclei decreases because a very few cells survive further overtoxic concentration. The decrease of micronuclei incidence at concentration above  $2 \times 10^{-6}$  M is accompanied with significant inhibition of cell proliferation and increased level of MDA.

Photodegradation product of diazinon, IMP, possesses stronger genotoxic potential than diazinon alone. In all samples treated with irradiated solutions of diazinon significant increase of the level of MDA and micronuclei incidence was observed, particularly for product yielded at 115 min of irradiation, where the level of MDA is 80% higher than that observed at the beginning of the irradiation (0 min). The same product induces almost 4-fold enhancement of micronuclei incidence. Incidence of micronuclei and level of MDA correlated positively, statistically significant ( $p = 0.005$ ).

In conclusion, despite no observed effect of diazinon (diazoxon) photodegradation products on the activity of the purified target enzymes, the experiments performed with human cell cultures indicate their significant toxicity which is particularly seen in significant enhancement of the MDA level, cytogenetic damage and reduction of AChE and  $\text{Na}^+/\text{K}^+$ -ATPase activity. The human lymphocyte micronucleus test is considered a well-established system to detect genotoxic activity of different agents, especially after development of FISH techniques to unequivocally distinguish the origin of the micronuclei. Therefore, determination of these parameters in exposed cell cultures could be recommended for toxicity evaluation of pesticides and their by-products.

### Conflict of interest statement

The authors declare that there is no conflict of interest.

### Acknowledgments

Authors would like to thank to the Ministry of Science and Technological Development of the Republic of Serbia for their financial support (Project No. 142051). The work was also supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia

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