Bulgarian Journal of Agricultural Science, 20 (Supplement 1) 2014, 9–14
Agricultural Academy

INFLUENCE OF [DIMETHYLPHOSPHINYLMETHYL)AMINO](PHENYL)-METHYLPHOSPHONIC ACID ON ATPase ACTIVITY OF RAT LIVER MITOCHONDRIA

M. SHKODROVA[1*], M. VYDEVSKA-CHICHOVA[1], H. DIMOV[1], D. GANCHEV[1], D. DONCHEVA-STOIMENOVA[1], Y. ZAGRANIARSKY[2], Ts. CHOLAKOVA[2] and S. VARBANOV[3]

[1] *University of Sofia "St. Kliment Ohridski", Department of Animal and Human Physiology, Faculty of Biology, BG – 1164 Sofia, Bulgaria*

[2] *University of Sofia "St. Kliment Ohridski", Department of Organic Chemistry, Faculty of Chemistry and Pharmacy, BG – 1164 Sofia, Bulgaria*

[3] *Bulgarian Academy of Sciences, Institute of Organic Chemistry with Center of Phytochemistry, BG – 1113 Sofia, Bulgaria*

Abstract

SHKODROVA, M., M. VYDEVSKA-CHICHOVA, H. DIMOV, D. GANCHEV, D. DONCHEVA-STOIMENOVA, Y. ZAGRANIARSKY, Ts. CHOLAKOVA and S. VARBANOV, 2014. Influence of [Dimethylphosphinylmethyl)amino](phenyl)-methylphosphonic acid on ATPase activity of rat liver mitochondria. *Bulg. J. Agric. Sci.,* Supplement 1: 9–14

Extensive studies on biological activity of α-aminophosphonic acids have been carried out because of their structural analogy with α-aminocarboxylic acids. Several α-aminophosphonic acid derivatives such as glyphosate, the fundamental substance of the herbicide Roundup, are widely used commercially. [(Dimethylphosphinylmethyl)amino](phenyl)-methylphosphonic acid (DMPPA) is an original α-aminophosphonic acid with dimethylphosphinyl substituent in the molecule. Its preparation has been reported recently. The knowledge of these types of α-aminophosphonic acids is scarce although its molecular structure might determine potential biological activity. The present work was undertaken to investigate the influence of DMPPA on ATPase activity of rat liver mitochondria. Three different mitochondrial preparations were used: intact mitochondria, mitochondria uncoupled by freezing/thawing and submitochondrial particles (SMPs). ATPase activity was determined by measurement of the inorganic phosphate increase in the reaction medium. Two approaches were applied to examine the effects of DMPPA on the ATPase activity of uncoupled mitochondria and SMPs. A set of experiments was conducted under conditions allowing simultaneous interactions of the substrate ATP and DMPPA with the active site of the enzyme. The second approach enabled to study the influence of DMPPA under conditions providing its interaction with the active site prior to saturation with the substrate. We found that DMPPA did not influence ATPase activity of both intact and 2,4-dinitrophenol-uncoupled mitochondria. This suggests that DMPPA does not possess uncoupling effect on intact liver mitochondria and is not able to pass the inner mitochondrial membrane. DMPPA inhibited ATPase activity of both freeze/thawed mitochondria and SMPs. These effects were demonstrated by both approaches of study. It is concluded that DMPPA effects on the ATPase activity of uncoupled mitochondria and SMPs are most probably due to direct interactions of the compound with the enzyme. This study provides a better insight into the mechanisms of the potential biological activity of the α-aminophosphonic acids and theirs derivatives.

Key words: α-aminophosphonic acids, dimethylphosphinyl-substituted phosphonates, mitochondria, submitochondrial particles, mitochondrial ATPase

Abbreviations: ATP – adenosine triphosphate; ATPase – adenosine triphosphatase; DMPPA – [(Dimethylphosphinylmethyl)amino](phenyl)-methylphosphonic acid; DNP – 2,4-dinitrophenol; Pi – inorganic phosphate; SMPs – submitochondrial particles

**E-mail: milshk@abv.bg*

Introduction

Extensive studies on biological activity of α-aminophosphonic acids have been carried out because of their structural analogy with α-aminocarboxylic acids (Hudson, 2000; Mastalerz and Kafarski, 2000; Oleksyzsyn, 2000). The most significant discoveries of the past 30 years have been those which led to the development of aminophosphonates as agrochemicals, especially the commercial development of the glyphosate (N-(phosphonomethyl) glycine) which is the fundamental substance of the herbicide Roundup (Chykaliuk et al., 1980; Naylor, 2002; Lolas and Coble, 2006). In addition, the discovery of aminophosphonic acids in the living systems stimulated the interest in this group of compounds. Synthesis of α-aminophosphonic acid analogues of protein and non-protein amino acids resulted in a new class of drugs and other bioactive compounds with a great variety of commercial applications, such as enzyme inhibitors, antifungal agents, herbicides, plant growth regulators and pesticides, immune system activators, neuroactive, antitumour, antiviral, and antibacterial compounds (Kafarski and Lejczak, 2000, 2001; Naydenova et al., 2007, 2010; Orsini et al., 2010). Along with this wide range of agricultural and medical applications, the necessity of profound research on possible adverse effects of these compounds on human health and the environment is growing.

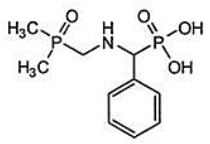

Fig. 1. Structure of [(Dimethylphosphinylmethyl)amino](phenyl)-methylphosphonic acid (DMPPA)

[(Dimethylphosphinylmethyl) amino](phenyl)-methylphosphonic acid (DMPPA) is an original α-aminophosphonic acid with dimethylphosphinyl-substituent in the molecule (Figure 1). Its synthesis from imine of aminomethyl-dimethylphosphine oxide has been reported recently (Zagraniarsky et al., 2008). The originality of DMPPA is based on its composition including two different phosphorus containing groups – phosphonyl and dimethylphosphinyl. This molecular structure might determine potential biological activity. The presence of a free phosphonyl group and/or a lipophilic cyclic part in the molecule of this compound creates the possibility of interaction with enzymes using adenosine triphosphate (ATP) as a substrate.

Mitochondrial ATP synthase/ATPase (ATP hydrolase: EC 3.6.1.34) is an enzyme complex, responsible for ATP synthesis in the cell. It is comprised of a membrane-spanning sector F_0 and a soluble sector F_1. The latter catalyzes the synthesis of ATP from adenosine diphosphate and inorganic phosphate (Pi) by utilizing the transmembrane proton gradient and membrane potential generated during substrate oxidation. This reaction can be reversed by pumping protons in the opposite direction resulting in ATP hydrolysis (Boyer, 1997). Although there are evidences that Roundup induces uncoupling of the oxidative phosphorylation of rat liver mitochondria associated to non-specific membrane permeabilization (Peixoto, 2005), there are no reports on direct or indirect effects of α-aminophosphonic acids on the mitochondrial ATPase.

The present work was undertaken to evaluate the *in vitro* effects of DMPPA on ATPase activity of rat liver mitochondria. This could contribute to the elucidation of the mechanisms underlying the potential biological activity of the α-aminophosphonic acids.

Materials and Methods

DMPPA was synthesized in the Department of Organic Chemistry, Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski" in cooperation with the Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences.

Liver mitochondria were isolated from male albino rats (Wistar strain, 120–150 g). All studies were performed in accordance with the institutional ethical guidelines. Intact mitochondria and submitochondrial particles (SMPs) were isolated as previously described (Shkodrova et al., 2013). ATPase activity was assayed at room temperature and continuous stirring in 5 ml reaction medium consisting of 200 mM sucrose, 10 mM KCl, 50 mM Tris-HCl, 100 μM EDTA-KOH, and 1 mM ATP (pH 7.5), and 50 μM 2,4-dinitrophenol (DNP) was included wherever indicated. Since SMP lose bound Mg^{2+} during isolation, their activity was assayed in the presence of 2 mM $MgCl_2$. Aliquots of the DMPPA stock solution were added to reach final concentrations of 10 and 100 μM, respectively. The reaction was started by adding the enzyme or ATP. Samples of 500 μl were taken after 15, 30, 60, 120, 180 and 300 s incubation and added to 200 μl of 3M perchloric acid for termination of the reaction. The concentration of Pi hydrolyzed from ATP was measured by the method of Fiscke and Subbarow (1925) with modifications. Blanks were assayed under the same conditions, except that the enzyme was omitted in order to determine the background Pi amount as a result of nonenzymatic hydrolysis of ATP.

ATPase activity was expressed as μmol Pi.mg $protein^{-1}$. The change of ATPase activity (Δ [%]) at each point during the reaction was calculated as the difference between the DMPPA-treated and the corresponding control samples values and normalized against the control samples values. Data are expressed as mean ± standard error of the mean (SEM). The difference between DMPPA-treated samples and the un-

treated control was tested by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered significant.

Results and Discussion

To test the possible uncoupling action of DMPPA a set of experiments with intact mitochondria was carried out. Figure 2A and 2B show the results from one representative experiment with intact and DNP-uncoupled mitochondria, respectively. DMPPA was applied in concentrations of 10 μM and 100 μM. ATPase activity remained low until the end of the registration under control conditions. Addition of DNP in final concentration of 50 μM powerfully stimulated ATP hydrolysis. DMPPA did not influence ATPase activity of both intact (Figure 2A) and DNP-uncoupled mitochondria (Figure 2B). This suggests that DMPPA does not possess an uncoupling effect on intact liver mitochondria and it is not able to pass the inner mitochondrial membrane.

For partial removal of the membrane barrier series of experiments with freeze/thawed mitochondria were carried out. Freezing/thawing disrupts the mitochondrial inner membrane leading to uncoupling and stimulation of initial ATPase activity. In parallel with this, mitochondria preserve their structure and the catalytic complex F_1 is partially protected from the reagents in the medium. In addition, for complete removal of the membrane barrier a set of experiments with SMPs was conducted. SMPs represent closed inverted vesicles derived from the inner mitochondrial membrane. As a result, the complex F_1 is located on the outside of the membrane and oriented to the solution, which allows direct access of the reagents in the medium to the enzyme.

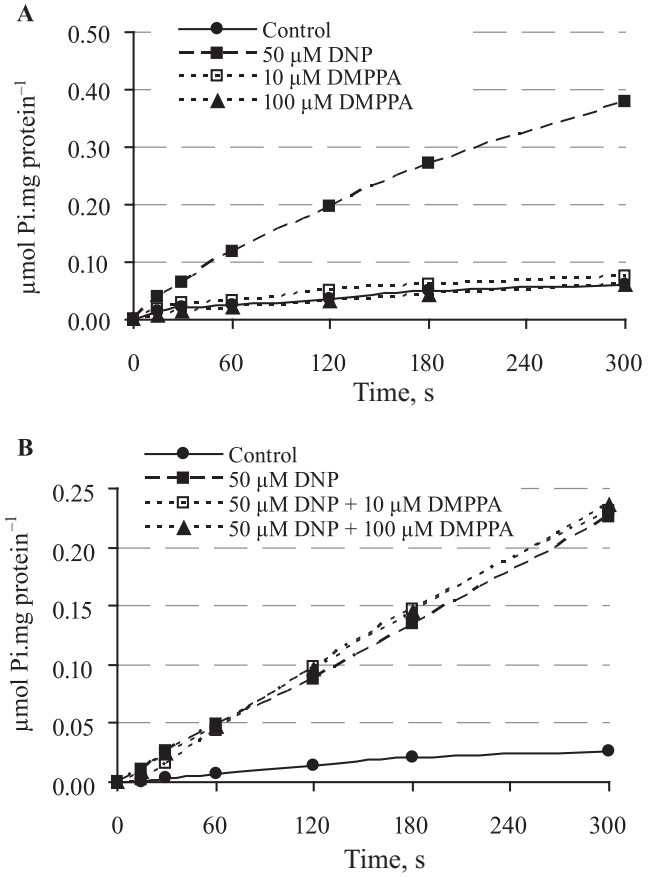

Fig. 2. Influence of DMPPA on ATPase activity of intact (A) and DNP-uncoupled (B) mitochondria. Data present curves from two representative experiments. The reactions were started by adding mitochondrial suspension (protein 4.93 mg/sample and 7.17 mg/sample for intact and DNP-uncoupled mitochondria, respectively)

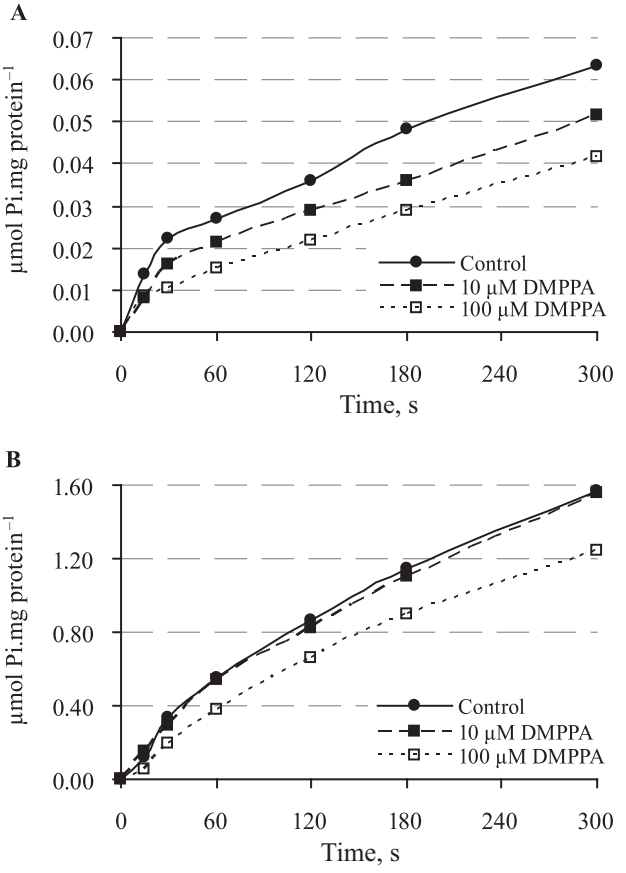

Fig. 3. Influence of DMPPA on ATPase activity of freeze/thawed mitochondria (A) and SMPs (B). Data present curves registered during one representative experiment with mitochondria and SMPs, respectively. The reactions were started by addition of 5.05 mg and 0.42 mg protein for the mitochondria and SMPs, respectively

Two approaches were applied to examine the effects of DMPPA on the ATPase activity of uncoupled mitochondria and SMPs. A set of experiments was conducted under conditions allowing simultaneous interactions of the substrate ATP and DMPPA with the active site of the enzyme. In this case DMPPA in concentrations of 10 μM and 100 μM and ATP (final concentration of 1 mM) were introduced into the medium prior to the reaction start by addition of the corresponding mitochondrial or SMPs suspensions. Figure 3A and 3B present the results from representative experiments with freeze/thawed mitochondria and SMPs, respectively. DMPPA reduced ATPase activity of uncoupled mitochondria in a dose-dependent manner. An inhibiting effect of DMPPA on the ATPase activity of SMPs was manifested only at the higher applied concentration (100 μM).

Table 1

Averaged normalized changes (Δ [%]) of ATPase activity of freeze/thawed mitochondria and SMPs at each point in the course of the reaction started by addition of mitochondrial and SMPs protein, respectively

Preparation	Time, s	DMPPA			
		10 μM		100 μM	
		Δ [%]	p	Δ [%]	p
Freeze/thawed mitochondria (n = 3)	15	–46.37 ± 10.71	0.259	–53.02 ± 9.37	0.130
	30	–32.40 ± 5.17	0.210	–47.68 ± 4.00	0.075
	60	–21.01 ± 4.20	0.255	–33.82 ± 5.09 *	0.045
	120	–19.56 ± 1.48 *	0.024	–26.19 ± 6.05 *	0.025
	180	–17.51 ± 3.45 **	0.007	–22.66 ± 10.60	0.100
	300	–14.27 ± 3.39 *	0.027	–15.07 ± 11.63	0.425
SMPs (n = 3)	15	15.02 ± 15.37	0.572	–83.33 ± 13.61 **	0.008
	30	–12.71 ± 0.98	0.295	–63.02 ± 10.11 **	0.006
	60	–4.40 ± 2.89	0.659	–46.18 ± 6.67 *	0.012
	120	–3.85 ± 3.14	0.743	–34.14 ± 4.43 *	0.026
	180	–2.79 ± 2.71	0.793	–30.84 ± 3.82 *	0.025
	300	–1.79 ± 3.09	0.839	–25.87 ± 2.13	0.050

Values are mean ± SEM of three independent experiments. Asterisks indicate significant differences (*p < 0.05, **p < 0.01) from the control.

Table 2. Averaged normalized changes (Δ [%]) of ATPase activity of freeze/thawed mitochondria and SMPs at each point in the course of the reaction started by addition of ATP

Preparation	Time, s	DMPPA			
		10 μM		100 μM	
		Δ [%]	p	Δ [%]	p
Freeze/thawed mitochondria (n = 5)	15	–53.33 ± 20.65	0.824	–68.96 ± 25.35	0.790
	30	–43.93 ± 16.65	0.461	–54.27 ± 22.28	0.825
	60	–43.10 ± 16.30	0.469	–50.28 ± 22.26	0.786
	120	–39.39 ± 10.25	0.430	–46.02 ± 19.00	0.694
	180	–33.47 ± 9.82	0.435	–41.95 ± 16.31	0.685
	300	–27.00 ± 11.54	0.533	–35.37 ± 12.77	0.673
SMPs (n = 3)	15	–2.28 ± 47.54	0.801	–51.05 ± 24.67	0.306
	30	–8.76 ± 21.75	0.480	–56.58 ± 19.27	0.154
	60	–2.45 ± 6.70	0.856	–33.96 ± 9.09	0.338
	120	4.36 ± 2.95	0.874	–19.99 ± 5.92	0.590
	180	–2.89 ± 3.72	0.917	–20.61 ± 1.40	0.571
	300	1.19 ± 4.03	0.993	–11.21 ± 2.01	0.769

ATP in final concentration of 1 mM was added after 1 min incubation of the mitochondria and SMPs in the presence of the corresponding concentrations of DMPPA. Values are mean ± SEM of five and three independent experiments for the mitochondria and SMPs, respectively.

Table 1 shows the averaged normalized changes (Δ [%]) of ATPase activity of freeze/thawed mitochondria and SMPs calculated at each point in the course of the reaction started by addition of mitochondrial or SMPs protein, respectively. The inhibiting effect of DMPPA on ATPase activity was most strongly manifested in the beginning of the reaction (15^{th} and 30^{th} s).

The observed effects of DMPPA could be due to direct interactions with the water soluble catalytic complex F_1 and/or with the membrane sector F_0.

The second approach of study enabled to investigate the influence of DMPPA under conditions providing its interaction with the active site of the enzyme prior to its saturation with the substrate. For that purpose the mitochondrial or SMPs suspensions were added to the reaction medium and incubated for 1 min in the presence of DMPPA. After that the reaction was started by addition of ATP. The results obtained with freeze/thawed mitochondria (Figure 4A) were similar to those from the previous set of experiments except that stronger effect was demonstrated. DMPPA in concentrations of 10 and 100 μM reduced ATPase activity of freeze/thawed mitochondria in a dose-dependent manner, but the differences between the groups were not statistically significant (Table 2). The stronger inhibiting effect is probably a result of DMPPA interactions with the enzyme before the reaction start with the substrate ATP. The results obtained with SMPs (Figure 4B, Table 2) showed a weaker inhibiting effect of DMPPA compared to those when reaction was started by the enzyme.

As it was pointed above, in SMPs the catalytic complex F_1 is located on the outside of the membrane and accessible to direct action of the reagents in the medium. Hence, stronger influences could be expected on SMPs compared to those on freeze/thawed mitochondria. This statement was not confirmed. On the contrary, a weaker inhibiting effect of DMPPA on ATPase activity of SMPs was found compared to uncoupled mitochondria. The differences in DMPPA effects on the two preparations could be due to the presence of Mg^{2+} in the reaction medium for SMPs (see section Materials and Methods), as well as to an additional influence of the mitochondrial membrane itself.

Taking into account the characteristics of the preparations used in this study (freeze/thawed mitochondria and SMPs) it could be assumed that the decreased ATPase activity is a result of a direct interaction of DMPPA with the enzyme. This is a possible explanation because the catalytic subunit F_1 in these preparations is either oriented to the medium (in SMPs) or it is linked to a disrupted membrane (in freeze/thawed mitochondria).

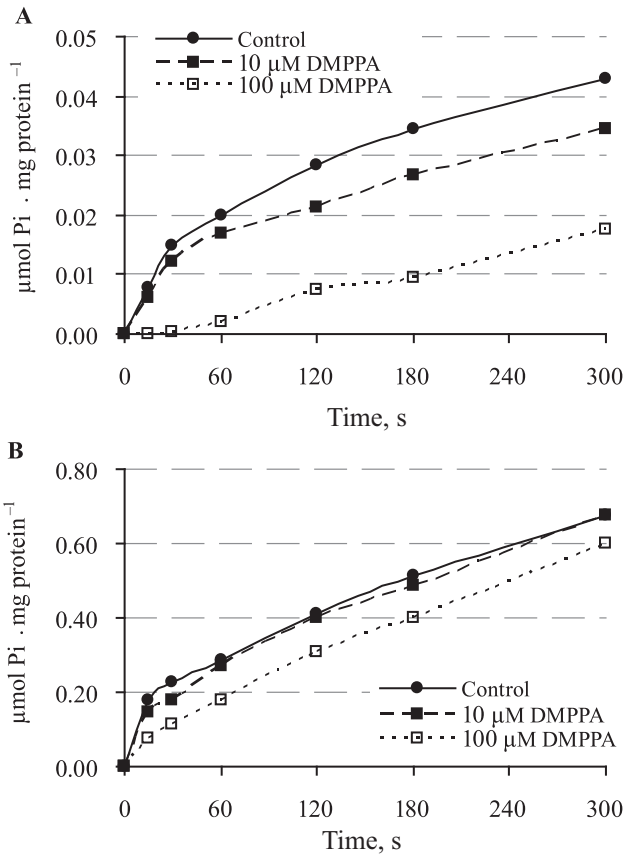

Fig. 4. Influence of DMPPA on ATPase activity of freeze/thawed mitochondria (A) and SMPs (B). Data present curves registered during one representative experiment with mitochondria and SMPs, respectively. The reactions were started by adding ATP in final concentration of 1 mM after 1 min incubation of the mitochondria and SMPs in the presence of the corresponding concentrations of DMPPA (protein 5.05 mg/sample and 0.75 mg/sample for the mitochondria and SMPs, respectively)

It should be noted that in the present study we demonstrate the inhibitory effects of DMPPA on the ATPase activity of a membrane-bound enzyme. There is a possibility that the compound affects the enzyme surroundings rather than enzyme itself. Further studies on DMPPA influence on the activity of isolated soluble ATPase (F_1) could provide opportunity to explain the mechanisms of the potential biological activity of the α-aminophosphonic acids.

Conclusions

Our findings demonstrate that DMPPA does not influence ATPase activity of both intact and DNP-uncoupled

mitochondria. This suggests that DMPPA does not possess uncoupling effect on intact liver mitochondria and is not able to pass the inner mitochondrial membrane. DMPPA inhibits ATPase activities of freeze/thawed mitochondria and SMPs. These effects are demonstrated under conditions allowing simultaneous interactions of the substrate ATP and DMPPA with the active site of the enzyme, as well as under conditions providing DMPPA interaction with the enzyme active site prior to saturation with the substrate. The effects of DMPPA on the ATPase activity of uncoupled mitochondria and SMPs are most probably due to direct interaction of the compound with the enzyme. Our results provide an insight into the mechanisms of the possible deleterious effects of α-aminophosphonic acids on the cells, in particular on mitochondria.

Acknowledgements

This work was supported by Grant № 24/2013 from Sofia University "St. Kliment Ohridski".

References

Boyer, P. D., 1997. The ATP synthase – a splendid molecular machine. *Ann. Rev. Biochem*., **66**: 717–749.

Chykaliuk, P., J. Abernathy and J. Gipson, 1980. Bibliography of glyphosate. The Texas Agricultural Experimental Station, Lubbock.

Fiscke, C. H. and Y. Subbarow, 1925. The colorimetric determination of phosphorus. *J. Biol. Chem*., **66**: 375–381.

Hudson, H. R., 2000. Aminophosphonic and Aminophosphinic Acids and their Derivatives as Agrochemicals. 13th chapter in: V. P. Kukhar and H. R. Hudson (Editors), *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity*, *John Wiley & Sons*, Chichester-Toronto, pp. 443–482.

Kafarski, P. and B. Lejczak, 2000. The Biological Activity of Phosphono- and Phosphinopeptides. 12th chapter in: V. P. Kukhar and H. R. Hudson (Editors), *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity*, *John Wiley & Sons*, Chichester-Toronto, pp. 407–442.

Kafarski, P. and B. Lejczak, 2001. Aminophosphonic acids of potential medical importance. *Curr. Med. Chem. Anticancer Agents,* **1**: 301–312.

Lolas, P. and H. Coble, 2006. Translocation of ^{14}C-glyphosate in johnsongrass (*Sorghum halepense* L. Pers.) as affected by growth stage and rhizome length. *Weed Res*., **20** (5): 267–270.

Mastalerz, P. and P. Kafarski, 2000. Naturally Occuring Aminophosphonic and Aminophosphinic Acids. 1st chapter in: V. P. Kukhar and H. R. Hudson (Editors), *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity*, *John Wiley & Sons*, Chichester-Toronto, pp. 1–32.

Naydenova, E. D., P. T. Todorov and K. D. Troev, 2010. Recent synthesis of aminophosphonic acids as potential biological importance. *Amino Acids*, **38**: 23–30.

Naydenova, E., K. Troev, M. Topashka-Ancheva, G. Haegele, I. Ivanov and A. Kril, 2007. Synthesis, cytotoxicity and clastogenicity of novel α-aminophosphonic acids. *Amino Acids*, **33**: 695–702.

Naylor, R., 2002. Weed management handbook, 11th chapter ''Herbicide-resistant weeds'', 9th edn. Blackwell, Banchory.

Oleksyzsyn, J., 2000. Aminophosphonic and Aminophosphinic Acid Derivatives in the Design of Transition State Analogue Inhibitors: Biomedical Opportunities and Limitations. 15th chapter in: V. P. Kukhar and H. R. Hudson (Editors), *Aminophosphonic and Aminophosphinic Acids, Chemistry and Biological Activity*, *John Wiley & Sons*, Chichester-Toronto, pp. 537–558.

Orsini, F., G. Sello and M. Sisti, 2010. Aminophosphonic Acids and Derivatives. Synthesis and Biological Applications. *Current Medicinal Chemistry*, **17**: 264–289.

Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere,* **61**: 1115–1122.

Shkodrova, M., M. Chichova, D. Ganchev, I. Sazdova and I. Minkov, 2013. Influence of sodium nitroprusside as a donor of nitric oxide on ATPase activity of rat liver mitochondria. *Bulg. J. Agricult. Sci.,* **19**: 167–170.

Zagraniarsky, Y., B. Ivanova, K. Nikolov, S. Varbanov and T. Cholakova, 2008. Synthesis of Dimethylphosphinyl-substituted ***α***-Amino(aryl)methylphosphonic Acids and Their Esters, *Z. Naturforsch.*, 63b: 1192–1198.