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Toxicovigilance: New biochemical tool used in sulfonylurea herbicides toxicology studies

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In vitro toxic effects of sulfonylurea herbicides (thifensulfuron-methyl and metsulfuron-methyl) were evaluated according to a new protocol. Physiological conditions were reproduced in order to boost toxicovigilance. Sulfonylureas and their hydrolysis products were added to biological substrates such as urea, alanine, aspartic acid, α-ketoglutarate, oxaloacetate, pyruvate and then incubated with some specific enzymes. Addition of these sulfonylureas and their degradation products did not significantly change the enzymatic activity of the urease, aspartate-aminotransferase, glutamate dehydrogenase, malate dehydrogenase and lactate dehydrogenase. However, the acid hydrolysis products inhibited up to 95% of the activity of the alanine-aminotransferase at low concentrations (0.27 μmol L^{-1}). Inhibition did not affect the mitochondrial aspartate-aminotransferase.

Keywords: sulfonylureas, herbicides, toxicovigilance, toxic agent, alanine-aminotransferase, aspartate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, urease

Sulfonylureas are herbicides (Fig. 1) which will find increased use due to their wide-spectrum effects on weeds and their low toxic effects on mammals (1). The effects of sulfonylureas on plants, micro-algae and bacteria are due to the inhibition of acetolactate synthase (ALS) involved in the synthesis of acetolactic and butyric acids, which are the precursors of the branched-chain amino acids: isoleucine, leucine and valine (2). Indeed, ALS catalyzes the condensation of pyruvate with acetaldehyde by means of amine hydroxy-ethyl-thiamine diphosphatase. The final amino acid results from the transfer of the glutamate amino group to the α-ketonic acid through transaminase (Fig. 2).

Thus far, possible effects on humans have not been described and hence the interest in toxicovigilance, which consists of monitoring and listing possible incidents caused by new toxic agents such as pesticides. *In vitro* predicting biological tools are essential for estimating exogenous molecular toxicity at the subcellular level and enable to establish links between molecules and *in vivo* toxic effects (3). The aim of this work is to develop

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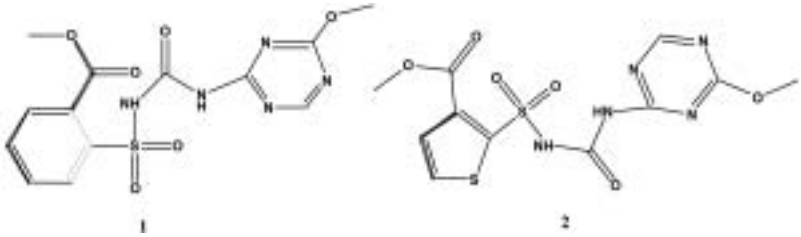

Fig. 1. Sulfonylurea herbicide structures: metsulfuron-methyl (1) and thifensulfuron-methyl (2).

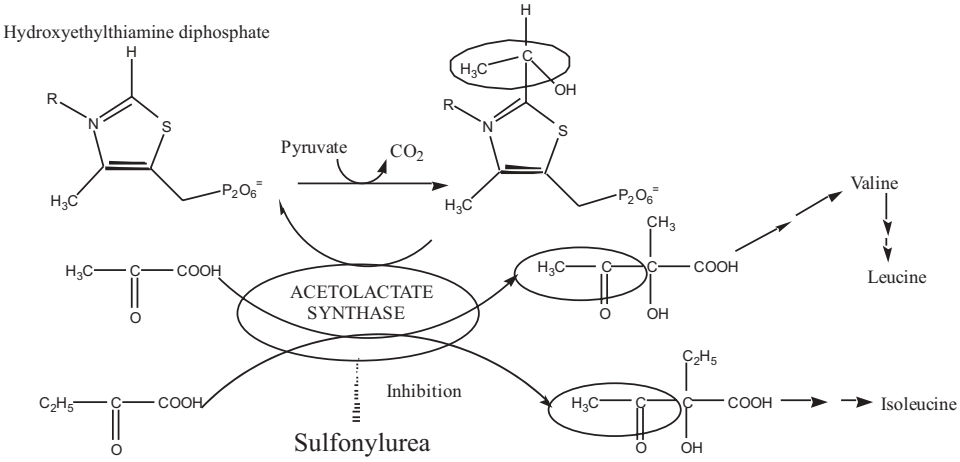

Fig. 2. Herbicide effects of sulfonylureas.

an i*n vitro* method in order to evaluate the effects of metsulfuron-methyl and thifensulfuron-methyl as well as their hydrolysis products on enzymatic and energetic cellular mechanisms. In this work, we describe the *in vitro* effects of sulfonylurea pesticides on the enzymatic activity involved in the metabolism of α-ketonic acid (Fig. 3).

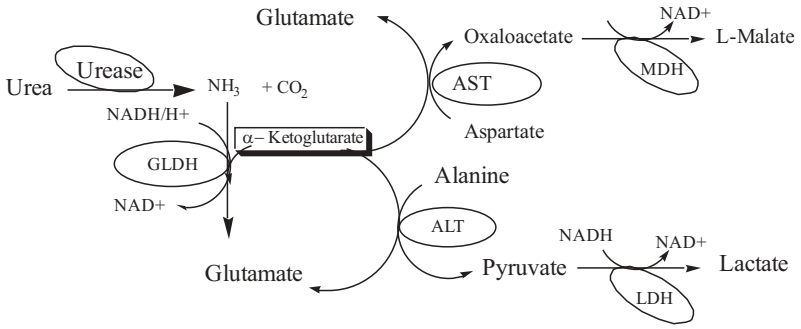

Fig. 3. Explored biochemical panel.

EXPERIMENTAL

Enzymatic activities were measured on the multiparametric analyzer Hitachi 911 (Boehringer, Germany) using aqueous solutions at 37 ± 0.1 °C.

The reagents used in this study and the lyophilized Universal Control Serum Precipath U were provided by Boehringer. Metsulfuron-methyl and thifensulfuron-methyl were provided by the Dupont de Nemour laboratory (France).

Hydrolysis

Sulfonylureas (20 μmol L^{-1}) were incubated in HCl medium at pH 1 and 40 °C for 15 minutes and then neutralized to pH 7 with NaOH (1 mol L^{-1}). Sulfonylureas (20 μmol L^{-1}) in sodium hydroxide solution at pH 11 were incubated at 40 °C for 15 minutes and neutralized to pH 7 with HCl.

Enzymatic activity tests

The folloving enzymes were studied: alanine aminotransferase (ALT), aspartate dehydrogenase (AST), glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and urease. The respective enzymatic reactions are described elsewhere (4–5).

Typically, enzymes and their corresponding substrates, were introduced in the reactive cells to which sulfonylureas were added at different concentrations (from 0.27 μmol L^{-1} to 0.4 mmol L^{-1}). Final concentrations obtained in the reactive cells are given in Table I. The mixtures were incubated at 37 °C. Enzimatic reactions involved in ketoglutarate metabolism are tested by measuring the NAD^+/NADH optical density at 340 nm.

Data were statistically analyzed by Student's *t*-test as described by Fischer. The values are expressed as mean ± SD.

Table I. Summary of biological reactions

	R_I		R_{II}		R_{III}		R_{IV}	
Substrate (mmol L^{-1})	Urea[b]	12.1	Alanine	625	Pyruvate	3.64	Aspartate	300
	Sulfonyl-urea	variable	Sulfonyl-urea	variable	Sulfonyl-urea	variable	Sulfonyl-urea	variable
	Keto-glutarate	5.5	Keto-glutarate	94	–	–	Keto-glutarate	75
	NADH/H^+	0.19	NADH/H^+	0.23	NADH/H^+	0.22	NADH/H^+	0.23
Enzyme (IU L^{-1})	Urease	347	ALT[b]	64	–	–	AST[b]	133
	GLDH	900	LDH	119	LDH[b]	249	MDH	420

ALT – alanine aminotransferase, AST – aspartate dehydrogenase, GLDH – glutamate dehydrogenase, LDH – lactate dehydrogenase, MDH-malate dehydrogenase.

[a] In the reactive cells (R_I, R_{II}, R_{III} and R_{IV}), enzymes and their corresponding substrates were incubated with sulfonylurea added at different concentrations (varying from 0.27 μmol L^{-1} to 0.4 mmol L^{-1}).

[b] Urea, ALT, AST and LDH derived from PPU (Universal Control Serum U) based on human serum.

RESULTS AND DISCUSSION

Enzymatic activities of LDH, ALT, AST, GLDH and MDH in the presence of native sulfonylureas or sulfonylureas previously incubated in basic or acid media, or without them, are compared and outlined in Table II. There is no significant difference between enzymatic activities of ALT, AST, GLDH, LDH, MDH and urease in the presence and in the absence of sulfonylureas in neutral or basic media.

Table II shows ALT activity in the presence of sulfonylureas previously incubated in acid medium. The ALT activities with and without sulfonylureas are significantly different ($p < 0.001$) and estimated at 64 ± 5 IU L^{-1} without sulfonylurea *versus* 5 ± 2 IU L^{-1} for thifensulfuron-methyl and 3 ± 1 IU L^{-1} for metsulfuron-methyl.

Table II. Enzymatic activities observed in the native and in the incubated samples of sulfonylureas[a]

Sulfonylurea (0.4 mmol L^{-1})	Urease (IU L^{-1})	GLDH (IU L^{-1})	ALT (IU L^{-1})	LDH (IU L^{-1})	AST (IU L^{-1})	MDH (IU L^{-1})
METSULFURON-METHYL						
Native	391 ± 25	858 ± 55	59 ± 6	246 ± 15	129 ± 07	401 ± 22
Incubated in basic medium	410 ± 16	900 ± 34	62 ± 5	252 ± 12	130 ± 12	404 ± 37
Incubated in acidic medium	302 ± 30	695 ± 55	3 ± 1[b]	260 ± 10	118 ± 08	367 ± 25
THIFENSULFURON-METHYL						
Native	388 ±22	851 ± 48	64 ± 5	246 ± 12	126 ± 06	392 ± 19
Incubated in basic medium	410 ± 10	972 ± 21	64 ± 5	243 ± 10	135 ± 12	420 ± 37
Incubated in acidic medium	347 ± 12	761 ± 27	5 ± 2[b]	249 ± 18	126 ± 05	394 ± 16
Control serum (no sulfonylurea added)	381 ± 26	838 ± 60	64 ± 5	250 ± 20	133 ± 09	413 ± 28

[a] Values are expressed as mean ± SD ($n = 3$).
[b] Significantly different from control serum ($p < 0.001$).

Table III reveals that ALT activity remained significantly lower ($p < 0.001$) than that in the control serum, even after successive dilutions of sulfonylureas; alanine concentration at 625 mmol L^{-1} was kept constant. ALT activities were found 9 ± 3 IU L^{-1} for 0.27 μmol L^{-1} of metsulfuron-metyl (corresponding to 13% of free ALT activity) and 12 ± 5 IU L^{-1} for 0.27 μmol L^{-1} thifensulfuron-methyl (corresponding to 17% of free ALT activity), at the alanine/sulfonylurea ratio close to 10^6.

The marked decrease of the ALT activity in acidic medium seems to be the result of hydrolysis products of sulfonylureas. In fact, the sulfonylureas are stable at neutral or basic pH but undergo acid hydrolysis in a medium comparable to a gastric one (6–8). Furthermore, the studies carried out by different teams have shown that, on one hand, acid hydrolysis was rapid at 40 °C and, on the other hand, the degradation products were benzoic-sulfonamide methyl ester (BCS for metsulfuron-methyl) and thiophen-carboxyl-sulfonamide methyl ester (TCS for thifensulfuron-methyl), and triazine which is common to both (9).

Table III. ALT activity in acid hydrolysed sulfonylurea medium[a,b]

Added sulfonylurea (mol L^{-1})	Alanine/sulfonylurea molar ratio	ALT (IU L^{-1})	Free ALT activity (%)
METSULFURON-METHYL			
2.7×10^{-7}	2.3×10^{6}	9 ± 3^{c}	13 ± 4
1.4×10^{-6}	4.6×10^{5}	10 ± 2^{c}	16 ± 6
2.7×10^{-6}	2.3×10^{5}	11 ± 4^{c}	16 ± 6
1.4×10^{-5}	4.6×10^{4}	10 ± 5^{c}	16 ± 7
2.7×10^{-5}	2.3×10^{4}	11 ± 2^{c}	16 ± 6
1.3×10^{-4}	4.8×10^{3}	3 ± 1^{c}	4 ± 1
2.7×10^{-4}	2.3×10^{3}	6 ± 2^{c}	9 ± 3
4.0×10^{-4}	1.6×10^{3}	5 ± 2^{c}	7 ± 3
THIFENSULFURON-METHYL			
2.7×10^{-7}	2.3×10^{6}	12 ± 5^{c}	17 ± 7
1.4×10^{-6}	4.6×10^{5}	11 ± 4^{c}	16 ± 6
2.7×10^{-6}	2.3×10^{5}	12 ± 2^{c}	17 ± 3
1.4×10^{-5}	4.6×10^{4}	10 ± 4^{c}	16 ± 6
2.7×10^{-5}	2.3×10^{4}	12 ± 5^{c}	17 ± 7
1.3×10^{-4}	4.8×10^{3}	4 ± 1^{c}	6 ± 1
2.7×10^{-4}	2.3×10^{3}	3 ± 2^{c}	4 ± 3
4.0×10^{-4}	1.6×10^{3}	3 ± 1^{c}	4 ± 1
CONTROL SERUM (no added sulfonylurea)	–	64 ± 5	93 ± 7

[a] Values are expressed as mean ± SD ($n = 3$).
[b] Constant concentration of alanine $c = 625$ mmol L^{-1}.
[c] Significantly different from PPU control serum ($p < 0.05$).
ALT activity was measured in the absence (with control serum) and presence of sulfonylureas at different concentrations (varying from 0.4 mmol L^{-1} and 0.27 μmol L^{-1}) previously incubated in acid medium and constant concentration of alanine at 625 mmol L^{-1}. Values are expressed as mean ± SD of three determinations.

The examined biochemical panel enables us to locate the effects of sulfonylureas and their hydrolysis products on the enzymatic mechanism involved in the branched--chain amino acids catabolism (Fig. 3).

There is no significant difference between the activities of urease, GLDH, AST, MDH and LDH incubated with native and hydrolyzed sulfonylureas. Lack of interference with urease and GLDH shows that the hydrolysis releases neither urea nor ammonia. Moreover, BCS, TCS and triazine do not disturb the enzymatic activities of AST, MDH and LDH and do not interact with their subtrates, namely α-ketoglutarate, aspartate, oxaloacetate and pyruvate.

A sharp significant ALT activity decrease was observed in the presence of sulfonylurea acid hydrolysis products at 0.27 μmol L^{-1} (see Table III). This inhibition of ALT could be a result of the specific competition of the hydrolysis product of sulfonylureas with alanine in particular since it does not affect the AST. The inhibition of ALT results from the resistance of ALT-BCS (or ALT-TCS) sulfonamide bond to hydrolysis leading to

accumulation of ALT in an imine form, unfit for transamination (Fig. 4). This ALT inhibition could result from the specific competition of the hydrolysis product of sulfonylureas with alanine in particular since it does not affect AST.

Bearing this in mind, the cytosolic ALT plays an important role for the hepatic cells

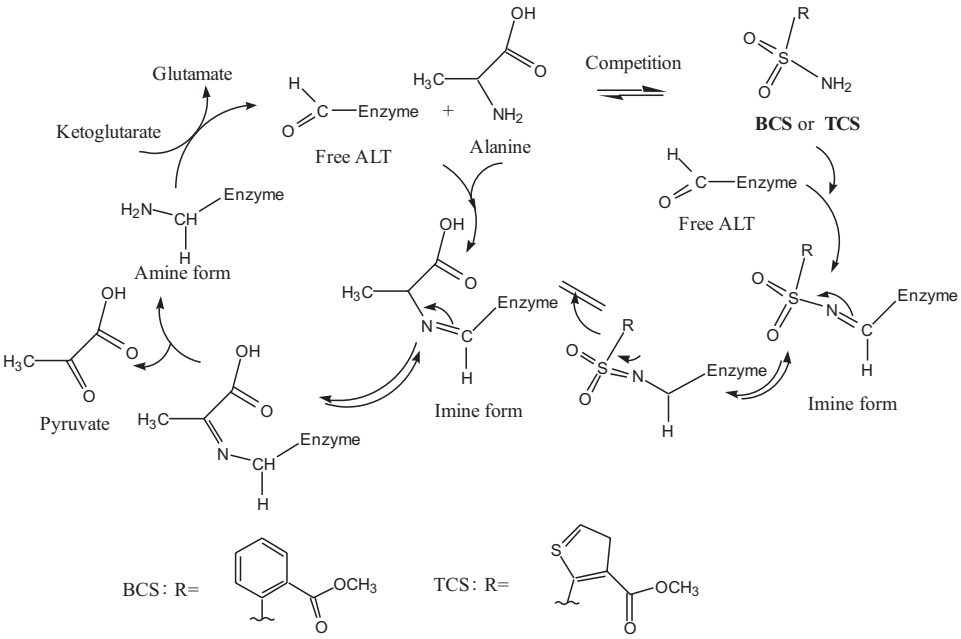

Fig. 4. Mechanism of ALT inhibition by hydrolyzed sulfonylureas.

in branched-chain amino acid catabolism, which constitutes a source of energy exploited by the cell and generates very toxic ammonia, then converted into urea by mitochondrial enzymes. Inhibition of ALT by hydrolyzed sulfonylureas leads to disturbance of aminoacid catabolism and mitochondrial urea cycle with accumulation of cytosolic ketoglutarate (Fig. 5).

The first step of the catabolism which generates ketonic components that undergo, inside the mitochondria, an oxidizing degradation occurs in the cytosol. Inhibition by the sulfonylurea hydrolysis products generates suppression of the penetration of α-ketogluratate and pyruvate as well as the intracytoplasmic accumulation of ketonic metabolites. This may explain the cell's impairment along with a cytosolic enzymatic release as well as the hepatic steatosis observed in animals (10). The quite moderate signs of the hepatic impairment compared with the deep *in vitro* inhibition of hydrolyzed sulfonylureas may be related to its low bioavailability due to the low lipophilicity of the degradation products (11).

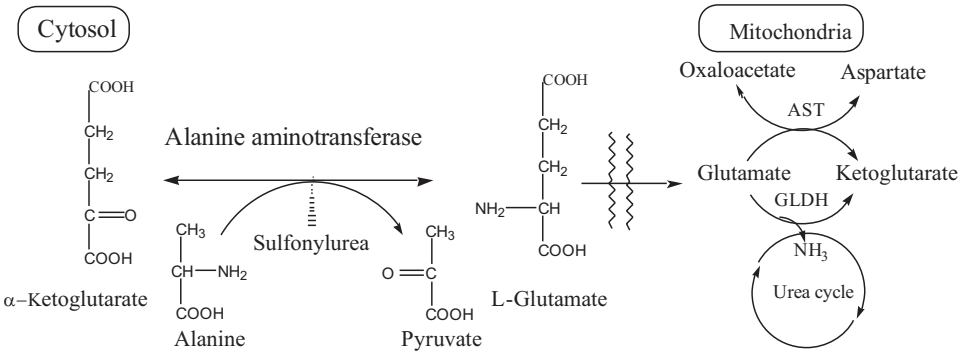

Figure 5. Biochemical toxic effects of sulfonylureas.

CONCLUSIONS

A new method of toxicovigilance, estimating the *in vitro* effects of toxic agents on the energetic and metabolic cellular mechanism as well as the competition between endogenous substrates have been explored. On one hand, this study shows the inherent toxicity of sulfonylurea herbicides for ALT by a significant sharp drop in the enzymatic activity, which persists even at very low concentrations of sulfonylurea hydrolysis products. This inhibition is specific for ALT since it does not affect the intramitochondrial AST. Comparatively, toxicity to humans and herbicide effects are due to ALT and acetolactate synthase inhibitions, respectively.

These biochemical studies estimating the toxic effects of sulfonylureas on the human enzymatic mechanism complement the toxicological knowledge of plants, algae and bacteria (1, 2 and 9). Other studies of the toxicological mechanism of sulfonylureas are in progress.

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S A Ž E T A K

Toksikološka kontrola: Nova biokemijska metoda u toksikološkim istraživanjima herbicida iz skupine sulfonilurea

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Procjenjeni su *in vitro* toksikološki učinci herbicida iz skupine sulfonilurea (tifensulfuron-metil i metsulfuron-metil) pod simuliranim fiziološkim uvjetima prema novom protokolu. Sulfoniluree i produkti njihove hidrolize dodani su u biološke supstrate kao što su urea, alanin, asparaginska kiselina, α-ketoglutarat, oksalacetat, piruvat te su inkubirani sa specifičnim enzimima. Dodatak ispitanih sulfonilurea i njihovih razgradnih produkata nije značajno mijenjalo enzimsku aktivnost ureaze, aspartat-aminotransferaze, glutamat dehidrogenaze, malat dehidrogenaze i laktat dehidrogenaze. Produkti kisele hidrolize inhibirali su u niskim koncentracijama (0,27 μmol L^{-1}) do 95% aktivnosti alanin-aminotransferaze, ali ne i aspartat-aminotransferazu iz mitohondrija.

Ključne riječi: sulfoniluree, herbicidi, toksikološka kontrola, alanin-aminotransferaza, aspartat dehidrogenaza, glutamat dehidrogenaza, laktat dehidrogenaza, malat dehidrogenaza i ureaza

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