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DAMINOZIDE – LACK OF THE GENOTOXIC ACTIVITY IN THE SHORT-TERM BACTERIAL TESTS

DAMINOZYD – BRAK AKTYWNOŚCI GENOTOKSYCZNEJ W KRÓTKOTERMINOWYCH TESTACH BAKTERYJNYCH

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Daminozide [ALAR] a plant growth regulator has been widely used on apples since the late 1960s. It has been identified as a possible carcinogen. Restrictions were ordered to reduce both application rates and allowable daminozide residue levels. Since conclusive scientific data necessary to characterize the risk of daminozide were not available, additional information on mutagenic activity of this compound was needed.

INTRODUCTION

In the present work, within a project of re-evaluation of authorised pesticides coordinated by PZH (National Institute of Hygiene) we aimed at investigation of the genotoxic effects of daminozide [ALAR; succinic acid mono (2,2-dimethyl-hydrazide)], a plant growth regulator. Daminozide has been widely used on apples since the late 1960s to enhance storability and colour [7]. It penetrates the apple skin and cannot be washed off. Daminozide may be also applied as a foliar spray in water. It hastens the ripening of fruits and vegetables, but because of its chemical stability and ability to form the residues in food products [6, 9, 19, 30] a substantial part of human population could be exposed to the compound [13, 16].

Daminozide has been identified as a possible carcinogen [23, 32]. Even small amounts of hydrolysis or oxydation lead to metabolites of toxicological significance. Hydrolysis of daminozide will yield the carcinogen 1,1-dimethylhydrazine [10, 24, 31]. Metabolic N-oxidation via N-oxide intermediates may yield products of high reactivity, on analogy with studies on monoalkyl- and monoacylhydrazines, dialkylhydrazines and nitrosoamines [4, 8, 11]. On the other hand, daminozide can be oxidated by photochemically generated signlet oxygen to dimethylnitrosamine and succinic anhydride [5]. Recently restrictions were ordered to reduce both application rates and allowable daminozide residue levels.

Since conclusive scientific data necessary to characterize the risk of daminozide were not available [28], additional information on mutagenic activity of this compound was needed.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from the sources listed below: Daminozide (ALAR 85) (purity 99%) was a gift from Uniroyal Chemical Ltd., (CAS No 1596-84-5); Tris (hydroxymethyl) -aminoethane (Tris), methyl methanesulphonate (MMS), 2-amonofluorene (2-AAF) and mitomycin C were from Sigma; 1-oxide-4-nitroquinoline (NQO) from Fluka AG; aflatoxin B_1 (AFB1), O-nitrophenyl-β-D-galactopyranoside (ONPG), L-tryptophan were from Calbiochem; p-nitrophenyl disodium phosphate (PNPP) from Merck; Aroclor 1254 from Analabs Inc.; sodium dodecylsulphate (SDS), Bacto trypton and Bacto yeast extract from Koch Light Lab. Ltd.; D-Biotin was from Serva. Uracil, L-histidine were from Ciech (Gliwice, Poland). All control mutagens and daminozide were dissolved in dimethyl sulphoxide (DMSO) - spectrophotometric grade, obtained from Serva. Fresh solution of all chemicals were prepared immediately before use.

Bacterial strains

The *S.typhimurium strains* TA97, TA98, TA100 and TA102 *his*G428 *rfa* pQA1 used in the *Ames* test, as well as TA1538, *his*D3052 *rfa uvrB*, and TA1978, *his*D3052 *rfa*, used in the repair test were gifts from Professor *B. N. Ames*, Biochemistry Department, University of California, Berkeley, California (USA).

Strain *E.coli* K-12 PQ37 (*sfiA: :Mud*) AP *lac/cts*, *lac*U169, *mal*$^+$, *uvrA*, *galE, galY, PhoC, rfa, F', thr, leu, his, pyrD, thi, trp: :Muc*$^+$, *sr1300: :Tn10, rpoB* used in SOS Chromotest was a gift from *P. Quillardet* and *M. Hofnung* U.B.M.T.G. Institut Pasteur, Paris, France.

Bacterial tests

a) *Ames* test was performed with *S. typhimurium* strains: TA97, TA98, TA100, TA102 and liver homogenate fraction (S9) which was prepared according to *Maron* and *Ames* [12, 21].

b) DNA repair-test was performed according to the method described by *Ames* et al. [2]. The culture media used for the assay with *Salmonella* were as described by *Ames* et al. [1].

c) β-Galactosidase assay. The induction of the SOS response by daminozide was measured in *E.coli* PQ37 strain by means of a *sfiA: : lacZ* operon fusion according to the principle of the SOS Chromotest [25, 26]. Cultivation of bacteria and measurements of β-galactosidase and alkaline phosphatase activities were performed as described Quillardet and Hofnung [26]. Bacteria were exposed to daminozide for 2h with vigorous agitation.

Each assay was accompanied by positive controls: 4NQO (20 ng/assay) was used for estimation without metabolic activation, and aflatoxin B_1 (30 ng/assay) for estimation with metabolic activation. The S9 mix used in the SOS Chromotest was prepared according to *Quillardet* and *Hofnung* [26].

β-Galactosidase activity was measured at 30°C and calculated in conventional units [22] referred to cell density measured at 600 nm.

The results are expressed as a ratio of the activities in the treated to non-treated bacteria at each time point (Induction factor).

Liver homogenate fraction (S9)

The liver homogenate fraction was prepared according to *Maron* and *Ames* [21] using Aroclor 1254 - induced *Wistar* male rats.

RESULTS

In order to reveal possible damages in the DNA induced by daminozide we have used several bacterial tests. Bacterial mutation assay (*Salmonella*/microsome assay - Ames test) have become the most extensively used *in vitro* short-term test in the screening for mutagenicity. In the Ames test the *S. typhimurium* strains TA97, TA98, TA100 and TA102 were used in the presence and absence of metabolic activation (Table I). In none of the tested strains did we find a threshold 2-fold increase in the number of his^+ revertants. This, according to the Ames criterion, does not allow the qualification of daminozide and its metabolites as mutagenic for *S. typhimurium* strains. Thus, it has not frame-shift, point mutation or oxidative and cross-linking mutagenic activity [20].

Table I. Mutagenic evaluation of daminozide in the *Ames* test
Oznaczenie aktywności mutagennej daminozydu testem *Amesa*

	N° of his^+ revertants/plate ± SD			(µg/plate)
	TA 97	TA 98	TA 100	TA 102
-S9				
0	166 ± 37	32 ± 4	155 ± 38	346 ± 48
100	158 ± 16	34 ± 6	153 ± 22	304 ± 50
250	150 ± 24	41 ± 5	149 ± 25	315 ± 50
500	163 ± 15	39 ± 4	153 ± 49	328 ± 52
+S9				
0	152 ± 25	36 ± 3	159 ± 27	320 ± 48
100	167 ± 30	34 ± 6	174 ± 20	340 ± 58
250	170 ± 37	31 ± 4	170 ± 34	320 ± 60
500	160 ± 29	39 ± 9	165 ± 29	290 ± 68

Revertants expressed as x ± SD, average of 12 plates.
– S9 – without metabolic activation,
+ S9 – with metabolic activation, 50 µl S9/plate were added,
NT – not tested.
H – Positive controls: TA97 and TA98 without S9: 4–NQO (10 µg/plate)
900 ± 49 and 500 ± 60, respectively; with S9: 2–AAF (10 µg/plate)
1600 ± 60 and 5300 ± 100, respectively.
TA 100 without S9: MMS (1 µg/plate) 2600 ± 120;
with S9: 2–AAF (10 µg/plate) 3200 ± 310.
TA 102 without S9: mitomycin C (0.5 µg/plate) 4700 ± 340.

Salmonella typhimurium TA1538 (*uvrB*) and TA1978 (uvr^+) allowed to detect the chemicals bound covalently to DNA, when used in the repair test [1]. The results of the repair test are shown in Table II. As can be seen there was not an appreciable difference in the zones of growth inhibition, produced by daminozide, between the two strains tested, both in the presence and absence of metabolic activation.

Table II. The zones of growth inhibition produced by daminozide in TA1538 *(uvrB)* and TA1978 *(uvr$^+$)* strains of *S. typhimurium* in the absence and presence of the metabolic activation system.
Strefy zahamowania wzrostu szczepów *S. typhimurium* TA1538 *(uvrB)* i TA1978 *(uvr$^+$)* przez daminozyd w obecności i nieobecności frakcji S9.

Compound	Dose (μg/plate)	Diameter of zone of growth inhibition (mm)			
		– S9		+ S9	
		TA1538	TA1978	TA1538	TA1978
daminozide	50	7 ± 2	6 ± 2	5 ± 1	5 ± 1
	100	7 ± 2	6 ± 1	5 ± 1	5 ± 1
	200	8 ± 1	6 ± 1	5 ± 2	5 ± 1
	500	8 ± 3	8 ± 1	6 ± 1	5 ± 2
mitomycin C	1	22 ± 2	15 ± 2	NT	NT
2-aminofluorene	50	NT	NT	15 ± 3	6 ± 2

Data are mean values from 9 plates (± SD),
NT – not tested,
where indicated 200 μl of S9 mix was added per plate.

Escherichia coli PQ37 (*uvrA*) was used to reveal the induction of the SOS response [14, 33, 34]. The expression of one of the SOS genes, *sfiA* gene [15] was monitored in bacterial SOS Chromotest to reveal induction of the SOS-response. In the bacterial strain used, the *sfiA* gene is fused with *lacZ*, a structural gene for β-galactosidase [25, 26]. Daminozide was used at the monotoxic concentration range, i.e. it did not affect the alkaline phosphatase activity. Slight induction of β-galactosidase (IF=1.4) by tested chemical was observed only without metabolic activation.

DISCUSSION

The results presented in this work demonstrate the lack of genotoxic activity of daminozide and/or its metabolites in all used short-term bacterial tests. Alar (daminozide) breaks down to UDMH (unsymmetrical dimethylhydrazine), exposure to which according to NDRC (Natural Resources Defence Council) calculations possess a cancer risk of 1:4200 [27]. Both daminozide (2% in drinking water) [32] and UDMH (0.01% in drinking water) [31] have been reported to induce angiosarcomas in mice in various organs, as well as tumors of the lungs, kidney and for UDMH only, the liver and colonic carcinoma [18, 29]. However, UDMH had very high toxicity for mice.

Our negative data according to mutagenic activity do not correlate with mentioned carcinogenicity of daminozide, but also data obtained from *in vitro* test on the genotoxicity of alkylhydrazines do not correlate with carcinogenicity [17], probably because the activation to the ultimate electrofiles does not proceed quantitatively. *Avakian* and *Marukchian* [3] have observed that daminozide elevates lipid peroxidation and activity of superoxide dismutase in a liver cytosol from treated rats. It could be possible, that long-term daminozide carcinogenic activity is correlated with its cytoplasmic metabolism, rather than direct reaction with DNA.

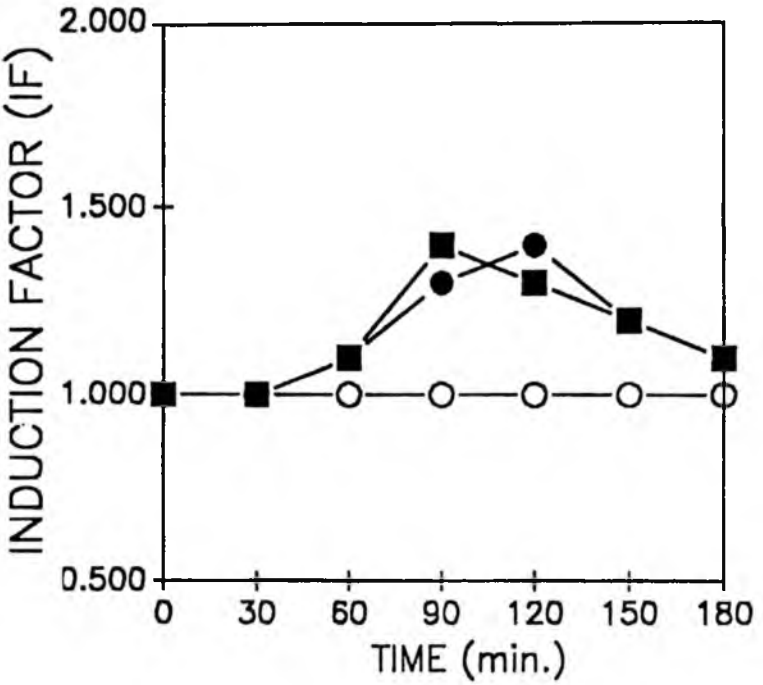

Fig. 1. Effect of daminozide and its metabolites on the SOS system induction in PQ37 strain of *E. coli* K–12.

Ryc. 1. Wpływ daminozydu i jego metabolitów na indukcję systemu SOS w szczepie *E. coli* K–12 PQ 37.

(■—■) – daminozide 417 mg/ml; (● —●) – daminozide 834 mg/ml; (o — o) – daminozide 834 mg/ml + S9.

IF without S9 (20 ng NQO/assay) – 14

IF with S9 (30 ng AFB1/assay) – 12

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Streszczenie

W pracy badano genotoksyczne działanie daminozydu i jego metabolitów powstających pod wpływem frakcji mikrosomalnej S9 z wątroby szczura. Aktywność mutagenną i genotoksyczną daminozydu określono stosując: szczepy *S. typhimurium* TA1538 i TA1978 (test reperacji) pozwalające na wykrycie związków kowalencyjnie związanych z DNA.

Stwierdzono, że ani daminozyd ani jego metabolity nie indukują mutacji rozpoznawanych testem *Amesa* w szczepach *S. typhimurium*. Nie wiążą się również kowalencyjnie z DNA, a bardzo słabą indukcję systemu SOS obserwuje się jedynie w obecności daminozydu, a nie jego metabolitów.

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Summary

In the recent paper genotoxic effects of daminozide and its metabolites were tested. Evaluation of the mutagenic effect of daminozide was by: (i) the *Salmonella*/mammalian microsome *Ames* test with *S.typhimurium* TA97, TA98, TA100 and TA102. (ii) *E. coli* PQ37 strain to reveal an induction of the SOS response, (iii) *S. typhimurium* TA1538 (*uvrB*) and TA1978 (uvr^+) to detect the chemicals bound covalently to DNA (repair test).

Daminozide was not mutagenic in any of the *S.typhimurium* strains and did not induce damages in DNA recognized by correndonuclease II, as shown by the repair test. Only metabolites of daminozide induced the SOS system.

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