Antimutagenic Effect of Resveratrol

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Abstract

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Evidence exist from population-based and laboratory studies that some phytochemicals have protective effects against tumors or other diseases and reveal antimutagenic activity. We studied the protective effect of the plant phytoallexin resveratrol on the mutagenic activity of three mutagens, i.e. aflatoxin B_1 (AFB_1), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and N-nitroso-N-methylurea (MNU) using the Ames and the micronucleus tests. In the Ames test, we proved a significant antimutagenic activity only against the indirect mutagens AFB_1 and IQ, not against the direct mutagen MNU. A significant decrease of mutagenicity of all three mutagens was detected by the micronucleus test.

Keywords: resveratrol; aflatoxin B_1; 2-amino-3-methylimidazo[4,5-f]quinoline; N-nitroso-N-methylurea

The use of natural chemicals allowing the suppression, retardation, or inversion of carcinogenic process is a promissing approach for the prevention of tumors. Most of the chemopreventive agents are plant extracts which prevent carcinogenic activation as blocking agents or inhibit malignant cell proliferation as suppressing agents which have low or none side effects or toxicity.

One of these phytochemicals with such a pleitropic effects is phytoallexin resveratrol-apolyphenolic compound present in grapes and other plants. Resveratrol (3,5,4'-trihydroxystilbene) is produced in the plant as a response to injury, ultraviolet irradiation, or fungal attack, and can be found mainly in the skin of grapes and in derived products such as red wine. It has a range of biological and pharmacological activities *in vitro* as well as *in vivo*. Resveratrol is well known as anti-inflammatory, anti-oxidative, and anti-carcinogenic agent and has also a positive effect on the immune system (Fremont 2000).

Multiple lines of evidence from epidemiological studies indicate an inverse relationship between the red wine consumption and the risk of cardiovascular diseases (Lin & Tsai 1999).

Resveratrol as a proven antioxidant which "quenches" peroxyl radicals (Sato *et al.* 2000; Rimando *et al.* 2002; Boyce *et al.* 2004) can prevent oxidative DNA damage, which plays an important role in the activity of many genotoxic substances (Damianaki *et al.* 2000; Sgambato *et al.* 2001). Resveratrol participates in the prevention of carcinogenesis by the inhibition of P450, an enzyme of phase I (Chang *et al.* 2001; Gusman *et al.* 2001), and through the induction of phase II xenobiotic metabolising enzymes (Savouret & Quesne 2002; Kundu & Surh 2004). Resveratrol can induce the activation of p53 and the subsequent apoptosis

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occurring through p53-dependent pathway (Dong 2003; Bode & Dong 2004), but it can also induce apoptosis independently of p53 (Mahyar-Roemer & Roemer 2001). Resveratrol can modulate signal transduction pathways by the inhibitory effect on the activation of transcription factors such as NF-κB and AP1 (Kundu & Surh 2004). Resveratrol, as a selective inhibitor of cyclooxygenase 2 (COX-2), is also a strong inhibitor of the dioxygenase activity of lipoxygenase (LOX) (Pinto *et al.* 1999).

Many authors have described the effect of resveratrol in the prevention of cardiovascular diseases (so-called French paradox). Resveratrol modulates lipid metabolism, inhibits the oxidation of LDL and thrombocyte aggregation (Pace-Asciak et al. 1995), and has a vasodilatation activity (Fremont 2000; Gusman *et al.* 2001). Lin and Tsai (1999) studied the balance between free oxygen radicals and endogenous antioxidants. This effect is connected with the cardioprotective action of resveratrol which results from the inhibition of the formation of the oxidised form of LDL (Wu *et al.* 2001).

In our work, we studied chemopreventive effects of resveratrol on the mutagenicity of three known mutagens, two indirect mutagens aflatoxin B_1 (AFB_1) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and a direct mutagen N-nitroso-N-methylurea (MNU), using the Ames test on *Salmonella typhimurium* and the micronucleus test on mice.

MATERIAL AND METHODS

Chemicals. The following chemicals were used for both tests: AFB_1 (Alexis Corporation, USA), IQ (ICN Biomedicals, Inc., Germany), MNU (Sigma-Aldrich Co, Lousiana, USA), resveratrol (Sigma-Aldrich Co, Lousiana, USA). Chemicals were diluted with dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co, Lousiana, USA).

Ames test. The Ames test with *Salmonella typhimurium* TA98 and TA100 (Ames 1971; Ames *et al.* 1975; Černá *et al*. 1989) was used for the evaluation of the antimutagenic effect of resveratrol *in vitro*.

The mutagenic substances were used in the following concentrations: AFB_1 in concentrations of 10 μg, 1 μg, and 0.1 μg per plate with both strains TA98 and TA100, IQ in concentrations of 0.1 μg, 0.01 μg, and 0.001 μg per plate with the strain TA98, in concentrations of 10 μg, 1 μg, and 0.1 μg with the strain TA100, MNU in concentrations of 1000 μg, 100 μg, and 10 μg only with the strain TA100 as these concentrations had no effect on the strain TA98. Each concentration of each mutagen was combined with four different concentrations of the antimutagen (300 μg, 30 μg, 3 μg, and 0.3 μg of resveratrol per plate). All chemicals were diluted with DMSO. For the metabolic activation, the S9 fraction of liver homogenate from laboratory rats induced by a mixture of polychlorinated biphenyls Delor was used (Maron & Ames 1983). Each combination of mutagen and antimutagen was tested in two separate experiments with three plates in each experiment.

Percentage of inhibition of mutagenicity was calculated by the formula:

$$\text{No of revertants of mutagen} - \frac{\text{No of revertants of mixture of mutagen and resveratrol}}{\text{No of tevertants of mutagen}} \times 100$$

For statistical analysis, Student's *t*-test was used.

Experimental animals. *In vivo* experiments (bone marrow micronucleus test) were carried out on ten-week-old male Balb C mice, each weighing 22–26 g (purchased from BIOTEST, Konárovice, CZ). The animals were housed under controlled light regime of 12/12 h, temperature of 20 ± 2°C, relative humidity of 60 ± 10%, and complete air recirculation 10–14-times per hour. The animals were supplied with water *ad libitum* and were fed with a commercial granulated mixture for laboratory rodents. These animals were divided into groups of 10 mice each.

Micronucleus test. The mouse bone marrow micronucleus test was carried out according to Schmid (1975). An increased frequency of micronuclei in polychromatophilic erythrocytes in comparison with the control groups indicates that the substance tested induces chromosomal damage in nucleated erythrocytes in the bone marrow. A total of 1000 polychromatophilic erythrocytes were scored per animal by the same observer for evaluating the frequencies of micronucleated polychromatophilic erythrocytes. Each experiment was run three times. As the negative control, mice were treated orally by a 7% solution of DMSO. For statistical analysis, Student's *t*-test was used.

Substances tested. The following concentrations of mutagens were used for *in vivo* test: AFB_1 1 mg per kg of body weight (b.w.), IQ 20 mg/kg b.w., MNU 20 mg/kg b.w. Resveratrol was applied to mice at the dose of 5 mg/kg b.w. by the gavage for three sequential days. Carcinogens were applied in

one dose on the third day. The controls received 7% DMSO. All of the substances (diluted with DMSO) were applied in volumes of 100 µl/10 g b.w.

RESULTS

Ames test

The results of the Ames test are summarised in Tables 1–3. All results presented in tables are expressed also as percentage of inhibition of the mutagenic activity of a sample of mutagen with antimutagen in comparison with the mutagenic activity of the respective mutagen.

Resveratrol at concentrations of 30 and 300 µg per plate revealed to possess a remarkable and dose dependent antimutagenic effect against all concentrations of AFB_1 (10, 1, and 0.1 µg per plate) in both strains TA98 and TA100. The only exception was the concentration of 30 µg per plate of resveratrol in combination with 10 µg per plate of AFB_1 in TA100. This concentration, similarly as other lower doses of resveratrol in both strains, did not reduce the mutagenic activity of AFB_1, except the lower resveratrol dose – 3 µg per plate which significantly reduced the activity of 1 µg of AFB_1 in the strain TA100 (Table 1).

A strong dose dependent effect of resveratrol was discovered also against the second indirect mutagen IQ used in concentrations of 0.1, 0.01, and 0.001 µg per plate with the strain TA98, and 10, 1, and 0.1 with TA100. In this case not only two highest resveratrol concentrations, but also the concentration of 3 µg per plate was antimuta-

Table 1. Effect of resveratrol on mutagenicity of AFB_1 – Ames test *(S. typhimurium* TA98, TA100*)*

AFB_1 + RES dose (µg/plate)	*S. typhimurium* TA98 + S9		*S. typhimurium* TA100 + S9	
	revertants ± SD	% inhibition	revertants ± SD	% inhibition
10 + 0	1407 ± 144		1540 ± 236	
10 + 0.3	1460 ± 103	+4	1550 ± 273	+1
10 + 3	1561 ± 49	+11	1835 ± 205	+19
10 + 30	1195 ± 71*	–15	1850 ± 318	+20
10 + 300	177 ± 26**	–87	586 ± 33**	–62
1 + 0	896 ± 242		1490 ± 417	
1 + 0.3	902 ± 152	+1	1084 ± 371	–27
1 + 3	757 ± 88	–16	869 ± 341*	–42
1 + 30	324 ± 21**	–64	626 ± 241**	–58
1 + 300	67 ± 9**	–93	205 ± 33**	–86
0.1 + 0	252 ± 130		504 ± 124	
0.1 + 0.3	275 ± 103	+9	511 ± 175	+1
0.1 + 3	217 ± 56	–14	397 ± 92	–21
0.1 + 30	87 ± 13*	–65	225 ± 17**	–55
0.1 + 300	36 ± 5**	–86	145 ± 6**	–71
Control (DMSO)	37 ± 7		116 ± 19	
0 + 0.3	36 ± 2		139 ± 4	
0 + 3	33 ± 2		139 ± 8	
0 + 30	31 ± 5		139 ± 7	
0 + 300	29 ±5		142 ± 15	

statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone *$P \leq 0.05$; ** $P \leq 0.01$
SD – standard deviation

Table 2. Effect of resveratrol on mutagenicity of IQ – Ames test *(S. typhimurium* TA98, TA100*)*

IQ + RES dose (μg/plate)	*S. typhimurium* TA98 + S9		IQ + RES dose (μg/plate)	*S. typhimurium* TA100 + S9	
	revertants ± SD	% inhibition		revertants ± SD	% inhibition
0.1 + 0	830 ± 143		10 + 0	1877 ± 73	
0.1 + 0.3	702 ± 150	–15	10 + 0.3	1584 ± 244	–18
0.1 + 3	508 ± 87**	–39	10 + 3	1400 ± 264*	–25
0.1 + 30	219 ± 16**	–74	10 + 30	592 ± 126**	–69
0.1 + 300	30 ± 7**	–96	10 + 300	129 ± 11**	–93
0.01 + 0	174 ± 44		1 + 0	1100 ± 157	
0.01 + 0.3	181 ± 47	+4	1 + 0.3	734 ± 170**	–33
0.01 + 3	115 ± 24*	–34	1 + 3	401 ± 67**	–64
0.01 + 30	57 ± 15**	–67	1 + 30	174 ± 11**	–84
0.01 + 300	26 ± 6**	–85	1 + 300	106 ± 8**	–90
0.001 + 0	67 ± 31		0.1 + 0	278 ± 51	
0.001 + 0.3	65 ± 32	–3	0.1 + 0.3	176 ± 51**	–37
0.001 + 3	44 ± 8*	–34	0.1 + 3	150 ± 39**	–46
0.001 + 30	36 ± 7*	–46	0.1 + 30	108 ± 15**	–61
0.001 + 300	25 ± 6*	–63	0.1 + 300	104 ± 14**	–63
Control (DMSO)	21 ± 3		Control (DMSO)	102 ± 15	
0 + 0.3	27 ± 4		0 + 0.3	112 ± 17	
0 + 3	24 ± 5		0 + 3	110 ± 7	
0 + 30	24 ± 2		0 + 30	110 ± 12	
0 + 300	23 ± 4		0 + 300	115 ± 14	

statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $^*P \leq 0.05$; $^{**}P \leq 0.01$
SD – standard deviation

genic and significantly decreased the activity of IQ (Table 2). The lowest concentration of 0.3 μg per plate was effective only against 1 and 0.1 μg of IQ per plate with TA100.

However, resveratrol was not effective against any of the tested concentrations of the direct mutagen MNU (Table 3), the decrease of mutagenic activity was only insignificant, even with the highest dose of resveratrol.

Micronucleus test

In the controls treated with 7% DMSO no significant increase in the frequency of micronuclei was observed in comparison with the intact animals.

The numbers of micronuclei in the animals influenced by resveratrol alone did not differ from those of the control group. On oral application of the combination of resveratrol (3 times 5 mg/kg b.w.) and AFB_1 in one dose (1 mg/kg b.w.), the number of micronuclei in polychromatophilic erythrocytes was lower in a statistically significant degree in comparison with the laboratory mice treated with AFB_1 alone.

A similar effect appeared on the application of the IQ mutagen. Resveratrol (3 times 5 mg/kg b.w.) in combination with one dose of IQ (20 mg/kg b.w.) significantly reduced its mutagenic effect.

Similarly, the treatment of mice with the combination of the same doses of resveratrol and one dose of MNU (50 mg/kg b.w.) led to a significant reduction of the number of micronuclei in comparison with the number of micronuclei elicited by MNU alone. The results are presented in Table 4.

Table 3. Effect of resveratrol on mutagenicity of MNU – Ames test (*S. typhimurium* TA100)

MNU + RES dose (μg/plate)	*S. typhimurium* TA100	
	revertants ± SD	% inhibition
1000 + 0	2675 ± 187	
1000 + 0.3	2577 ± 237	–4
1000 + 3	2591 ± 208	–3
1000 + 30	2590 ± 140	–3
1000 + 300	2496 ± 179	–7
100 + 0	2356 ± 65	
100 + 0.3	2098 ± 305	–11
100 + 3	2103 ± 172	–11
100 + 30	2185 ± 131	–7
100 + 300	2160 ± 183	–8
10 + 0	452 ± 191	
10 + 0.3	439 ± 74	–3
10 + 3	429 ± 83	–5
10 + 30	408 ± 109	–10
10 + 300	357 ± 64	–21
Control (DMSO)	146 ± 35	
0 + 0.3	151 ± 32	
0 + 3	142 ± 31	
0 + 30	144 ± 29	
0 + 300	151 ± 31	

SD – standard deviation

Table 4. Effect of resveratrol on mutagenicity of the mutagens $AFB_{1,}$ IQ and MNU – micronucleus test

Substance	Number of micronuclei	SD
Control 7% DMSO	0.6	0.4
Resveratrol	0.8	0.7
AFB1	3.8*	1.1
AFB1 + resveratrol	2.8**	0.9
IQ	4*	1.6
IQ + resveratrol	2.5**	1.6
MNU	42.4*	5.7
MNU + resveratrol	31.8**	9.3

*significantly higher number of micronuclei as against the negative control (DMSO)

**significantly higher number of micronuclei as against mutagen alone

SD – standard deviation

DISCUSSION

New information about chemoprotective effects of phytochemicals from the field of molecular biology and biochemistry increases the interest of scientists and clinicians in the chemoprevention of malignant diseases and stimulate significant progress in experimental genotoxicology (Morse & Stoner 1993; Kelloff *et al.* 1996; Mettlin 1997). Anticarcinogenic and antimutagenic activities of plant substances were proved by many authors in both *in vitro* and *in vivo* systems. Boyce *et al.* (2004) found resveratrol to be potent in blocking the mutagenicity of the food-derived heterocyclic amins: 2-amino-1-methyl-6 phenylimidazol[4,5-*b*]pyridine (MeIQx) and 2-amino-3,8dimethylimidazo[4,5-*f*] quinoxaline (PhIP) in the Ames test and in chinese hamster V 79 cells. Also, in our previous experimental studies we demonstrated antimutagenic and immunomodulatory effects of some substances occuring in plants (Šmerák *et al.* 2002).

It is probable that multiple mechanisms including the effect on the metabolic activation of mutagens or the influence on detoxification enzymes and blocking DNA-adducts formation are involved in the antimutagenic effect of resveratrol.

It has previously been shown using the Ames assay that resveratrol can suppress the induction of mutations by 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (TRP-p-1) (Uenobe *et al.* 1997), N-methyl-N'-nitro-N-nitrosoguanidin (MNNG) (Kim *et al.* 2002), or by methylmethansulphonate and benzopyrene (Fu *et al.* 2004).

In our experiments on *Salmonella typhimurium*, resveratrol revealed a much stronger effect on the mutagenicity of the indirect mutagens AFB_1 and IQ in comparison with the effect on the mutagenicity of the direct mutagen MNU. Our results support the fact that resveratrol is very active in the suppression of metabolising phase I enzymes (Gusman *et al.* 2001) and are in agreement with the results of Boyce *et al.* (2004) who proved the antimutagenic effect of resveratrol on the mutagenicity of the indirect food mutagen MeIQx. It has been also stated that high concentrations of resveratrol can inhibit cellular proliferation (Boyce *et al.* 2004), but this was not detected in our experiments on prokaryotic cells.

A significant decrease of the mutagenicity of all three mutagens, AFB_1, IQ, and MNU, by resveratrol was detected in micronucleus test. Also Fu *et al.* (2004) proved that resveratrol prevents cyclophosphamide-induced micronucleus formation in the dose dependent manner.

Diet (nutrition) may be considered as a very important factor influencing favourably pathophysiological processes in organism and may be a very effective factor in the prevention strategy against various diseases, including diseases with genotoxicological ethiology.

The study of chemoprotective effects of phytochemicals or the study of their interactions and knowledge of the mechanisms of their effect shoud lead us to a wider use of these substances as dietary supplements or as a part of functional foods in the prevention of many diseases including tumors.

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Antimutagenic Effect of Curcumin and Its Effect on the Immune Response in Mice

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Abstract

Šmerák P., Polívková Z., Šestáková H., Štětina R., Bárta I., Langová M., Turek B., Bártová J. (2006): **Antimutagenic effect of curcumin and its effect on the immune response in mice**. Czech J. Food Sci., **24**: 72–83.

A wide array of antioxidative and anti-inflammatory substances derived from edible plants have been reported to possess chemopreventive and chemoprotective activities. Among the most extensively investigated and well-defined dietary chemopreventives is curcumin. Using the Ames test and *in vivo* micronucleus test, chemiluminescence test, blastic transformation test, and comet assay, we examined the antimutagenic effects of the chemically identified chemoprotective substance curcumin (diferuloylmethane) in the pure form on mutagenicity induced by three reference mutagens: aflatoxin B_1 (AFB_1), 2-amino-3-metylimidazo[4,5-f]quinoline (IQ), and *N*-nitroso-*N*-metylurea (MNU), and the effect of curcumin on the immunosuppression caused by these mutagens. Curcumin in the pure form showed a clear antimutagenic and immunomodulatory activities on mutagenicity and immunosuppression induced by reference mutagens.

Keywords: curcumin; antimutagenic effects; response of immune; Ames test; micronucleus test; comet assay; chemiluminescence; blastic transformation tests

It has been known that most human cancers are induced by environmental factors including chemical, radioactive, and biological factors that exist in our life environment. Epidemiological studies have indicated significant differences in the incidence of cancers among ethnic groups who have different lifestyles and have been exposed to different environmental factors. It has been estimated that some human cancers could be prevented by the modification of lifestyle including the dietary modification (Surh 2003).

The use of natural chemicals allowing suppression, retardation, or inversion of carcinogenic process is a promising approach, especially for

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the prevention of tumours. According to a large number of epidemiological studies, a high consumption of fruits and vegetables is consistently associated with a low incidence of most human cancers (Dorai & Aggarwal 2004). The dietary components as chemopreventive agents have received much attention in the public and the medical community.

Curcuma longa L., which belongs to the *Zingiberaceae* family, is a perennial herb that grows up to 1 m of height with a short stem, and is distributed throughout the tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China. As a powder, called turmeric, has been used in Asian cookery for thousands of years. Current traditional Indian medicine claims the use of its powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism, and sinusitis.

Curcumin – diferuloylmethane, an aromatic yellow compound isolated from the root of *Curcuma longa*, is a chemopreventive agent with a multiple mechanism of action (Surh 2002; Duvoix *et al.* 2004). It has anti-inflamatory and anti-oxidative effects (Surh *et al.* 2001), reduces lipid peroxidation, thus affecting the risk of cardiovascular diseases. Many authors proved its anticarcinogenic effects on the inhibition of tumour formation and tumour promotion of tumours induced chemically or by radiation in laboratory animals (Inano & Onoda 2002); this effect was also proved *in vitro* on human carcinoma cell lines (Ellatar & Virji 2000).

The presumption of the anti-carcinogenic effect of curcumin is also supported by the findings demonstrating the increased activation of macrophages and NK cells and the modulation of the glutathione detoxification mechanism (Bhaumik *et al.* 2000).

Curcumin was described as a good anti-angiogenesis agent in the prevention of tumour promotion and was able to inhibit human telomerase activity in MCF-7 cells (Ramachandran *et al.* 2002). The ability of curcumin to support apoptosis in cancer cells without cytotoxic effects on healthy cells is also important and was described in different cell lines (Tourkina *et al.* 2004), but in same pathologies the effect of curcumin was negative because it reduced the effect of chemotherapy on the induction of apoptosis in breast cancer cells (Somasundaram *et al.* 1997, 2002). A very important role of curcumin is in altering the metabolic activation or detoxification of mutagens and carcinogens. It inhibits cytochrome P450 enzymes involved in the activation of mutagens and carcinogens (Duvoix *et al.* 2004). There is some evidence on the induction effect of curcumin on phase II metabolising enzymes – the dietary supplementation of curcumin enhances antioxidant and phase II metabolising enzymes in ddY male mice (Igbal *et al.* 2003), but the inhibition of GST activity by high doses of curcumin was also described (Piper *et al.* 1998).

Its antioxidative effect was proven in many different systems (Betancor-Fernandez *et al.* 2003). Curcumin exerts a variety of immunomodulatory effects (Churchil *et al.* 2000). Curcuminoids have been shown to be free radical scavengers that suppress the production of superoxide by macrophages, and a significant increase of macrophage phagocytic activity was also observed in curcumin treated animals. These results indicate the immunostimulatory activity of curcumin (Lukita-Atmadja *et al.* 2002).

Given its pleiotropic effects, curcumin represents an important factor for chemoprevention of human tumours and is under great interest of research studies. In our study, we combined the methods of studying the antimutagenic effects of chemopreventive agents with the methods of testing their effects on the immune response.

The effect of curcumin as a chemically identified chemoprotective substance in the pure form on the mutagenicity or immunosuppression caused by three known mutagens AFB_1, IQ, and MNU was studied using the Ames bacterial mutagenicity test and *in vivo* micronucleus, chemiluminescence and blastic transformation tests, resp. comet assay.

In our study, we chose two different representants of mutagenes which commonly occur in the human diet – mycotoxin AFB_1, food mutagen IQ (promutagens) and, third, MNU, a direct mutagen of endogenous origin.

Aflatoxin B_1 (AFB_1) – contaminating foodstuffs is one of the most thoroughly studied and a well known mycotoxin with carcinogenic activity. In the present study, it is used as a reference mutagen which exerts mutagenic activity in all prokaryotic and eukaryotic testing systems (Bárta *et al.* 1998; Šmerák *et al.* 2001 and others).

The another reference mutagen, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), one of the toxic substances contaminating foods, is formed by heat processing of foodstuffs. It is one of the most serious amino acid pyrolysates (heterocyclic amines)

with mutagenic, carcinogenic, and immunosuppressive activity (Loprieno *et al.* 1991).

N-nitroso-*N*-methylurea (MNU) is an important carcinogenic *N*-nitroso compound; unlike nitrosamines and AFB_1, it is a directly acting carcinogen requiring no metabolic activation (IARC 1978).

MATERIAL AND METHODS

Ames test. Antimutagenic activity of curcumin was tested by the Ames test (Maron & Ames 1983; Černá *et al*. 1989) on the auxotrophic hisbacterial strains of *Salmonella typhimurium* TA98 and TA100.

The mutagenic substances were applied in the following concentrations: AFB_1 in the concentrations of 10 μg, 1 μg and 0.1 μg per plate with both strains, TA98 and TA100, IQ in the concentrations of 0.1 μg, 0.01 μg and 0.001 μg per plate with the strain TA98, and in the concentrations of 10 μg, 1 μg and 0.1 μg with the strain TA100, MNU in the concentrations of 1000 μg, 100 μg and 10 μg with the strain TA100 only as these concentrations had no effect on the strain TA98. Each concentration of each mutagen was combined with four different concentrations of the antimutagen (300 μg, 30 μg, 3 μg and 0.3 μg of curcumin per plate). For the metabolic activation of the indirect mutagens (AFB_1 and IQ), the S9 fraction was used of liver homogenate from laboratory rats induced by a mixture of polychlorinated biphenyls Delor 103.

Each combination of mutagen and antimutagen was tested in two separate experiments with three plates in each experiment. Percentage of the inhibition of mutagenicity was calculated as [(No of revertants of mutagen – No of revertants of mixture of mutagen and curcumin)/No of revertants of mutagen] × 100. For the statistical analysis Student's *t*-test was used.

Experimental animals. All *in vivo* experiments (bone marrow micronucleus test, chemiluminescence test, blastic transformation method and comet assay) were carried out on ten-week-old male Balb C mice, each weighing 22–26 g (purchased from BIOTEST, Konárovice, CZ). The animals were housed under controlled light regime of 12/12 h, temperature of 20 ± 2°C, relative humidity of 60 ± 10%, and complete air recirculation 10–14-times per hour. The animals were supplied with water *ad libitum* and were fed with a commercial granulated mixture for laboratory rodents. For each group 7–10 mice were used.

Preparation of substance tested

Curcumin was applied to mice per os for three days in succession by gavage. The reference mutagens were applied once on the third day, 1–1.5 h after the application of antimutagens, in the same way.

In all experiments, each respective substance was dissolved in 7% DMSO and applied in volumes of 0.1 ml/10 g of murine body weight. Equal amounts of solvent (7% DMSO) were applied to the control group.

In the experiments testing the antimutagenic effect, the following concentrations of curcumin and its combinations with mutagens were used.

Micronucleus test. Curcumin (Sigma-Aldrich) was applied in the dose of 5 g/kg and 1 g/kg of the murine body weight in the combinations of curcumin and aflatoxin B_1. In the combinations of curcumin and IQ or MNU, the dose was 1 g/kg b.w. in 0.1 ml/10 g of the murine body weight.

Aflatoxin B_1 (AFB_1) (Alexis Corporation) was applied to mice in the dose of 5 mg/kg b.w.

2-amino-3-methylimidazo[4,5-f]quinoline (IQ), (ICN) in the dose of 20 mg/kg b.w.

N-nitroso-*N*-methylurea (MNU), (Sigma-Aldrich) in the dose of 50 mg/kg b.w.

Comet assay. Curcumin was applied in the dose of 1 g/kg of the murine body weight in 0.1 ml/10 g of the murine body weight.

Aflatoxin B_1 (AFB_1) (Alexis Corporation) was applied to mice in the dose of 5 mg/kg b.w.

2-amino-3-methylimidazo[4,5-f]quinoline (IQ), (ICN) in the dose of 20 mg/kg b.w.

N-nitroso-*N*-methylurea (MNU), (Sigma-Aldrich) in the dose of 50 mg/kg b.w.

Chemiluminescence test and blastic transformation method. Curcumin was applied in the dose of 1 g/kg of the murine body weight in 0.1 ml/10 g of the murine body weight.

Aflatoxin B_1 was applied to mice in the dose of 1 mg/kg b.w.

2-amino-3-methylimidazo[4,5-f]quinoline (IQ), (ICN) at the dose of 20 mg/kg b.w.

N-nitroso-*N*-methylurea (MNU) (Sigma-Aldrich) at the dose of 20 mg/kg b.w.

The micronucleus test

The mouse bone marrow micronucleus test was carried out according to Schmid (1975). A total of 1000 polychromatophilic erythrocytes were scored per animal for the evaluation of the frequencies of

micronucleated polychromatophilic erythrocytes. Polychromatophilic erythrocytes were observed 24 h after the aplication of the reference mutagens to the animals pre-treated with curcumin. Each experiment was run three times. For the statistical analysis Student's *t*-test was used.

Single strand breaks – Comet assay

The alkaline version of the comet assay according to Singh *et al.* (1988) modified by Collins *et al.* (1997) was used. Briefly, the cells were embedded in agarose on a microscope slide, lysed with nonionic detergent and left to unwind the DNA in alkaline electrophoresis solution (0.3M NaOH, 10mM EDTA) for 40 min. The electrophoresis (25 V, 300 mA, 4°) results in the attraction of DNA to the anode. However, if the DNA is intact, the supercoiling prevents any significant movement. The relative amount of DNA in the tail of the comet formed reflects the number of breaks in the DNA. The per cent of DNA in the tail was converted to the number of SSB/10^9 Da using the calibration of the method by X-ray irradiation (Collins *et al.* 1996). The statistical significance of the difference between the treated and the control animals was tested by Mann-Whitney *U* test.

Chemiluminescence test

The chemiluminescence test was performed according to the modification of Šestáková *et al.* (1997). In the chemiluminescence test, the degree is determined to which phagocytes are capable of liquidating the ingested material. Well known are the mechanisms in which hydrogen peroxide participates in killing (Klebanoff 1980). H_2O_2 is synthesised in phagocytes upon receiving a signal by their membrane. The finding of a higher or lower activity of the complex H_2O_2-MPO-Cl^- (I^-) speaks of the readiness of the first defense line against bacteria, tumour cells, as well as carcinogen-altered cells. The results are presented in maximum values (mV) of the chemiluminescence

Table 1. Effect of curcumin on mutagenicity of AFB_1 – Ames test – TA98, TA100

AFB_1 + curcumin dose (μg/plate)	*S. typhimurium* TA98 + S9		*S. typhimurium* TA100 + S9	
	revertants ± SD	% inhibition	revertants ± SD	% inhibition
10 + 0	836 ± 130		1147 ± 107	
10 + 0.3	859 ± 159	+3	1128 ± 88	–2
10 + 3	846 ± 138	+1	1208 ± 71	+5
10 + 30	444 ± 146**	–47	945 ± 14*	–18
10 + 300	46 ± 9**	–95	146 ± 6**	–87
1 + 0	299 ± 125		1117 ± 161	
1 + 0.3	215 ± 28	–28	1065 ± 157	–5
1 + 3	203 ± 26	–32	1061 ± 169	–5
1 + 30	72 ± 7**	–74	551 ± 166**	–51
1 + 300	19 ± 3**	–94	61 ± 7**	–95
0.1 + 0	70 ± 16		477 ± 148	
0.1 + 0.3	72 ± 18	+3	464 ± 169	–3
0.1 + 3	74 ± 16	+6	312 ± 95	–35
0.1 + 30	37 ± 11**	–47	143 ± 32**	–70
0.1 + 300	19 ± 3**	–73	62 ± 16**	–87
Control – DMSO	21 ± 1		118 ± 9	
0 + 0.3	22 ± 2		143 ± 5	
0 + 3.0	18 ± 3		142 ± 12	
0 + 30.0	19 ± 2		101 ± 16	
0 + 300.0	18 ± 1		79 ± 6	

SD – standard deviation; *$P \leq 0.05$; **$P \leq 0.01$

response of polymorphonuclear leucocytes in the dependence on time.

The blastic transformation method

For the study of the readiness of cells in acquired immunity, we selected a functional test assessing T-lymphocytes, the blastic transformation method (Field 1996). This test of lymphocyte activation determines the functional capacity of T-lymphocytes to react to a mitogen by proliferation, and as such it is a more direct examination of the immune competence than just determining the numbers of various lymphocyte populations (Stites & Terr 1994).

RESULTS

Ames test

Curcumin showed protective effects against mutagenicity of three different concentrations of AFB_1 (10 μg, 1 μg and 0.1 μg per plate) in both tested strains of *Salmonella typhimurium* TA98 and TA100. The effective concentrations were 30 μg and 300 μg of curcumin per plate. The inhibition of mutagenicity was dose dependent and is expressed as percentage of inhibition of mutagenic activity in Table 1. Lower concentrations 0.3 μg and 3 μg of curcumin per plate were not effective, with the exceptions of its effects on 1 μg AFB_1 per plate in TA98 and the effect of 3 μg of curcumin per plate on mutagenicity of 0.1 μg of AFB_1 per plate.

Similar strong and dose dependent inhibition effects of two highest concentrations of curcumin were detected also against mutagenicity of all concentrations of IQ (0.1 μg, 0.01 μg and 0.001 μg per plate in TA98, 10 μg, 1 μg and 0.1 μg per plate in TA100). Lower concentrations, i.e., 0.3 and 3 μg of curcumin per plate, only slightly reduced the effect of IQ, mostly not significantly (Table 2).

The effect of curcumin on the direct mutagen MNU on TA100 was less effective as two highest concentrations of curcumin reduced the muta-

Table 2. Effect of curcumin on mutagenicity of IQ – Ames test – TA98, TA100

IQ + curcumin dose (μg/plate)	*S. typhimurium* TA98 + S9		IQ + curcumin dose (μg/plate)	*S. typhimurium* TA100 + S9	
	revertants ± SD	% ihibition		revertants ± SD	% inhibition
0.1 + 0	1210 ± 99		10 + 0	1302 ± 91	
0.1 + 0.3	1109 ± 46	–8	10 + 0.3	1182 ± 132	–9
0.1 + 3	1058 ± 74*	–13	10 + 3	1186 ± 147	–9
0.1 + 30	791 ± 62**	–35	10 + 30	1169 ± 98	–10
0.1 + 300	67 ± 15**	–95	10 + 300	125 ± 26**	–90
0.01 + 0	389 ± 80		1 + 0	545 ±128	
0.01 + 0.3	350 ± 76	–10	1 + 0.3	468 ± 95	–14
0.01 + 3	329 ± 114	–15	1 + 3	362 ± 34*	–34
0.01 + 30	236 ± 69**	–39	1 + 30	263 ±45**	–52
0.01 + 300	40 ± 7**	–89	1 + 300	98 ± 5**	–82
0.001 + 0	102 ± 5		0.1 + 0	186 ± 30	
0.001 + 0.3	101 ± 11	–1	0.1 + 0.3	159 ± 19	–15
0.001 + 3	85 ± 13*	–17	0.1 + 3	157 ± 38	–16
0.001 + 30	61 ± 12**	–40	0.1 + 30	136 ± 23*	–27
0.001 + 300	28 ± 3**	–73	0.1 + 300	85 ± 12**	–54
Control – DMSO	37 ± 5			112 ± 22	
0 + 0.3	42 ± 9			144 ± 3	
0 + 3	33 ± 5			138 ± 7	
0 + 30	36 ± 1			114 ± 11	
0 + 300	26 ± 4			76 ± 4	

SD – standard deviation; *$P \leq 0.05$; **$P \leq 0.01$

Table 3. Effect of curcumin on mutagenicity of MNU – Ames test – TA98, TA100

MNU + curcumin dose (μg/plate)	*S. typhimurium* TA100	
	revertants ± SD	% inhibition
1000 + 0	1856 ± 99	
1000 + 0.3	1849 ± 154	0
1000 + 3	1864 ± 116	0
1000 + 30	1662 ± 126*	–11
1000 + 300	1202 ± 184**	–35
100 + 0	1977 ± 83	
100 + 0.3	1877 ± 100	–5
100 + 3	1895 ± 70	–4
100 + 30	1770 ± 74**	–11
100 + 300	1536 ± 92**	–22
10 + 0	783 ± 157	
10 + 0.3	775 ± 134	–1
10 + 3	772 ± 176	–1
10 + 30	516 ± 98**	–34
10 + 300	386 ± 50**	–51
Control – DMSO	64 ± 16	
0 + 0.3	71 ± 9	
0 + 3	74 ± 13	
0 + 30	60 ± 15	
0 + 300	51 ± 17	

SD – standard deviation; *P ≤ 0.05; **P ≤ 0.01

genicity of MNU to a lower extent in comparison with the indirect mutagens. The highest effect was found in the combination of 300 μg per plate with a lower concentration of MNU – 10 μg per plate (Table 3).

Micronucleus test

In the group of animals treated with aflatoxin B_1 a statistically significant higher number was found of micronuclei in polychromatophilic erythrocytes of the bone marrow as compared with the control group. The number of micronuclei in animals influenced by curcumin alone did not differ from that of the control group. On oral application of the combination of curcumin and aflatoxin B_1 at a dose of 5 g/kg, 1 g/kg and 0.5 g/kg b.w., the number of micronuclei in polychromatophilic erythrocytes was lower in a statistically significant degree in comparison with laboratory mice treated with AFB_1 alone.

Table 4. Numbers of micronuclei in polychromatophilic erythrocytes in the bone marrow of mice treated with a combination of curcumin and the mutagens

Substance studied	Number of micronuclei ± SD
Control – 7% DMSO	2.2** ± 0.82
Curcumin – 1 g/kg	2.6** ± 0.49
Curcumin – 5 g/kg	3.7** ± 1.3
AFB1 – 5 mg/kg	9.8* ± 2.4
Curcumin + AFB1 – 5 g/kg + 5 mg/kg	4.0** ± 1.2
Curcumin + AFB1 – 1 g/kg + 5 mg/kg	3.2** ± 1.1
IQ – 20 mg/kg	6.6* ± 1.02
Curcumin + IQ – 1 g/kg + 20 mg/kg	3.4** ± 0.80
MNU – 50 mg/kg	36.5* ± 5.85
Curcumin + MNU – 1 g/kg + 50 mg/kg	18.4** ± 5.20

*significantly higher number of micronuclei as against the negative control (DMSO); **significantly lower number of micronuclei as against mutagen alone; SD – standard deviation

A similar effect came about on the application of the IQ mutagen: curcumin in combination with the IQ mutagen reduced its mutagenic effect to a statistically significant degree. Likewise, on the treatment of mice with the combination of curcumin and MNU a significant reduction of the number of micronuclei in comparison with the number of micronuclei elicited by MNU alone was observed. The results are presented in Table 4.

Comet assay

The effect of curcumin on the induction of DNA damage by AFB_1, MNU or IQ was followed with bone marrow cells, liver cells, and with epithelium cells of the colon using the comet assay. In our experiment, AFB_1 did not induce any significant amount of SSB in bone marrow cells, therefore no effect of curcumin on this induction could bee seen. No induction of SSB was seen either in liver cells of animals treated with AFB_1 alone, however, some slight but significant increase of DNA breaks was observed 2 h after the application of AFB_1 to animals pre-treated with curcumin. A significant induction of the DNA damage was found in colon epithelium cells where the number of SSB increased from the value 1.33 to 2.77 SSB/10^9 Da

2 h after the aplication of AFB_1. This number of SSB decreased again to the level of the control 24 h after the application. No effect of the pretreatment of mice with curcumin was observed, either after 2 h or 24 h from the application.

In contrast to AFB_1, MNU induced DNA damage in all cell types. In bone marrow cells the level of SSB increased from the value of 0.49 SSB/10^9 Da that was found in controls to 1.7 SSB/10^9 Da. The previous application of curcumin decreased this value significantly to 1.37 SSB/10^9 Da. Twenty four hours after the applicaton of MNU, the value of SSB decreased to 0.8 SSB/10^9 Da approximately both in curcumin treated and non-treated animals. In contrast to bone marrow cells, no effect of curcumin on the induction of DNA damage by MNU was observed in liver cells. A significant number of SSB was induced in these cells within 2 h after the application of MNU (2.61 SSB/10^9 Da), which dropped to 1.2 SSB/10^9 Da after 24 hours.

Quite a high number of SSB was induced by MNU in colon epithelium cells. The number of SSB rose from the value of 1.33 SSB/10^9 Da found in the controls to 3.1 SSB/10^9 Da and this amount of SSB remained practically unchanged after 24 hours. In the mice pre-treated with curcumin, we found a significant reduction of SSB (to 2.46 SSB/10^9 Da). This amount also did not change during the 24 h interval after the treatment.

Similarly to AFB_1, IQ did not induce remarkable DNA damage in bone marrow and in liver cells. Only in liver the amount of SSB rose to 0.77 SSB/10^9 Da, which was about twice as much as the control level. However, IQ was quite efficient in inducing DNA breaks in colon epithelium cells, where we found a significant number of breaks (3.11 and

Table 5. The effect of curcumin on the DNA damage (DNA strand breaks) induced by MNU or IQ in bone marrow cells, liver cells and colon epithelium cells

Tissue	Time (h)	MNU			Curcumin + MNU			IQ			Curcumin + IQ		
		mean	SE	*P*	mean	SE	*P*	mean	SE	*P*	mean	SE	*P*
Bone marrow	2	1.70	0.06	< 0.001**	1.37	0.09	0.012§§	0.31	0.05		0.55	0.15	
	24	0.80	0.06	0.03**	0.85	0.10		0.29	0.05		0.10	0.06	
Liver	2	2.61	0.26	< 0.001**	2.57	0.07		0.77	0.04		0.56	0.11	
	24	1.20	0.17	0.02**	1.66	0.22		0.66	0.03		0.47	0.13	
Colon	2	3.12	0.11	< 0.001**	2.46	0.27	0.046§§	3.11	0.05	< 0.001**	2.26	0.50	0.036§§
	24	3.29	0.10	< 0.001**	2.43	0.24	0.04§§	2.85	0.34	< 0.001**	1.85	0.29	0.022§§

§§ the statistical significance of difference between values found in animals treated only with a mutagen and animals treated both with a mutagen and curcumin

**the statistical significance of difference between values found in control and treated animals

Table 6. The effect of curcumin on the DNA damage (DNA strand breaks) induced by AFB_1 in bone marrow cells, liver cells and colon epithelium cells

Tissue	Time (h)	Control		Curcumin		AFB_1			Curcumin + AFB_1		
		mean	SE	mean	SE	mean	SE	*P*	mean	SE	*P*
Bone marrow	2	0.49	0.08	0.43	0.05	0.34	0.06		0.53	0.12	
	24	0.45	0.04	0.46	0.09	0.40	0.05		0.18	0.06	
Liver	2	0.33	0.06	0.42	0.08	0.39	0.07		0.59	0.08	0.029**
	24	0.39	0.05	0.43	0.05	0.37	0.05		0.39	0.08	
Colon	2	1.33	0.25	1.92	0.31	2.77	0.25	0.002**	2.96	0.23	0.001**
	24	1.57	0.00	1.15	0.16	1.39	0.26		1.58	0.22	

**the statistical significance of difference between values found in control and treated animals

2.85 SSB/10^9 Da at 2 h or 24 h after treatment, respectively). These values were decreased significantly by the previous application of curcumin to 2.26 and 1.85 SSB/10^9 Da.

The results are presented in Tables 5 and 6.

Chemiluminescence test

In all the investigations, the carcinogens (AFB_1, IQ, MNU) lowered the chemiluminescence values in a statistically significant degree when compared with the control.

In the experiment with curcumin (CRC) and AFB_1, the chemiluminescence values in the CRC group of mice and the combination of CRC and AFB_1 group did not differ from the control group until the 12th day. From the 12th day of the investigation, the chemiluminescence values decreased to a statistically significant degree (day 12 $P < 0.05$; days 21–27 $P < 0.01$) only in the group of animals treated with the combination of CRC and AFB_1 in comparison with the control group of mice. Over the whole 27-day period of testing, curcumin repaired the negative (suppressive) effect of AFB_1 on the lethal phase of phagocytosis in murine granulocytes (Table 7).

In the experiment with CRC and IQ, we obtained results analogous to those in the AFB_1 experiment. The chemiluminescence values in the groups of mice treated with CRC only or with CRC and IQ did not differ from the controls up to the 12th day of follow-up. From day 20, the chemiluminescence values fell in the group of mice treated with CRC or CRC and IQ (day 20 $P < 0.05$; day 26 $P < 0.01$) as compared with the control group of mice. Curcumin repaired the suppressive effect of IQ on the function of murine granulocytes (Table 7) throughout the whole period of follow-up.

In the experiments with CRC and MNU, the chemiluminescence values in the group with CRC alone did not differ statistically to any significant degree from the values found in the control group of mice. The values in the group of mice treated with CRC and MNU, over the whole period of testing, were significantly lower (days 1, 5, and 12, $P < 0.05$; days 21 and 27, $P < 0.01$), however, over that whole period this animal group showed statistically significant higher values of chemiluminescence in comparison with the reaction of animals treated with MNU only. Curcumin significantly suppressed the negative effect of MNU on phagocytosis in murine granulocytes (Table 7).

Table 7. Chemiluminescence values after administration of curcumin and mutagens (means ± S.D.)

Day after of mutagen administration	Control (7% DMSO)	AFB1 (1 mg/kg)	Curcumin (1 g/kg)	Curcumin + AFB_1
1st	700 ± 17.2	470 ± 8.6	656 ± 11.6	740 ± 10.9
6th	697 ± 15.0	320 ± 7.2	652 ±14.4	656 ± 11.3
12th	690 ± 14.1	232 ± 9.3	644 ± 13.2	565 ± 12.1
21st	688 ± 12.3	186 ± 6.4	640 ± 11.7	541 ± 10.6
27th	691 ± 11.8	150 ± 7.8	641 ± 12.6	520 ± 12.2
		IQ 20 mg/kg	Curcumin 1 g/kg	Curcumin + IQ
1st	792 ± 13.3	506 ± 10.2	799 ± 12.1	805 ± 15.2
6th	788 ± 12.9	489 ± 9.8	810 ± 11.8	775 ± 12.0
12th	785 ± 15.2	396 ± 11.3	766 ± 14.5	723 ± 14.4
20th	790 ± 14.9	215 ± 10.8	683 ± 14.9	651 ± 13.8
26th	782 ± 15.4	192 ± 8.6	600 ± 15.0	615 ± 14.6
		MNU 20 mg/kg	Curcumin 1 g/kg	Curcumin + MNU
1st	654 ± 10.5	500 ± 7.2	632 ± 14.5	595 ± 12.6
5th	649 ± 13.5	402 ± 9.1	630 ± 12.9	580 ± 11.8
12th	646 ± 14.8	312 ± 7.5	615 ± 14.9	545 ± 15.3
21st	652 ± 15.1	222 ± 8.8	604 ± 13.4	510 ± 12.2
27th	650 ± 12.2	203 ± 5.0	589 ± 15.9	491 ± 10.5

Table 8. Blastic transformation values after administration of curcumin and mutagens (means ± S.D.)

Day after of mutagen administration	Control (7% DMSO)	AFB_1 (1 mg/kg)	Curcumin (1 g/kg)	Curcumin + AFB_1
5th	18.85 ± 3.66	4.79 ± 1.05	16.25 ± 4.0	17.05 ± 3.25
13th	22.2 ± 5.03	5.46 ± 0.72	18.88 ± 4.86	15.0 ± 2.02
19th	27.51 ± 4.5	2.97 ± 0.45	22.33 ± 3.22	18.87 ± 2.87
		IQ (20 mg/kg)	Curcumin (1 g/kg)	Curcumin + IQ
5th	20.13 ± 2.8	5.32 ± 1.32	21.45 ± 3.05	20.2 ± 3.22
13th	25.06 ± 3.12	3.98 ± 0.66	23.2 ± 2.55	19.82 ± 4.14
19th	19.88 ± 2.15	2.83 ± 0.4	17.15 ± 2.48	13.75 ± 2.25
		MNU (20 mg/kg)	Curcumin (1 g/kg)	Curcumin + MNU
5th	18.85 ± 3.2	5.14 ± 1.06	16.25 ± 2.52	19.07 ± 3.83
13th	22.2 ± 4.34	6.94 ± 1.27	18.88 ± 2.91	13.5 ± 2.54
19th	27.51 ± 4.22	4.13 ± 0.7	22.33 ± 3.74	18.77 ± 2.75

Blastic transformation method

In all the blastic transformation experiments, all the carcinogens (AFB_1, IQ, MNU) lowered the capacity of T-lymphocytes to transform into blasts in a statistically significant degree ($P < 0.01$).

In the experiment with CRC and AFB_1 over three weeks of follow-up, the stimulation indexes (SI) were found to be higher to a statistically significant degree ($P < 0.01$) in the groups of mice treated with CRC and AFB_1 in comparison with the stimulation indexes in the groups treated with AFB_1 only (Table 8). The same results were obtained in the experiment with CRC and IQ (Table 8).

In the experiment with CRC and MNU, statistically significant higher SI were found (days 5 and 19, $P < 0.01$; day 13, $P < 0.05$) in the groups of mice treated with the combination of CRC and MNU in comparison with the groups of mice treated with MNU only (Table 8).

Also in the assessment of the functional capacity of T-lymphocytes regarding blastic transformation, curcumin revealed the ability to compensate significantly the suppressive effects of all three carcinogens.

DISCUSSION

Epidemiological studies have indicated a significant difference in the incidence of cancers among ethnic groups, who have different lifestyles and have been exposed to different environmental factors (Smith-Warner *et al.* 2003). Many authors focused their attention on the study of anticarcinogenic and antimutagenic effects of phytochemicals in *in vivo* and *in vitro* tests (Anto *et al.* 2002; Park & Surh 2004).

Curcumin is an extremely potent inhibitor of mutagenicity of indirect mutagens AFB_1 and IQ in the concentration of 300 μg per plate, and a potent inhibitor in the concentration of 30 μg per plate as it was proved in Ames test with *Salmonella typhimurium* TA98 and TA100 after metabolic activation. The effect on the mutagenic activity of the direct mutagen MNU was also detected but to a much lower extent in comparison with the effect of the indirect mutagens. This is in agreement with the fact that curcumin inhibits metabolic activation of mutagens especially by inhibiting phase I enzymes (Duvoix *et al.* 2004) or affecting the detoxification enzymes (Igbal *et al.* 2003).

Similar results were achieved by several authors using the Ames test. Nagabushan *et al.* (1987) described the inhibition of mutagenicity of several indirect mutagens by curcumin but no inhibition of mutagenicity of direct mutagens sodium azide, monoacetylhydrazine, streptozocin, and 4-nitrophenylendiamine. Soni *et al.* (1997) proved a decrease of mutagenicity of AFB_1 and Shishu *et al.* (2002) the inhibition by curcumin of mutagenicity of several indirect cooked food mutagens including IQ using the Ames assay. Contrary to Nagabushan's negative results with curcumin and direct mutagens, De Flora *et al.* (1994) proved the inhibitory effect of curcumin on mutagenicity of the direct-acting mutagen 4-nitroquinoline 1-oxide (4NQO). Also in our experiments the ef-

fect of curcumin against mutagenicity of direct mutagen MNU was significant, but its effects against AFB_1 and IQ were much stronger.

In micronucleus test, we detected antimutagenic effect of curcumin against all three mutagens. Treshiama *et al*. (1998) described similar effects of curcumin on chromosomal aberrations or micronuclei induced by irradiation, and Shukla *et al*. (2002) on chromosomal aberrations induced by cyclophosphamide.

The immune system plays a significant role in the combination with prooxidative and antioxidative processes in the organism, especially in the initial stages of oncogenesis. Many substances, for example all the above mentioned mycotoxins, are important immunosupressors and play an important and critical role in the initial stages of cancer progession, because in the final stage of oncogenesis, in which it is decided whether a tumour cell shall progress or shall be eliminated, namely the controlling and liquidating action of the immune system is decisive.

Curcumin also significantly suppresses the induced oxidative stress by scavenging free radicals, and its antioxidative activity seems to be derived from its suppressive effects on the increase of the peroxisome content and the decrease of the glutathione peroxidase and D-glucose-6-phosphate dehydrogenase activities (Watanabe & Fukui 2000).

Many authors proved anticarcinogenic effects of curcumin on the inhibition of tumour formation and tumour promotion of the tumours in laboratory animals induced chemically or by radiation (Ikezaki *et al*. 2001; Inano & Onoda 2002); antiproliferative effect was also proved *in vitro* on human carcinoma cell lines (Elattar & Virji 2000). The antimutagenic effect of curcumin against various mutagens in *in vivo* and *in vitro* test systems was proved by different authors (El-Hamss *et al*. 1999; Polasa *et al*. 2004).

Curcumin blocks the tumour initiation induced by benzo(a)pyrene (B(a)P) and dimethylbenz(a)anthracene via the inhibition of the formation of DNA adducts (Huang *et al.* 1992). It is also known to inhibit skin carcinogenesis, carcinogenesis of the forestomach and colon in mice (Huang *et al.* 1994). The mechanism of anticarcinogenic effect is not fully understood. Curcumin may inhibit BaP-induced forestomach cancer in mice by affecting the activation as well as inactivation pathways of BaP metabolism in the liver (Singh *et al*. 1998). However, the induction of SSB in V79 cells *in vitro* with directly acting methylating agent MNNG was also reduced by curcumin (Chakraborty *et al*. 2004). The authors found that curcumin not only has a chemoprotective action but may also decrease the DNA damage via the stimulation of DNA repair. Besides its chemoprotective and antimutagenic effects, curcumin induces apoptosis by multiple mechanisms (Anto *et al*. 2002). Curcumin was also found to be able to induce DNA damage in human gastric mucosa cells and in isolated human lymphocytes (Blasiak *et al.* 1999).

Our results are in good agreement with those describing the antitumourigenic action of curcumin (see above). However, a significant reduction of DNA damage induced by MNU or IQ was observed only in colon epithelium cells. These cells were probably protected by curcumin present in the colon, which remained there after *per os* application in a higher concentration compared to liver or bone marrow. In the two latter organs the concentration of curcumin was probably not high enough to reduce the amount of the DNA damage induced by MNU doses used in this study.

AFB_1 and IQ induced DNA damage only in colon epithelium cells, while no significant DNA damage was found in liver or bone marrow cells. It is interesting that curcumin inhibits the DNA damage induction by IQ but it did not reduce DNA breaks induced in colon cells with AFB_1. The possibility that curcumin inhibits only some of the specific cytochromes or stimulates only certain detoxifying enzymes may not be excluded (Singh *et al*. 1998).

Functional foods and nutraceuticals constitute great promise to improve health and prevent aging-related chronic diseases (Ferrari 2004). The study of phytochemicals with chemopreventive effects and a better understanding of their health-related interactions should lead to a better use of the dietary intervention in the prevention of cancers.

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Antimutagenic Effect of Genistein

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Abstract

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A great variety of health benefits including the protection against breast and prostate cancers has been attributed to the soya consumption, because of the presence of soy beans isoflavones, genistein, and others. We investigated the antigenotoxic effect of genistein on the genotoxicity of three mutagens and carcinogens – aflatoxine B_1 (AFB_1), 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), and *N*-nitroso-*N*-methylurea (MNU), using the Ames bacterial mutagenicity test and the micronucleus test. In the Ames test on *Salmonella typhimurium*, a significant antimutagenic effect was determined against the indirect mutagen AFB_1 in two strains, TA98 and TA100. However, the effect on the IQ indirect mutagenicity was more pronounced in the test with TA98 than with TA100. The mutagenicity of the direct mutagen MNU was suppressed by genistein only at its highest concentration used (300 μg/plate). The protective effect of genistein against all three mutagens was proved in the micronucleus test as the treatment of mice with the combinations of genistein and mutagens resulted in a significant reduction of the number of micronuclei in comparison with the number of micronuclei induced by the individual mutagens alone.

Keywords: chemoprevention; aflatoxin B_1; 2-amino-3-methylimidazo [4,5-f]quinoline; *N*-nitroso-*N*-methylurea; Ames test; micronucleus test

Genistein is a phytoestrogen belonging to the group of isoflavons with a wide variety of pharmacological effects. Genistein is synthetised in plants from flavanone naringenin. The major dietary source of genistein are soya products (Dixon & Ferreira 2002).

There exist numerous data showing the protective effects of soya products in animal models and in epidemiological studies (Rosenberg Zand *et al.* 2002). A cross-national study involving 50 countries identified soya products as foods with a protective effect against prostate cancer (Herbert *et al.* 1998). The low incidence of breast cancer in Asian women compared to women in western countries was attributed to a high consumption of soya products (Adlercreutz *et al.* 1991; Lee *et al.* 1991). This correlation is less evident among the second generations of Asian immigrants to USA adopting a western style diet (Ziegler *et al.* 1993). Urinary levels of soya derived isoflavones

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including genistein were lower in breast cancer patiens as compared with controls (Zheng *et al.* 1999). An association between high soya intake and lower incidence of endometrium cancer were also described (Goodman *et al*. 1997). Epidemiologic data are summarised in Park and Surh (2004). In a study of Lamartiniere *et al*. (2002), genistein was confirmed as an agent protecting animals against experimentally induced mammary and prostate cancers.

The antiestrogenic activity of genistein is the probable mechanism of its chemopreventive effect. Genistein shares the structural similarity with estrogen estradiol 17 β and the ability to be tied up with estrogen receptors. Thus, genistein exerts both estrogenic and antiestrogenic activities, the latter one by competing for estradiol receptors. The opposite effects are attributed to different responses provoked by different doses of genistein, which in lower concentrations exerts estrogen-like activity, in higher concentrations may act as an antiestrogen and an inhibitor of the tumour-promoting effects of estrogens (Sarkar & Li 2002, 2004).

Genistein has been shown to be an inhibitor of several intracellular enzymes such as tyrosin kinases, topoisomerase II, phosphatidylinositol kinases, ABC transporters, where genistein ligates their ATP-binding domain, thus involving cell signalling cascades and cell cycle progression. These effects on the molecular and cellular levels are summarised in Polkowski and Mazurek (2000); Sarkar and Li (2002, 2004); Park and Surh (2004). On the cellular level, it induces cellular differentiation, alters cell cycle progression, inhibits cell proliferation, and induces apoptosis. Genistein has an antiangiogenic effect, inhibits proteins involved in the multidrug resistance of cancer cells. Genistein exerts an antioxidant effect, protects cells against the reactive oxygen free radicals, and inhibits osteoclastic function.

There also exist information on genotoxicity of genistein detected by studies *in vivo* and *in vitro* using higher concentrations (for review see Stopper *et al.* 2005).

Messina and Loprinzi (2001), while reviewing the literary data, came to uncertainity that the comsumption of soya may affect the risk of breast cancer or the survival of breast cancer patiens. Because of this conflicting results, genistein is under intensive study.

In our work, we present the effect of genistein on the mutagenicity of two indirect mutagens, i.e. aflatoxin B_1 (AFB_1) and 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), and on the mutagenicity of the direct mutagen *N*-nitroso-*N*-methylurea (MNU), using the Ames test *in vitro,* and the micronucleus test *in vivo*.

MATERIAL AND METHODS

The Ames test

For the evaluation of the antimutagenic effect of genistein *in vitro*, the Ames test was performed using *Salmonella typhimurium* TA98 and TA100 strains (Ames 1971; Ames *et al.* 1975; Maron & Ames 1983; Černá *et al*. 1989).

The mutagenic substances were used at the following concentrations: AFB_1 at the concentrations of 10 μg, 1 μg, and 0.1 μg per plate in both strains, TA98 and TA100; IQ at the concentrations of 0.1 μg, 0.01 μg, and 0.001 μg per plate in the strain TA98, and at the concentrations of 10 μg, 1 μg, and 0.1 μg in the strain TA100; MNU at the concentrations of 1000 μg, 100 μg, and 10 μg in the strain TA100 only. These MNU concentrations had no effect upon the strain TA98. Each concentration of the individual mutagens was combined with four different concentrations of genistein (300 μg, 30 μg, 3 μg, and 0.3 μg per plate). All chemicals were diluted in DMSO. For the metabolic activation the S9 liver homogenate fraction from laboratory rats treated with a mixture of polychlorinated biphenyls Delor was used (Maron & Ames 1983). All combinations of the mutagens and antimutagen were tested in two separate experiments, with three plates in each experiment.

The percentage of the inhibition of mutagenicity was calculated using the following formula:

[(No. of revertants of mutagen – No. of revertants of mixture of mutagen and genistein)/No. of revertants of mutagen] × 100

The micronucleus test

The experiments *in vivo* (bone marrow micronucleus test) were carried out on ten-week-old male Balb C mice, weighing 22–26 g. The animals were housed under standard conditions in groups of 10 mice for the treatment.

The following concentrations of mutagens were used for *in vivo* tests: AFB_1 5 mg/kg of body weight (b.w.), IQ 20 mg/kg b.w., MNU 50 mg/kg b.w.

Genistein was administered at the dose of 20 mg per kg b.w. to mice by gavage for three consecutive days. Carcinogens were administered at one dose on the third day. All the substances (diluted in DMSO) were administered in the volumes of 100 µl/10 g b.w. The control mice were orally treated with 7% solution of DMSO.

The mouse bone marrow micronucleus test was carried out according to Schmid (1975). A total of 1000 polychromatophilic erythrocytes were scored per animal by the same observer for evaluating the frequencies of micronucleated polychromatophilic erythrocytes. Each experiment was run three times.

The statistical significance of the differences between two means defined for the respective mutagen and its mixture with genistein was tested by the Student's *t*-test.

RESULTS

The results of the Ames test (Tables 1–3) are expressed as a number of revertants, and also as the percentage of inhibition of the mutagen activity of the sample consisting of a mixture of the respective mutagen and genistein in comparison with the mutagenicity of the individual mutagen, according to the formula presented in Methods.

The number of revertants induced by genistein did not differ from the control values. Two highest concentrations of genistein (300 and 30 µg per plate) revealed a significant dose – dependent antimutagenic effect upon all concentrations of AFB_1 in the TA98 and TA100 strains. The lower concentration of genistein, 3 µg per plate, was significantly antimutagenic only in combination with 1 and 0.1 µg of AFB_1 per plate in both strains (Table 1).

Table 1. Effect of genistein on mutagenicity of AFB_1 – Ames test

AFB_1 + genistein dose (µg/plate)	*S. typhimurium* TA98 + S9			*S. typhimurium* TA100 + S9		
	No of revertants	± SD	% of inhibition	No of revertants	± SD	% of inhibition
10 + 0	807	113		989	50	
10 + 0.3	784	104	–3	937	50	–5
10 + 3	733	118	–9	916	52	–7
10 + 30	519**	51	–36	815**	71	–18
10 + 300	182**	16	–77	310**	98	–69
1 + 0	254	45		567	36	
1 + 0.3	231	28	–9	534	27	–6
1 + 3	185*	19	–27	498*	32	–12
1 + 30	124**	14	–51	322**	34	–43
1 + 300	47**	7	–81	118**	21	–79
0.1 + 0	79	10		212	14	
0.1 + 0.3	81	15	+3	186	31	–12
0.1 + 3	57**	2	–28	161**	11	–24
0.1 + 30	45**	4	–43	129**	12	–39
0.1 + 300	29**	3	–63	103**	10	–51
Control – DMSO	25	3		95	8	
0 + 0.3	24	5		103	6	
0 + 3	23	3		101	6	
0 + 30	24	3		106	12	
0 + 300	27	4		94	11	

SD – standard deviation; *$P \leq 0.05$; **$P \leq 0.01$

Table 2. Effect of genistein on mutagenicity of IQ – Ames test

IQ + genistein dose (μg/plate)	*S. typhimurium* TA98 + S9			IQ + genistein dose (μg/plate)	*S. typhimurium* TA100 + S9		
	No. of revertants	± SD	% of inhibition		No. of revertants	± SD	% of inhibition
0.1 + 0	1113	98		10 + 0	1142	206	
0.1 + 0.3	1022	87	–8	10 + 0.3	1125	350	–1
0.1 + 3	1019	102	–8	10 + 3	964	215	–16
0.1 + 30	953*	27	–14	10 + 30	964	280	–16
0.1 + 300	491**	66	–56	10 + 300	549*	354	–52
0.01 + 0	387	72		1 + 0	380	128	
0.01 + 0.3	370	77	–4	1 + 0.3	370	85	–3
0.01 + 3	338	65	–13	1 + 3	349	113	–8
0.01 + 30	249**	43	–36	1 + 30	345	122	–9
0.01 + 300	117**	15	–70	1 + 300	171*	62	–55
0.001 + 0	114	23		0.1 + 0	155	42	
0.001 + 0.3	96	21	–16	0.1 + 0.3	147	39	–5
0.001 + 3	71**	15	–38	0.1 + 3	157	44	+1
0.001 + 30	63**	11	–45	0.1 + 30	131	29	–15
0.001 + 300	34**	5	–70	0.1 + 300	106	32	–32
Control – DMSO	25	4		Control – DMSO	106	32	
0 + 0.3	27	5		0 + 0.3	113	16	
0 + 3	27	4		0 + 3	108	29	
0 + 30	24	4		0 + 30	114	30	
0 + 300	26	4		0 + 300	106	35	

SD – standard deviation; *$P \leq 0.05$; ** $P \leq 0.01$

The effect of genistein on IQ mutagenicity was more pronounced in the tests with TA98 strain than in those with TA100 strain. Two highest genistein concentrations were antimutagenic in combinations with all doses of IQ in TA98, while the concentration of 3 μg per plate only in combination with the lowest IQ dose (0.001 μg per plate). The effect was dose dependent. However, in the strain TA100 only the highest concentration (300 μg per plate) significantly suppressed the mutagenicity of 10 and 1 μg of IQ per plate (Table 2).

The mutagenicity of the direct mutagen MNU was suppressed by 300 μg of genistein per plate in combinations with 100 and 10 μg of MNU per plate in the strain TA100. Other concentrations of genistein were without the antimutagenic effect (Table 3).

In the micronucleus tests all three mutagens revealed significant mutagenic activities. The number of micronuclei in animals influenced by genistein alone did not differ from that of the control group. Oral administration of the combination of genistein (at a dose of 20 mg/kg b.w.) and aflatoxin B_1 revealed a lower number of micronuclei in polychromatophilic erythrocytes in a statistically significant degree in comparison with laboratory mice treated with AFB_1 alone.

Similarly, a significant suppression of IQ mutagenicity was reached with the combination of genistein and the IQ mutagen.

The treatment of mice with the combination of genistein and MNU resulted in a significant reduction of the number of micronuclei in comparison with the number of micronuclei induced

Table 3. Effect of genistein on mutagenicity of MNU – Ames test

MNU + genistein dose (µg/plate)	*S. typhimurium* TA100		
	No. of revertants	± SD	% of inhibition
1000 + 0	1726	240	
1000 + 0.3	1775	115	+3
1000 + 3	1746	139	+1
1000 + 30	1699	136	–2
1000 + 300	1350	420	–22
100 + 0	1811	122	
100 + 0.3	1866	153	+3
100 + 3	1883	157	+4
100 + 30	1758	229	–3
100 + 300	1334**	138	–26
10 + 0	1050	262	
10 + 0.3	1011	290	–4
10 + 3	1021	302	–3
10 + 30	932	244	–11
10 + 300	598*	336	–43
Control – DMSO	110	8	
0 + 0.3	107	15	
0 + 3	117	14	
0 + 30	108	7	
0 + 300	98	11	

SD – standard deviation; *$P \leq 0.05$; **$P \leq 0.01$

by MNU alone. The results of the micronucleus test are presented in Table 4.

Table 4. Efect of genistein on mutagenicity of AFB_1, IQ and MNU – micronucleus test

Substance studied	Number of micronuclei	± SD
Control 7% DMSO	2.0**	0.82
Genistein 20 mg/kg	2.6**	0.49
AFB1 5 mg/kg	8.2*	2.5
Genistein + AFB1 20 mg/kg + 5 mg/kg	4.0**	1.6
IQ 20 mg/kg	11.0*	2.45
Genistein + IQ 20 mg/kg + 20 mg/kg	3.6**	1.62
MNU 50 mg/kg	21.8*	5.2
Genistein + MNU 20 mg/kg + 50 mg/kg	9.2**	2.9

*significantly higher number of micronuclei as against the negative control (DMSO)
**significantly lower number of micronuclei as against mutagen alone
SD – standard deviation

DISCUSSION

It is well known that damage to the genome or aberrant DNA methylation, resulting in aberrant gene expression (suppression of tumour suppressor genes and inappropriate expression of oncogenes), is fundamental to tumorigenesis. The variability in cancer expression is due to the differences in the amount of DNA damage and the capacity to repair DNA damage, both being influenced by the genetic predisposition (gene polymorphism) and the dietary factors. Metabolism including the detoxification of genotoxic chemicals is influenced by the dietary factors, and the dietary intervention offers us a good oportunity for the cancer prevention.

Many protective compounds were discovered in plants; among them, genistein has been extensively investigated for its chemopreventive ability, especially against tumours of breast and prostate. Its effect involves antioxidant properties, modulation of key enzymes and inhibitors of the cell cycle (Choi *et al.* 1998a, b), and induction of apoptosis in transformed cells (Kumi-Kiaka *et al*. 2000; Sarkar & Li 2004).

On the other hand, there are also studies on genotoxicity of this compound. In experimental animals, Dingley *et al.* (2003) detected an increase of PhIP (2-amino-1-methyl-6 phenyl-imidazo[4,5-b] pyridine) adducts but not IQ adducts after genistein treatment. In the study by Tsutsui *et al*. (2003) genistein was shown to induce chromosomal aberrations, aneuploidies, DNA adducts, and transformation of Syrian hamster embryonal cells.

Misra *et al*. (2002) described a significant increase in the frequency of micronucleated erythrocytes, but this effect was small and not dose related; genistein had no effect on the incidence of tumours developed in p53 knockout mice. Misra did not prove the genotoxic effect in the Ames test without metabolic activation; after metabolic

activation, only a small increase in the number of revertants was detected in TA100 strain of *Salmonella typhimurium*, however, not in other tester strains.

We did not detect any genotoxic effect of genistein either in the Ames test or in the micronucleus test, because the number of revertants or the number of micronuclei did not differ significantly from the controls. We proved a clear dose-dependent antimutagenic effect of genistein upon the indirect mutagenicity of AFB_1 and IQ in the Ames test. The effect upon AFB_1 mutagenicity was similar in TA98 and TA100 strains. The effect upon IQ mutagenicity was stronger in TA98 strain than in TA100 strain. The direct mutagenicity of MNU was suppressed only by the highest concentration of genistein in TA100 strain. Clear antimutagenic effects of genistein upon the mutagenicity of all three mutagens were also proved in the micronucleus test.

Similar results were achieved by Weisburger *et al*. (1998) in the Ames test, a dose-related inhibition of the mutagenicity of heterocyclic amine PhIP by genistein was detected. Miyazava *et al*. (1999) proved antimutagenic activity of genistein against Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) and furylfuramide in the Ames and umu tests.

Antigenotoxic effect of genistein and also its opposite effect are discussed in excellent reviews by Polkowski and Mazurek (2000), Sarkar and Li (2002) and Park and Surh (2004), altogether with the explanation of its effects at molecular and cellular levels.

It is supposed that genotoxicity of genistein can be caused by the inhibition of DNA topoisomerase II resulting in stabilisation of DNA double strand breaks at topoisomerase II-DNA binding sites (Boos & Stopper 2000; Snyder & Gillies 2002; Stopper *et al*. 2005).

It is evident that the understanding of the effects of genistein and other phytoestrogens is far from being clear. Their bipolar response – protective in lower doses and possible genotoxicity of higher doses, especially in *in vivo* experiments, needs additional studies in this field. It is also obvious that the concentrations necessary for the genotoxic effect in *in vitro* studies and in experimental studies *in viv*o can hardly be reached by our usual diet. But we must be carefull with the use of phytoestrogen concentrates in menopause. Phytoestrogens are often presented as non-hormonal. This may be dangerous for the women under the risk of re-occurrence of estrogen dependent cancers. In addition, the review of 105 clinical studies has not brought any clear proof that phytoestrogens lower the risk of breast cancer and cardiovascular diseases, but it seems to be evident that they decrease the risk of osteoporosis (Cornwell *et al*. 2004).

Since phytoestrogen genistein has a lower afinity to the estrogen receptors than the physiological ligand estradiol, it may act as an enhancer of the cell proliferation in the absence of hormone, but may be anti-estrogenic in the presence of estradiol and reduce estradiol-mediated cell proliferation. Interactions of different compounds with genotoxic and antigenotoxic effects may change the final effect of the individual compounds. Additionally, other environmental or life style factors may be related to the risk of cancers (Bouker & Hilakivi-Clarke 2000).

The reviewed data indicate that the intake of concentrated phytoestrogens as supplements should not be advised to menopausal women while diet containing high amounts of plant substances is important for the health maintenance (Stopper *et al*. 2005).

Further studies are needed on the effects of genistein and other phytoestrogens, especially studies on the combined effects of different plant substances, because of the possible interactions of genotoxic and antigenotoxic compounds. In a diet, this interactions may result in potentiation, as well as in antagonistic effects.

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Current trends and perspectives in nutrition and cancer prevention*

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There is an increasing evidence that dietary phytochemicals may play important roles as chemopreventive or chemotherapeutic agents in prevention of many diseases, including tumors. The purpose of this study was to examine antimutagenic effects and effect on the immune response of representative series of substances which commonly occur in human diet.

Using the Ames bacterial mutagenicity test and *in vivo* chemiluminescence test, we investigated antigenotoxic and immunomodulatory effects of juices and vegetable homogenates (carrot + cauliflower, cauliflower, red cabbage, broccoli, onion, garlic) on the genotoxicity of AFB_1 and pyrolysates of aminoacids. Using the Ames test and *in vivo* micronucleus, the chemiluminescence test, the blastic transformation test and the comet assay we examined antimutagenic effects of chemically identified chemoprotective substances in the pure form (resveratrol, diallylsulphide, phenethyl isothiocyanate, ellagic acid, epigallocatechin gallate, genistein and curcumine) on mutagenicity induced by three reference mutagens: aflatoxin B1 (AFB_1), 2-amino-3-metylimidazo[4,5,-f] chinolin (IQ) and N-nitroso-N-metylurea (MNU) and effect of phytochemicals on the immunosuppression caused by these mutagens.

All complete vegetable homogenates and substances of plant origin tested, showed a clear antimutagenic and immunomodulatory activities on mutagenicity and immunosuppression induced by reference mutagens. Only in the Ames test the effect of some phytochemicals against direct mutagen MNU was lower compared to indirect mutagens AFB_1 and IQ. Similarly, resveratrol and epigallocatechin gallate had no inhibitory effect on mutagenicity MNU in the Ames test.

Key words: phytochemicals, antimutagenic effects, effect on the immune response, Ames test, micronucleus test, chemiluminescence and blastic transformation tests

Tumors in man are largely the result of the action of environmental factors [1]. These environmental factors include chemical carcinogens and radiation almost exclusively originating from human activities. Besides carcinogenic substances, anthropogenic in character, a group of natural carcinogens exists.

Progress in understanding the biological basis of cancer revealed that damage to the genome or aberrant DNA methylation resulting in aberrant gene expression (suppression of tumor suppressor genes and inappropriate expression of oncogenes) is fundamental to tumorigenesis. The inter-individual variability in cancer expression is due to differences in the amount of DNA damage and capacity to repair that damage, both being influenced by genetic predisposition (gene polymorphism) and by dietary factors. Dietary factors play also important role in metabolization and detoxification of genotoxic chemicals. This situation offers us good oportunity for dietary intervention in cancer prevention.

It is generally known that high intake of fruits and vegetables can decrease the risk of cancer development [2]. Secondary metabolites presented in many plants as phenols, polyphenols, carotenoids, flavonoids, isoflavonoids, alkyl sulphides, isothiocyanates, alcaloids, phytoallexins and others are known for their anticancer properties. The mechanism of the protection includes a great variety of effects such as antioxidant activity against endogenous and exogenous oxidative damage to biomolecules, inhibition of metabolic activa-

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tion of carcinogens, increasing of enzyme activity participating in the detoxification of carcinogenic compounds, as glutathione-S-transpherase [3], blocking of nitrosamines, inhibition of synthesis of N-nitroso-compounds, altered estrogen metabolism, altered colonic milieu, effect on DNA methylation, DNA repair, effect on cell signaling cascades, increased apoptosis of cancer cells, decreased cell proliferation, positive effect on immune functions, etc. [4]. Carcinogenicity via the generation of free radicals may be modulated or prevented by scavenging free radicals with antioxidants, such as vitamins, curcumin, isoflavonoids, resveratrol and plant polysaccharides [5].

Consumption of certain fruit and vegetables is inversely associated with incidence of many cancers especially carcinoma of stomach, colon, esophagus, bladder, pancreas, lung, breast, cervix, endometrium, prostate and others as was proved in epidemiologic studies [6]. A low incidence of breast cancer in Asian women [7] is also attributed to the soya consumption. Several epidemiological studies have shown inverse association between increased green tea intake and cancers of many organs, especially of stomach, colorectum, liver and lung [8].

The biochemically and biologically active substances found in vegetables with regard to anticarcinogenic effects have been studied by many authors [9]. We have also demonstrated the antimutagenic and immunomodulatory effect of substances found in raw vegetables in mice conditioned with AFB_1, and pyrolysates of aminoacids [10, 11].

For these effects the named substances may be used as effective chemopreventive and chemoprotective agents. While mutagenic and co-mutagenic substances may occur in foodstuffs as well as in ambient air, antimutagens can act only through their intake in foodstuffs [12].

Diallyl sulphide belongs to the group of thiolic substances, (found mostly in garlic and onions), and these have been particularly assessed for their antibacterial and antiatherogenic activity; however, also their antioncogenic effects have been observed [13]. Diallyl sulphide and similar substances also influence the endogenic formation of nitrosamines and decrease the risk of gastric carcinoma. They inhibit carcinogens activation and accelerate carcinogens detoxification, influence signal transduction pathways and induce apoptosis [14]. Ellagic acid belongs to the polyphenols and is found in fruit (strawberries, raspberries, grapes, black currant, walnuts). It decreases the metabolic activation of carcinogenic substances by its anti-oxidative effect and by decreasing the activity of cytochrome P450 [15]. It also increases the activity of glutathione-S-transferase and protects the DNA against damage [16]. It markedly inhibits the mutagenic activity of aflatoxin B_1 [17].

Phytoallexin resveratrol, found mainly in the skin of grapes and in derived products such as red wine, has many important effects (anti-inflammatory, anti-oxidative, anti-carcinogenic) and influences activity of the immune system [18]. It can prevent oxidative damage to DNA which plays an important role in activity of many genotoxic substances [19]. Many authors have described the effect of resveratrol in the prevention of cardiovascular diseases (so-called French paradox). Resveratrol modulates lipid metabolism, inhibits oxidation of LDL and thrombocyte aggregation [20]. Molecular mechanisms of chemopreventive effects of resveratrol were described by BODE and DONG [21].

Phenethyl-isothiocyanate belongs to the group of aryl-isothiocyanates which decrease the activity of cytochrome P450 1A2 and influence the metabolic activity of carcinogenic substances [22]. Like several other aryl-isothiocyanates it increases the activity of glutathione-S-transferase, NAD(P)H, chinone oxidoreductase, and UDP-glucuronosyl transferase as was proved in laboratory mice, rats, and humans [23]. Possible targets for chemoprevention by PEITC and other phytochemicals are discussed by LEE and SURH [24].

Isoflavone genistein, a phenolic compound, present in high concentration in soybeans as a natural isoflavonoid, phytoestrogen [25], has anticancer activities, including influence on differentiation, apoptosis, inhibition of cell growth and inhibition of angiogenesis [8]. Two antibody-genistein conjugates, B43-genistein and EGF-genistein, are in clinical development for the treatment of acute lymphoblastic leukemia and breast cancer. Genistein is considered as effective chemotherapeutic agent against carcinoma with antioxidative and anti-inflammatory effects [26].

The therapeutic potential of genistein and epigallocatechin gallate (polyphenolic compound and major ingredient in green tea) was described by WANG [26], PARK and SURH [8]. Chemopreventive effects of green tea against human tumors were also confirmed by CONNEY et al [27]. Mechanisms of its anticarcinogenic activity on molecular level were discussed by BODE and DONG [21].

Yellow pigment curcumin – diferuloyl methane, isolated from the root of turmeric (*Curcuma longa)*, commonly used as a spice and food coloring material (E 100), is a chemopreventive agent with multiple mechanism of action [28]. Very important role of curcumin is in altering metabolic activation or detoxification of mutagens and carcinogens. It inhibits cytochrome P450 enzymes involved in activation of mutagens and carcinogens [28] and has effect on detoxifications enzymes [29]. Supressive effects on human brest carcinoma cells were also described [34] by inhibiting genes through influence of estrogen receptors. Curcumin exerts a variety of immunomodulatory effects [30].

For our study we chose two different representants of mutagenes which commonly occur in the human diet – mycotoxin AFB_1, food mutagen IQ (promutagens) and third MNU, direct mutagen with endogenous origin.

Aflatoxin B_1 (AFB_1) – contaminating foodstuffs is one of the most thoroughly studied and the well known mycotoxin with carcinogenic activity. In the present study it is used as a reference mutagen which exerts mutagenic activity in all prokaryotic and eukaryotic testing systems [31].

Another reference mutagen 2-amino-3-methylimida-

zo[4,5-f]quinoline (IQ), one of toxic substances contaminating foods, is formed by heat processing of foodstuffs. It is one of the most serious amino acid pyrolysates (heterocyclic amines) with mutagenic, carcinogenic and immunosuppressive activity [32].

N-nitroso-N-methylurea (MNU) is an important carcinogenic N-nitroso compound; and contrary to nitrosamines and AFB_1 it is a directly acting carcinogen requiring no metabolic activation [33].

In our study we combined methods of study of antimutagenic effects of chemopreventive agents with methods of testing of their effect on immune response.

Material and methods

Material. We studied chemopreventive effects of carrote with cauliflower, cauliflower, red cabbage, broccoli, onion, garlic as juices and complete vegetable homogenates, using the Ames test and chemiluminescence test, The effect of resveratrol, diallylsulphide, phenethyl isothiocyanate, ellagic acid, epigallocatechin gallate, genistein and curcumine as individual chemically identified chemoprotective substances in the pure form (Sigma-Aldrich Co, Lousiana, USA) on the mutagenicity or immunosuppression caused by three known mutagens AFB_1 (Alexis Corporation, USA), IQ (ICN Biomedicals, Inc., Germany) and MNU (Sigma-Aldrich Co, Lousiana, USA), was studied using the Ames bacterial mutagenicity test and *in vivo* micronucleus test, chemiluminescence and blastic transformation tests. Chemicals were diluted in DMSO (Sigma-Aldrich Co, Lousiana, USA).

Ames bacterial mutagenicity test was performed on auxotrophic his- bacterial strains of *Salmonella typhimurium* TA98 and TA100 [35].

Mutagenic substances were applied in the following concentrations: IQ in concentrations of 0.1 mg, 0.01 mg and 0.001 mg per plate in strain TA98; in concentrations of 10 mg, 1 mg and 0.1 mg in strain TA100. AFB1 in concentrations of 10 mg, 1 mg and 0.1 mg per plate in both strains, TA98 and TA100. MNU in concentrations of 1000 mg, 100 mg and 10 mg only in strain TA100 as these concentrations had no effect in strain TA98. Each concentration of any mutagen was combined with four different concentrations of antimutagen. For metabolic activation of indirect mutagens (AFB1 and IQ) the S9 fraction of liver homogenate from laboratory rats induced by a mixture of polychlorinated biphenyls Delor was used.

Each combination of mutagen and antimutagen was tested in two separate experiments with three plates in each experiment.

Percentage of inhibition of mutagenity was calculated as follows: (No of revertants of mutagen – No of revertants of mixture of mutagen and curcumin /No of revertants of mutagen) x 100. For statistical analysis Student's t-test was used.

Experimental animals. All *in vivo* experiments (bone marrow micronucleus test, chemiluminescence test, blastic transformation method and commet assay) were carried out on ten-week-old male Balb C mice, of weight 22–26 g (BIOTEST, Konárovice, CR). For each group 7–10 mice were used. All experiments were performed on the same animals.

Statistical evaluation of all methods was carried out by the t-test.

Methods. The mouse bone marrow micronucleus test was carried out according to SCHMID [36]. A total of 1000 polychromatophilic erythrocytes were scored per animal for evaluating the frequencies of micronucleated polychromatophilic erythrocytes. Each experiment was run three times.

The chemiluminescence test was performed according to the modification of ŠESTÁKOVÁ [11]. In the chemiluminescence test the degree to which phagocytes are capable of liquidating ingested material is determined. Well known are the mechanisms in which hydrogen peroxide participates in killing. H2O2 is synthesized in phagocytes upon receiving a signal by their membrane. The finding of a higher or lower activity of the complex H2O2-MPO-Cl- (I-) speaks of the readiness of the first defense line against bacteria, tumor cells as well as carcinogen-altered cells. Results are presented in maximum values (mV) of the chemiluminescence response of polymorphonuclear leukocytes in the dependence on time.

For the study of the readiness of cells in acquired immunity we have selected a functional test assessing T-lymphocytes, the blastic transformation method [37]. This test of lymphocyte activation determines the functional capacity of T-lymphocytes to react to a mitogen by proliferation, and is a more direct examination of immune competence than just determining the numbers of various lymphocyte populations [38].

Results

Using the Ames test all vegetable homogenates revealed significant antimutagenic activity as they decreased mutagenicity of AFB_1. Juice of cauliflower showed a clear antimutagenic activity against mutagenicity of 2-amino-3-metylimidazo[4,5,-f] chinolin (IQ). Vegetable juice of carrot with cauliflower effectively prevented the negative effect of AFB_1 and aminoacids pyrolysates in one of the phases of phagocytosis as measured by chemiluminescence (Tab. 1).

Also plant substances (resveratrol, diallylsulphide, phenetyl isothiocyanate, ellagic acid, epigalocatechin galate, genistein and curcumine) showed a clear antimutagenic activity.

In the Ames test all phytochemicals revealed dose dependent antimutagenic effect against all concentrations of two indirect mutagen AFB_1 and IQ in both strains TA98 and TA100 (Tab. 2, 3). The most effective concentrations were 30 and 300 μg/plate. Only effect of DAS against all concentrations of AFB_1 and against some concentrations of IQ was not detected in TA100.

The effect of all phytochemicals against direct mutagen MNU was lower in comparison with indirect mutagen and

Table 1. Effects of juices and vegetable homogenates

Effects / tests	Antimutagenic (mutagen) Ames test	Effect on immune response chemiluminescence	Effect on immune response blastic transformation
Carrot +cauliflower	+ AFB_1	+ AFB_1 + pyrolysate	+ AFB_1 + pyrolysate
Cauliflower	+ AFB_1 + IQ	N	N
Red cabbage	+ AFB_1	N	N
Broccoli	+ AFB_1	N	N
Onion	+ AFB_1	N	N
Garlic	+ AFB_1	N	N

+: antimutagenic and immunostimulatory or immunomodulatory effects, N: not tested

Table 2. Ames test – effects of phytochemicals on mutagenicity of AFB_1, IQ and MNU on TA98 strain

		mutagen bacterial strain								
		AFB_1 TA98			IQ TA98			MNU TA98		
	µg/plate	10	1	0.1	0.1/10	0.01/1	0.001/0.1	1 000	100	10
Res	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	+	+	+	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
DAS	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–	–
	30	–	+	+	+	+	–	–	–	–
	300	+	+	+	+	+	+	–	–	–
PEITC	0.3	–	+	–	+	+	+	–	–	–
	3	+	+	+	+	+	+	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	t	t	t	t	t	t	–	–	–
EA	0.3	–	–	–	–	–	–	–	–	–
	3	+	–	–	–	–	–	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
EGCG	0.3	+	+	–	–	–	–	–	–	–
	3	+	+	–	–	–	–	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
Gen	0.3	–	–	–	–	–	–	–	–	–
	3	–	+	+	–	–	+	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
Cur	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	+	–	+	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–

+: significant decrease of mutagenic activity, –: without significant decrease of mutagenic activity, t: toxicity

resveratrol and EGCG was not antimutagenic at all. EA decreased mutagenic activity only in the lowest concentration of MNU (10 µg/plate), no effect against higher concentrations of MNU was detected. On the other hand PEITC and curcumine was antimutagenic against MNU. PEITC was toxic in the highest concentration (300 µg/plate), and was a very potent antimutagen also in lower concentrations against indirect mutagens. Concentration 30 µg/plate of PEITC was effective against all concentrations of MNU. The effect of curcumine against MNU was significant in concentrations 30 and 300 µg/plate but the level of inhibition of mutagenity was lower in comparison with the effect against indirect mutagens.

In the micronucleus test all plant substances had inhibitory effect on mutagenicity induced by reference mutagens, (IQ, AFB1, MNU), and reduced its mutagenic effect to a statistically significant degree (Tab. 4).

All tested substances of plant origin in combination with mutagens repaired the degree of blastic transformation after their administration.

The results show that all tested substances effectively prevented the negative effect of mutagens in one of the phases of phagocytosis as measured by chemiluminescence (Tab. 4).

Discussion

It has been estimated that some human cancers could be prevented by modification of lifestyle including dietary modification [39].

Epidemiological studies have indicated a significant difference in the incidence of cancers among ethnic groups who have different lifestyles and have been exposed to different environmental factors [40]. The consumption of fruits, soybean and vegetables has been associated with reduced risk of several types of cancers [41].

Many authors focused their attention on the study of anticarcinogenic and antimutagenic effects of phytochemicals in *in vivo* and *in vitro* tests [8].

Our studies focused on substances of plant origin, and on effects of the complex of substances contained in uncooked vegetables (carrot, cauliflower, garlic). We have proven antimutagenic and immunomodulatory properties of the selected compounds of plant origin in the form of chemically defined substances (resveratrol, diallyl sulphide, phenethyl isothiocyanate, ellagic acid, epigallocatechin gallate, genistein, curcumin) examined in combination with three selected strong carcinogens with proven mutagenic effect (aflatoxin B_1, 2-amino-3-methylinidazol[4,5] quinoline and N-nitroso-N-methylurea) [42].

Table 3. Ames test – effects of phytochemicals on mutagenicity of AFB_1, IQ and MNU on TA100 strain

		mutagen bacterial strain								
		AFB_1 TA100			IQ TA100			MNU TA100		
	µg/plate	10	1	0.1	0.1/10	0.01/1	0.001/0.1	1 000	100	10
Res	0.3	–	–	–	–	+	+	–	–	–
	3	–	+	–	+	+	+	–	–	–
	30	–	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
DAS	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–	–
	30	–	–	–	–	+	–	–	–	–
	300	–	–	–	–	+	–	+	+	+
PEITC	0.3	–	+	+	–	+	+	–	–	–
	3	+	+	+	+	+	+	–	–	–
	30	+	+	+	+	+	+	+	+	+
	300	t	t	t	t	t	t	t	t	t
EA	0.3	–	+	–	–	–	–	–	–	–
	3	–	+	–	–	–	–	–	–	+
	30	+	+	+	+	+	+	–	–	+
	300	+	+	+	+	+	+	–	–	+
EGCG	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–	–
	30	+	+	+	+	+	+	–	–	–
	300	+	+	+	+	+	+	–	–	–
Gen	0.3	–	–	–	–	–	–	–	–	–
	3	–	+	+	–	–	–	–	–	–
	30	+	+	+	–	–	–	–	–	–
	300	+	+	+	+	+	–	–	+	+
Cur	0.3	–	–	–	–	–	–	–	–	–
	3	–	–	–	–	+	–	–	–	–
	30	+	+	+	–	+	+	+	+	+
	300	+	+	+	+	+	+	+	+	+

+: significant decrease of mutagenic activity, –: without significant decrease of mutagenic activity, t: toxicity

Our results of *in vitro* Ames test discovered that antimutagenic effect of phytochemicals tested was less pronouncied against direct mutagen MNU than against indirect mutagens AFB_1 and IQ, both requiring metabolic activation. This was detected in all chemicals, and resveratrol and epigallocatechin galate were even ineffective against MNU.

Curcumin is a strong inhibitor of mutagenicity of indirect mutagens AFB_1 and IQ, and the effect on mutagenicity of MNU was also significant but somewhat lower than against indirect mutagens.

Similar results were achieved by several authors using the Ames test. NAGABUSHAN [43] described inhibition of mutagenicity of several indirect mutagens by curcumin, but inhibition of mutagenicity of direct mutagens sodium azide, monoacetylhydrazine, streptozocin and 4-nitrophenylendiamine was not observed. SONI [44] proved decrease of mutagenicity of AFB_1 and SHISHU [45] inhibition of mutagenicity of several indirect cooked food mutagens including IQ by curcumin in the Ames assay.

It is in agreement with the fact that the dominant reason for antimutagenicity of phytochemicals in *in vitro* tests is the interference with metabolic activation of mutagens, and another reason can be the cytotoxic effect of highest concentrations detected in our experiment in the Ames test using the highest concentration of phenetyl isothiocyanate. Also curcumin inhibits metabolic activation of mutagens especially by inhibiting phase I enzymes [28] or exerts effect on detoxifications enzymes [29], and this effect may be more extensive. Significant inhibitory activity of the tea catechins, ECG and EGCG, against the mutagenicity of Trp-P-2 and *N*-OH-Trp-P-2 has been found by OKUDA [46] using *Salmonella typhimurium* TA98 and TA100.

In micronucleus test we detected antimutagenic effect of the tested phytochemicals against all three mutagens. Similar effects of curcumine were described by TRESHIAMA [47] on chromosomal aberrations or micronuclei induced by irradiation. Induction of micronuclei and chromosomal aberrations produced by whole body exposure to ionizing radiation in mice was found to be significantly inhibited by oral administration of ellagic acid [47]. Hot water extracts of green tea effectively suppressed AFB_1-induced chromosome aberrations in bone marrow cells in rats [48].

Plant phytochemicals have a wide variety of effects. Their activity is pleiotropic and it concerns not only metabolic activation or detoxification of mutagen and carcinogens or free radical scavenging effects, but also exerts effects on DNA methylation, DNA repair, cell signalling cascade and apoptosis etc. [4]. That is why chemoprevetive agents work in all stages of carcinogenic processes as blocking agents in the stage of initiation or as supressing agents of promotion or progression stages [1, 8].

Immune system plays significant role in combination with prooxidative and antioxidative processes in organism, especially in the initial stages of oncogenesis. Many substances, for example the all above mentioned mycotoxins, are important immunosupressors and play important and critical role in initial stages of cancer progession. In the final stage of oncogenesis in which it is decided whether a tumor cell shall progress or shall be eliminated, the controlling and liquidating action of the immune system is namely decisive.

According to our experimental experiences a significant biological phenomenon is that the behavior of many substances depends on various conditions, particularly on dos-

Table 4. Effects of chemoprotective agents on mice

Effects	Antimutagenic effect	Effect on immune response	
Tests	Micronucleus	Chemilumine-scence	Blastic transformation
Resveratrol	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Dialylsulphide	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Phenetyl isothiocyanate	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Ellagic acid	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Epigallocatechin gallate	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Genistein	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU
Curcumin	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU	+ AFB_1 + IQ + MNU

+: antimutagenic, immunostimulatory or immunomodulatory effects

age. Such substances may either act as mutagenic or antimutagenic agents, or show no provable positive or negative mutagenic activity. In this case, the effect depends not only on a combined action of individual substances but also on the time. The time factor is therefore important not only in respect to the duration of the action of a particular substance but also in respect to the dosing regimen, i.e. the time at which the particular substance starts to act. This is analogous to the subsequent action of initiators and promotors in a multistage carcinogenic process in which the sequence of actions is critical [12].

The differences between the activities of extracts obtained from the particular plant (whole plant, roots, leaves, seeds) and separated substances were observed in some cases. In such cases the resultant effect depended on the amount of additional substances in the particular extract or leachate and on the activity of additional substances which may either show an antimutagenic effect by inhibiting the activity of a mutagenic substance (particularly if the mutagenic substance is present at a low concentration), or enhance the mutagenic effect of other substances. Quercetin in combination with hypericin may serve as an example [49]. Study of possible synergistic effects or other interactions of phytochemicals is necessary for understanding their effects [50].

Phytochemicals with chemopreventive effects can be used as dietary supplements, functional foods or even drugs. Interaction between phytochemical components can modify the effect on human health and can explain the health effects of regional differences in diets, unexpected and side effects of drugs or dietary supplements [50].

Functional foods and nutraceuticals constitute a great promise in efforts to improve health and prevent aging-related chronic diseases. Study of phytochemicals with chemopreventive effects and better understanding of their health-related interactions should lead to better use of dietary intervention in cancer prevention.

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Antimutagenic Effect of Diallyl Sulfide

Antimutagenní účinek dialyl sulfidu

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Abstract:

Naturally occurring organosulfur compounds, such as diallyl sulfide (DAS), are effective in protection against cancer induced by a variety of chemical carcinogens in laboratory animals.

We tested antimutagenic effect of DAS on mutagenic activity of indirect-acting carcinogens 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and aflatoxin B_1 (AFB_1) and direct-acting carcinogen N-nitroso-N-methylurea (MNU) by the Ames test and by the micronucleus test. Chemiluminescence test was used to evaluate an immunomodulation effect on immunosuppression induced by carcinogens.

In the Ames test, DAS at concentration 300 μg per plate strongly suppressed a number of IQ-induced revertants in *Salmonella typhimurium* strain TA98 and moderately in TA100, YG1021 and YG1024. Lower concentrations of DAS (30 μg, 3 μg and 0,3 μg per plate) did not reduce mutagenic activity of IQ in a such extent, the effect was dose dependent. Antimutagenic effect of the two highest doses of DAS upon mutagenic activity of AFB_1 was significant only in transgenic strains YG1021, YG1024 and to a less extent in the strain TA98, this effect was not significant in the strain TA100. DAS only at concentration 300 μg per plate was effective against direct-acting mutagen MNU in TA100.

In vivo experiments: mouse bone marrow micronucleus and chemiluminescence tests were carried out on BALB/c mice. DAS administered orally at the dose 3 x 100 mg/kg decreased significantly the number of micronuclei in polychromatophilic erytrocytes induced by AFB_1, IQ and MNU.

In all of the immunologic experiments, DAS significantly repaired the immunosuppressive effects of all three carcinogens tested. Results of this study indicate that in view of its capability to modulate

immunosuppression caused by carcinogens, DAS may play an important role in the prevention of carcinogenesis.

Keywords: chemoprevention, immunomodulation, Aflatoxin B_1, 2-amino-3-methylimidazo [4,5-f]quinoline, N-nitroso-N-methylurea

1. Introduction

Phytochemicals with potential chemoprotective properties are present in various foods in the human diet. Garlic and onion, members of the *Allium* genus, are rich in sulfur-containing components such as diallyl sulfide, diallyl disulfide, diallyl trisulfide. Naturally occurring organosulfur compounds are strong modifiers of action of chemical carcinogens and these substances are thought to be the most active in inhibiting the early stage of the carcinogenic process (Hermann-Antosiewicz and Singh 2004). Cancer risk is reduced by the inhibition of carcinogen activation, by the increase of carcinogens detoxification (Yang et al. 2001; Thompson and Ali 2003) and by the stimulation of immune function through activation of macrophages and induction of T cell proliferation (Lau et al. 1991; Lamm and Riggs 2000). Sulfur-containing compounds administration enhanced white blood cell count, bone marrow cellularity, weight of spleen and thymus in BALB/c mice (Kuttan 2000). Allyl sulfides have antigenotoxic potency through modulation of a drug-metabolizing enzyme. It is known that natural organosulfur compounds can inhibit Phase I enzymes (cytochrome $P450E_1$) and enhance detoxification process by activation Phase II enzymes (Yang et al. 1993). Diallyl sulfide modifies carcinogen detoxification enzymes after gastric intubation of rats. A single dose of DAS decrease hepatic CYP2E1 protein, increase hepatic CYP1A1 and CYP1A2 proteins levels (Devenport and Wargovich 2005). This compound is able to suppress proliferation of cancer cells by blocking cell cycle progression and by causing apoptosis. Epidemiological studies have shown an negative correlation between the consumption of allium vegetables and gastric cancer (Lau et al. 1990). This preventive effect of garlic has been reported in epidemiological studies from China (Khanum et al. 2004) and from certain areas in Italy (Buiatti et al. 1989). The oral consumption of garlic is effective in preventing mammary tumor in mice (Lau, 1990). The inhibition of diethylstilbestrol-induced DNA adducts by DAS may prevent the initiation of estrogen-induced cancer (Green et al. 2005). Wargovich and Goldberg (1985) described that DAS given orally can significantly reduce carcinogen - induced incidence of colorectal adenocarcinoma in mice colon.

The aim of the study was to investigate antimutagenic and immunomodulative effects

of diallyl sulfide (DAS) on genotoxicity and immunosuppression induced by carcinogenes aflatoxin B_1 (AFB_1), 2-amino-3-methylimidazo [4,5-f]quinoline (IQ), and N-nitroso-N-methylurea (MNU).

2. Methods

2.1. Chemicals

The following chemicals were used for both tests: AFB_1 (Alexis Corporation, USA), IQ (ICN Biomedicals, Inc., Germany), MNU (Sigma-Aldrich Co, Lousiana, USA), DAS (ICN Biomedicals, Inc., Germany). Chemicals were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co, Lousiana, USA).

2.2. Ames test

The Ames test was used for the evaluation of antimutagenic effect of DAS *in vitro*. The Ames test was performed with *Salmonella typhimurium* TA98, TA100, YG1021 and YG1024 strains (Maron and Ames 1983).

Mutagenic substances were used at the following concentrations: AFB_1 at concentrations of 10 μg, 1 μg and 0.1 μg per plate in both strains TA98 and TA100, IQ at concentrations of 0.1 μg, 0.01 μg and 0.001 μg per plate in the strain TA98, at concentrations of 10 μg, 1 μg and 0.1 μg in the strain TA100, MNU at concentrations of 1000 μg, 100 μg and 10 μg only in the strain TA100 as these concentrations had no effect in the strain TA98. Each concentration of each mutagen was combined with four different concentrations of antimutagen (300 μg, 30 μg, 3 μg, and 0.3 μg of DAS per plate). For metabolic activation the S9 fraction of liver homogenate from laboratory rats induced by a mixture of polychlorinated biphenyls Delor (Maron and Ames 1983) was used. Each combination of mutagen and antimutagen was tested in two separate experiments with three plates in each experiment.

Only one concentration of mutagen was chosen for tests with YG strains. 0.1 μg of IQ per plate and 10μg of AFB_1 per plate were combined with 30 and 300 μg of DAS per plate in the strains YG1021 and YG1024 . Tests with AFB_1 were performed also without metabolic activation, as this concentration of AFB_1 works also as a light direct mutagen.

Percentage of inhibition of mutagenicity was calculated according to the formula:

$$\frac{\text{No. of revertants of mutagen} - \text{No. of revertants of mixture of mutagen and DAS}}{\text{No. of revertants of mutagen}} \text{ x } 100$$

Student´s *t*-test was used for statistic evaluation.

2.3. Experimental animals

Male BALB/c male mice, ten weeks old, each weighting 22-25g were obtained from BIOTEST (Konarovice, CZ) and were allowed to acclimatize for at least one week. The mice were maintained on a 12-h light:dark cycle, temperature 20 ± 2°C, relative humidity of 60 ± 10% , complete air recirculation 10-14times per hour and were supplied with laboratory chow and water *ad libitum*. Mice were randomly distributed into groups of 5 mice/cage.

2.4. Substances tested

Following concentrations of mutagens were used for *in vivo* tests: AFB_1 1 mg/kg of body weight (b.w.), IQ 20 mg/kg b.w., MNU 50 mg/kg b.w. for micronucleus test and 20 mg/kg b.w. for chemiluminescence test. DAS was administered at the dose of 100 mg/kg b. w. to mice by the gavage on three consecutive days. Carcinogens were applied at one dose on the third day. Control mice were treated orally by a 7% solution of DMSO. All of the substances (diluted in DMSO) were administered in volumes of 100 μl/10 g b.w. Twenty-four hours after the last treatment the animals were killed by cervical dislocation.

2.4. Micronucleus test

The micronucleus test is aimed at the evaluation of the mutagenic (clastogenic) activity of chemical substances tested on an eucaryotic mammal model. The mouse bone marrow micronucleus test was carried out according to Schmid (1975). An increased frequency of micronuclei in polychromatophilic erythrocytes in comparison with control groups indicates that the substance tested induces chromosomal damage in nucleated erythrocytes in the bone marrow. The frequencies of micronucleated reticulocytes were determined by counting 1000 reticulocytes per mouse. Each experiment was run three times. For statistical analysis Student's t-test was used.

2.5. Chemiluminescence test

The influence of diallyl sulfide on the immune reaction of mutagen-treated mice, was studied by the chemiluminescence test (Thomas et al. 1983). In this test the degree to which phagocytes are capable

to liquidate ingested material was determined. The mechanisms in which hydrogen peroxide participates in killing are known (Klebanoff 1980). H_2O_2 is synthetized in phagocytes upon receiving signals by their membranes. The finding of a higher or lower activity of the complex H_2O_2-MPO-Cl^- (I^-) show us the readiness of the first defense line against bacteria, tumor cells as well as carcinogen-altered cells. Results are presented in maximum values of the chemiluminescence response of polymorphonuclear leucocytes in the dependence on time.

3. Results

3.1. Ames test

DAS at the dose 300 ug per plate significantly decreased IQ mutagenicity given at the doses of 0.1 μg, 0.01μg and 0.001μg per plate by 61%, 68% and 47%, respectively in the strain TA98, by 21%, 30%, and 26% in the strain TA100, and by 41% in the both transgenic strains YG1021 and YG1024. DAS at the dose 30 μg also reduced IQ mutagenicity but less markedly. DAS at this dose inhibited IQ mutagenicity in the strain TA98 by 31%, 27% and 24%, and by 10 to 23% in the strain TA100. Results are summarised in Table 1 and Table 2.

Antimutagenic effect of DAS on the activation-dependent mutagen AFB_1 was statistically significant in combination with 300 μg of DAS per plate and 10μg AFB_1 per plate as DAS inhibited the mutagenicity by 35%, in combination with 300μg and 30μg of DAS with 1μg AFB_1 per plate by 39% and 15% and in combination with 0.1μg AFB_1 by 42% and 32% in the strain TA98. In the present study DAS didn´t significantly modify the mutagenicity of AFB_1 in the strain TA100 (Table 3). In transgenic strains the effect was more pronounced, and both concentrations of DAS 300 and 30μg per plate were effective. DAS in concentration 300μg per plate reduced mutagenic activity of AFB_1 in the strain YG1021 by 57% and in YG1024 by 71%. In YG1021 antimutagenic action of the two highest concentrations of DAS was followed also against 10μg AFB_1 per plate without metabolic activation (Table 2).

DAS at the dose 300 μg per plate had antimutagenic effect on mutagenicity of direct mutagen MNU in the strain TA100. Other concentrations of DAS were not effective against any of tested concentrations of MNU (Table 4).

3.2. Micronucleus test

In the group of animals treated with AFB_1 a significantly higher number of micronuclei in reticulocytes was found as compared with control groups treated by DMSO only. DAS pretreatment at three consecutive doses of 100 mg/kg reduced mutagens-induced increase in a number of micronuclei markedly. The data presented here show that DAS is capable of inhibiting AFB_1, IQ and MNU induced micronucleated reticulocyte formation in mice. The results are presented in Table 5.

3.3. Chemiluminescence test

The chemiluminescence test followed up the relationship of chemiluminescence values with respect to time in each group of mice, and the maximum values in the curves which are presented in graphs. Between the group of mice treated with DAS alone and the control group no significant difference has been observed.

The effect of DAS (3x100 mg/kg) together with the effect of the three carcinogens in doses AFB_1 (1 mg/kg), IQ (20 mg/kg), and MNU (20 mg/kg) on the phagocytic function of murine peritoneal granulocytes, resulted in a significant reparation of suppression of chemiluminescence ($p < 0.01$) caused by all the carcinogens tested and has been found over the whole period of follow-up.

In the group of mice treated with the combination of DAS and AFB_1, values of chemiluminescence increased only in the day 13^{th} ($p< 0.05$) as compared with values of the control group. From the day 20^{th} those values did not differ significantly from the controls (Fig.1).

During the experiment with IQ, the chemiluminescence values of mice treated with DAS alone did not differ from controls significantly. Only in the day 6^{th} the chemiluminescence values increased ($p< 0.05$) and in the day 29^{th} decreased ($p< 0.05$) in comparison with the control values. The same result of the course of follow-up was observed even in the group of mice treated with DAS together with IQ (Fig. 2).

After administration of the combination of DAS altogether with MNU to mice the values of chemiluminescence increased in comparison with controls in days 6^{th} and 15^{th} only, and in days 27^{th} and 29^{th} the chemiluminescence values under DAS and MNU treatment decreased significantly ($p < 0.05$) below the values found in controls (Fig. 3).

4. Dicussion

Antimutagenic effect of DAS upon AFB_1 activity in the Ames test was described by Tadi et al. (1991). However, different works describe different action of DAS against various mutagens. Weak antimutagenic activity was described against 4-(methylnitrosamino)-1-(3pyridol)-1-butanon (Miller et al. 1994). Strong reduction of N-nitrosodimethylamin and weak reduction of AFB_1 mutagenicity was detected in the Ames test by Le Bon et al. (1997). The same authors also mentioned the reduction of single strand DNA breaks caused by AFB_1 but not MNU. DAS also decreased number of micronuceli induced by benzo(a)pyrene and cyclophosphamide (Shukla et al. 2003).

In our work, DAS in the Ames test revealed antimutagenic activity at the highest concentration 300 μg per plate against of all IQ concentrations, nevertheless only in the strain TA98. In the strain TA100 this effect was significant only in the DAS combination with 1 μg of IQ per plate. In summary, the effect of DAS was more pronounced in the strain TA98. The antimutagenic effect only of the highest concentration of DAS 300 μg per plate was the same in strains YG1021 and YG1024.

Similar effect, as to the combination DAS with IQ was also followed in combination of DAS with AFB_1. Significant antimutagenic effect was proved in the strain TA98 not in the TA100. Stronger effect of DAS on AFB_1 mutagenicity was seen in the transgenic strains with metabolic activation, where both concentrations tested were antimutagenic. This antimutagenic effect was even followed against AFB_1 without metabolic activation in the strain YG1021.

DAS was antimutagenic in the highest concentration against all of MNU doses. Anticlastogenic effect of three repeated doses of DAS on the mutagenicity of individual dose of mutagens AFB_1, IQ and MNU was also remarkable in the micronucleus test.

Different effects of DAS on the mutagenicity of various mutagens and carcinogens are explained by the different effects of DAS on the activity of CYP enzymes: weak induction of CYP1A, strong reduction of CYP2B, inhibition of CYP2E1 (Brady et al. 1988; Haber et al. 1995; Siess et al. 1997; Wargowich 2006). CYP modification by allylsulfides can lead to potentiation or reduction of mutagenicity of individual mutagens as was proved by the Ames test (Guyonet et al. 2000).

Potentiation of Phase II enzymes is probably a main mechanism of protective effect upon mutagenicity and carcinogenicity of allylsulfides (Wilkinson and Clapper 1997). In the Ames test this effect was proved (Guyonett et al. 2001). DAS has effect in the both phases of metabolisation of mutagens and carcinogens and the final effect is dependent on the balance between processes of activation and detoxification.

In our study, we proved antimutagenic effect of DAS on indirect mutagens in the Ames test, especially in the strains TA98 and in transgenic strains. DAS had also antimutagenic effect on direct mutagen MNU in the strain TA100 and against $AFB_{1,}$ without metabolic activation in the strain YG1021. It is clear that other mechanisms independent of mutagens metabolism are also involved in the antimutagenic effect. Hong et al. (2000) mentioned an effect of apoptosis by the increase of p53 induction in the cell culture of tumor cells.

The immunoprotective effect of DAS was assessed by the chemiluminescence method detecting the production of oxygen radicals, namely production of the complex of hydrogen peroxide-myeloperoxidase-halid cofactor during phagocytosis. The phagocytic function of murine peritoneal granulocytes was suppressed significantly by all three carcinogens tested. In all of the experiments, DAS decreased significantly the immunosuppressive effects of all three examined carcinogens. Results of this study indicate that DAS may play an important role in the prevention of carcinogenesis also by the capability to modulate immunosuppression caused by carcinogens. Suppression of antibody production and blastic transformation of T and B lymphocytes in mice treated with N-nitrosodimethyl-amine was described by Jeong and Lee (1998) and this suppression was repaired by DAS. Similarly Griffin et al. (2000) described protective effect of DAS in increase of blastic transformation of T lymphocytes in mice treated with trichlorethylen. DAS inhibited the production of activated cytokines, and such inhibition was associated with suppression of nitric oxide and prostaglandin E(2) production in stimulated macrophages (Chang et al. 2005).

Chemoprevention is one of the practical strategies how to reduce the risk of cancer. Results of our study indicate that DAS may play an important role in the prevention of carcinogenesis, because of a great variety of effects including antimutagenic and immunoprotective properties.

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Table 1: Effect of DAS on mutagenicity of IQ- Ames test (*S. typhimurium* TA98, TA100).

IQ + DAS	***S. typhimurium TA98* +S9**		**IQ + DAS**	***S. typhimurium TA100* +S9**	
dose (µg/plate)	revertants ± *SD*	**%**inhibition	**dose** (µg/plate)	revertants + *SD*	**%** inhibition
0.1 + 0	**1212** ± *167*		10 + 0	**1515** ± *320*	
0.1 + 0.3	**1179** ± *261*	**-3**	10 + 0.3	**1437** ± *467*	**-5**
0.1 + 3	**1076** ± *213*	**-11**	10 + 3	**1434** ± *397*	**-5**
0.1 + 30	**840** ± *169*♦	**-31**	10 + 30	**1295** ± *341*	**-15**
0.1 + 300	**467** ± *272*♦	**-61**	10 + 300	**1204** ± *181*	**-21**
0.01 + 0	**300** ± *57*		1 + 0	**871** ± *96*	
0.01 + 0.3	**245** ± *158*	**-18**	1 + 0.3	**794** ± *84*	**-9**
0.01 + 3	**247** ± *92*	**-18**	1 + 3	**681** ± *245*	**-22**
0.01 + 30	**220** ± *54*∗	**-27**	1 + 30	**667** ± *173*	**-23**
0.01 + 300	**97** ± *45*♦	**-68**	1 + 300	**607** ± *84*♦	**-30**
0.001 + 0	**93** ± *27*		0.1 + 0	**301** ± *71*	
0.001 + 0.3	**95** ± *20*	**+2**	0.1 + 0.3	**336** ± *84*	**+12**
0.001 + 3	**87** ± *35*	**-6**	0.1 + 3	**283** ± *40*	**-6**
0.001 + 30	**71** ± *13*	**-24**	0.1 + 30	**271** ± *72*	**-10**
0.001 + 300	**49** ± *12*♦	**-47**	0.1 + 300	**222** ± *75*	**-26**
Control(DMSO)	**30** ± *8*		Control(DMSO)	**111** ±*15*	
0 + 0.3	**29** ± *7*		0 + 0.3	**148** ±*25*	
0 + 3	**31** ± *14*		0 + 3	**178** ±*82*	
0 + 30	**29** ± *14*		0 + 30	**169** ±*79*	
0 + 300	**31** ± *13*		0 + 300	**127** ±*53*	

∗ : statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.05$

♦: statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.01$

SD: standard deviation

Table 2: Effect of DAS on mutagenicity of IQ and AFB_1 assessed by Ames test (*S. typhimurium* YG 1021, YG 1024).

Mutagen + DAS	dose (μg/plate)	*S. typhimurium* YG1021		*S. typhimurium* YG1024	
		revertants±***SD***	**%** inhibition	**revertants** ±***SD***	**%** inhibition
IQ +S9	0,1 + 0	**1189**±*98*		**1725**±*203*	
	0,1 + 30	**1086**±*72*	**-9**	**1543**±*186*	**-11**
	0,1 + 300	**696**±*321*∗	**-41**	**1023**±*463*∗	**-41**
+S9	control (DMSO)	**31**±*6*		**47**±*6*	
AFB_1 +S9	10 + 0	**1105**±*176*		**739**±*24*	
	10 + 30	**825** ± *200*∗	**-25**	**493**±*81*♦	**-33**
	10 + 300	**473** ± *143*♦	**-57**	**211**± *115*♦	**-71**
+S9	control (DMSO)	**26** ±*6*		**47**±*5*	
+S9	0 + 30	**36**±*1*		**52**±*8*	
	0 + 300	**28**±*1*		**54**±*5*	
+S9	control (DMSO)	**31**±*6*		**47** ±*6*	
AFB_1 -S9	10 + 0	**95**±*17*		**114**±*36*	
	10 + 30	**76**±*6*♦	**-20**	**131**±*31*	**+15**
	10 + 300	**71**± *12*♦	**-25**	**109**±*19*	**-4**
-S9	control (DMSO)	**21**±*6*		**30**±*6*	
-S9	0 + 30	**30**±*6*		**32**±*9*	
	0 + 300	**29**±*3*		**30**±*2*	
-S9	control (DMSO)	**26**±*7*		**30**±*2*	

∗ : statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.05$

♦: statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.01$

SD: standard deviation

Table 3: Effect of DAS on mutagenicity of AFB_1 assessed by Ames test (*S. typhimurium* TA98, TA100).

AFB_1 + DAS	***S. typhimurium TA98* +S9**		***S. typhimurium TA100* +S9**	
dose (μg/plate)	revertants ± *SD*	**%** inhibition	revertants ± *SD*	**%** inhibition
10 + 0	**1260** ± *77*		**1351** ±*211*	
10 + 0.3	**1277** ± *78*	**+1**	**1340** ±*225*	**-1**
10 + 3	**1298** ± *81*	**+3**	**1333** ±*226*	**-1**
10 + 30	**1272** ± *104*	**+1**	**1375** ±*229*	**+2**
10 + 300	**824** ± *275*∗	**-35**	**1371** ± *78*	**+2**
1 + 0	**606** ± *75*		**1095** ±*228*	
1 + 0.3	**556** ± *39*	**-8**	**1056** ±*310*	**-4**
1 + 3	**528** ± *110*	**-13**	**1006** ± *309*	**-8**
1 + 30	**518** ± *41*∗	**-15**	**1037** ± *315*	**-5**
1 + 300	**367** ± *136*♦	**-39**	**919** ± *246*	**-16**
0.1 + 0	**145** ± *39*		**473** ± *181*	
0.1 + 0.3	**154** ± *32*	**+6**	**421** ± *236*	**-11**
0.1 + 3	**132** ± *21*	**-9**	**425** ± *214*	**-10**
0.1 + 30	**99** ± *10*∗	**-32**	**404** ± *177*	**-15**
0.1 + 300	**84** ± *39*∗	**-42**	**351** ± *115*	**-26**
Control(DMSO)	**37** ± *6*		**110** ± *12*	
0 + 0.3	**34** ± *3*		**118** ± *7*	
0 + 3	**40** ± *1*		**127** ± *10*	
0 + 30	**35** ± *5*		**127** ± *24*	
0 + 300	**36** ±*4*		**114** ± *12*	

∗ : statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.05$

♦: statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.01$

SD: standard deviation

Table 4 : Effect of DAS on mutagenicity of MNU assessed by Ames test (*S. typhimurium* TA100).

MNU + DAS dose (µg/plate)	***S. typhimurium TA100*** revertants + *SD*	**%** inhibition
1000 + 0	**2749** ± *211*	
1000 + 0.3	**2722** ± *229*	**-1**
1000 + 3	**2670** ± *224*	**-3**
1000 + 30	**2669** ± *255*	**-3**
1000 + 300	**2164** ± *362*♦	**-21**
100 + 0	**2361** ± *277*	
100 + 0.3	**2242** ± *232*	**-5**
100 + 3	**2234** ± *202*	**-5**
100 + 30	**2242** ± *290*	**-5**
100 + 300	**1717** ± *244*♦	**-27**
10 + 0	**451** ± *51*	
10 + 0.3	**469** ± *82*	**+4**
10 + 3	**444** ± *78*	**-2**
10 + 30	**392** ± *40*	**-13**
10 + 300	**267** ± *49*♦	**-41**
Control (DMSO)	**146** ± *35*	
0 + 0.3	**167** ± *42*	
0 + 3	**178** ± *42*	
0 + 30	**175** ± *46*	
0 + 300	**173** ± *43*	

∗ : statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.05$

♦ : statistically significant difference of the sample with antimutagen and mutagen from the mutagen alone $p \leq 0.01$

SD: standard deviation

Table 5: Effect of DAS on mutagenicity of the mutagens AFB_1, IQ and MNU assesed by micronucleus test.

Substance studied	Dose	Frequency of micronuclei	SD
Control	**7% DMSO**	1.8	1.1
DAS	**3x 100 mg/kg**	2.6	0.8
AFB_1	**1 mg/kg**	9.1*	1.8
AFB_1 + DAS		3.0**	0.9
IQ	**20 mg/kg**	6.5*	1.6
IQ + DAS		3.4**	1.9
MNU	**50 mg/kg**	32.2*	4.4
MNU + DAS		15.4**	3.4

*): significantly decreased frequency of micronuclei as against the negative control (DMSO)

**): significantly decreased frequency of micronuclei as against mutagen alone

SD: standard deviation

Fig. 1: Chemiluminescence test – maximal values of chemiluminescence after DAS + AFB_1 administration

Fig 2: Chemiluminescence test – maximal values of chemiluminescence after DAS + IQ administration

Fig 3: Chemiluminescence test – maximal values of chemiluminescence after DAS + MNU administration