Reduction of genotoxic effects of N-nitrosomorpholine in human hepatoma cells and hamster lung cells by carboxymethyl chitin-glucan

D. SLAMENOVA[1], M. SRAMKOVA[1], I. CHALUPA[1], J. SMIGOVA[1], G. KOGAN[2*]

[1] Cancer Research Institute, Slovak Academy of Sciences, Vlárska 7, 833 91 Bratislava, Slovakia, exonslam@savba.sk; [2] Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia; [*] Present address: Directorate General Research, European Commission, B-1050, Brussels, Belgium

Received October 25, 2007

N-nitrosomorpholine (NMOR) belongs to the group of N-nitrosamines and represents a known hepatocarcinogen. Exposure to this compound is considered to be a potential health hazard to humans. There is, however, considerable evidence that the effect of many carcinogenic agents can be markedly influenced or altered by various natural substances. The objective of this study was to assess the DNA-protective and anticlastogenic effects of the derivative of a natural compound, carboxymethyl chitin-glucan (CM-CG), against genotoxicity of N-nitrosomorpholine (NMOR) in human hepatoma cells HepG2 and hamster lung cells V79 cultured *in vitro*. The exponentially growing cells were pre-treated during 24 h with three different concentrations of CM-CG (150, 750 and 1500 mg/ml) and then treated with different concentrations of NMOR. DNA-protective effects of CM-CG were evaluated by single-cell gel electrophoresis (SCGE, comet assay) and anticlastogenic effects by chromosomal aberration assay. At the SCGE assay a short-term (30 min) and at the chromosomal aberration assay a continuous treatment with NMOR was used. In both HepG2 and V79 cells pre-treated with CM-CG, a significant decrease of the percentage of DNA lesions induced by NMOR was observed along with a reduction of NMOR-induced chromosomal aberrations. We did not find any substantial differences between the genotoxic effects of NMOR on HepG2 and V79 cells, which have different histopathological origins and different levels of metabolizing enzymes. Three different concentrations of CM-CG exerted a similar protective effect against NMOR-induced DNA lesions and chromosomal aberrations in both HepG2 and V79 cells.

Key words: carboxymethyl chitin-glucan, HepG2 and V79 cells cultured in vitro, single cell gel electrophoresis, chromosomal aberrations, protective effects of glucans

N-nitroso compounds represent the most dangerous environmental mutagens and carcinogens related to food and nutrition, as their precursors nitrites and secondary amines are contained in many common foods. They are reactive under the acidic conditions of the stomach [23, 5]. Nitrites are generally used as inhibitors of bacterial contamination in meat products and they are also produced *in vivo* by reduction of nitrates by bacteria [11, 38]. The secondary amine morpholine is important as a chemical intermediate in the rubber industry. In the presence of nitrite, morpholine can be converted to NMOR both *in vitro* and *in vivo*. Alkylation of DNA is generally assumed to be the primary event in the carcinogenicity of nitrosamines and it is responsible for the overwhelming majority of NMOR-induced strand breaks in nuclear DNA [27] and mitochondrial DNA [14]. Robichová et al. [28] demonstrated that the fat-soluble vitamins A and E, which are dietary constituents, reduced the harmful effects of NMOR in human hepatoma cells HepG2. In this work we attempted to reduce the level of NMOR-induced DNA lesions and chromosomal aberrations in human HepG2 and hamster V79 cells by pre-incubation of the cells with the water-soluble derivative of a natural polysaccharide complex, CM-CG.

β-D-glucans containing (1→3)- and (1→6)-glycosidic linkages are the major skeletal component of the cell wall of yeasts and filamentous fungi. Their association with other skeletal components ensures rigidity of the cell wall and defines its morphology and stability against the environment. In the mycelia of the filamentous fungus *Aspergillus niger*, β-D-glucans create a covalently linked complex with chitin [34].

[*] Corresponding author

The content of chitin (a homopolymer of *N*-acetyl-(1→4)-β-D-glucosamine) in this complex represents approximately 30-40%. The chitin-glucan complex (CG complex) can be simply prepared from the waste material left during commercial production of chemical compounds by *Aspergillus niger* and represents a natural inexpensive source of a non-toxic protective and immunomodulating drug [36]. The structure of the CG complex was partially characterized by Stagg and Feather [34] and more recently by Machová et al. [19, 20]. It can be assumed that in the CG complex both components (chitin and glucan) may be involved in its protective effect [13]. Since the CG complex is water-insoluble, preparation of its water-soluble derivatives represents the crucial task that would enable its application with minimized adverse side effects. A procedure used most frequently for the preparation of the water-soluble derivatives of insoluble polysaccharides is carboxymethylation [30, 12]. Carboxymethyl chitin-glucan used in this study was prepared from the CG complex isolated from *Aspergillus niger* mycelium according to Machová et al. [19].

Materials and Methods

Cell lines. Human HepG2 hepatoma cells were obtained from A. R. Collins (University of Oslo, Oslo, Norway) and cultured at 37°C in humidified atmosphere of 5% CO_2 in William's medium supplemented with 10% fetal calf serum. Quasidiploid V79 Chinese hamster cells were obtained from A. Abbondanolo (National Institute for Cancer Research, Genoa, Italy). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g/l glucose and 10% fetal bovine serum. Antibiotics (penicillin 200 U/ml, streptomycin and kanamycin (100 μg/ml) were added to both types of media. All cell lines were cultured in glass or plastic Petri dishes or small glass vials in a CO_2 incubator at 37°C.

Chemicals. NMOR was prepared by nitrosation of morpholine. Morpholine (Slovak University of Technology, Bratislava, Slovakia) was kept at 4°C and diluted immediately before use in PBS (concentration of the stock solution was 0.2 ml/ml). The stock solution of sodium nitrite ($NaNO_2$, Lachema, Brno, Czech Republic) was prepared before use in PBS (100 mg/ml). The N-nitrosation reaction was carried out under standard conditions according to Brambilla et al. [3]. NMOR was determined according to Mirvish et al. [23]. The stock solution (10.2 mM) was kept at -20°C and diluted before use.

Carboxymethyl chitin-glucan (CM-CG) was prepared according to the procedure described by Machová et al. [19]. The degree of substitution of the product, determined by potentiometric titration, was 0.43 and its molecular weight, established by high performance liquid chromatography, was 220 kDa.

Single cell gel electrophoresis (SCGE; comet assay). The procedure of Singh et al. [31] was followed with minor modifications [33]. Briefly: the assayed cells were suspended in 0.75% LMP agarose and spread on a base layer (100 μl of 1.0% NMP agarose in Ca^{2+}- and Mg^{2+} -free PBS buffer) on a microscopic slide. When agarose solidified, the slides were placed in lysis mixture for 1 h at 4°C to remove cellular proteins. The slides were then transferred to an electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and kept in this solution for 40 min unwinding time at 4°C. A current of 25 V (300 mA) was then applied for 30 min. The slides were removed, neutralized with Tris-HCl (0.4 M, pH 7.5) and stained with 20 μl ethidium bromide (EtBr, 5 μg/ml). EtBr-stained nucleoids were evaluated with an Olympus fluorescence microscope. For each sample, 100 comets were scored by computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of DNA content in the tail, which is linearly related to the frequency of single strand DNA breaks (ss DNA breaks).

Chromosomal aberrations. Slides were prepared using the standard air-drying method and stained with 10% aqueous

Fig. 1a
HepG2 cells

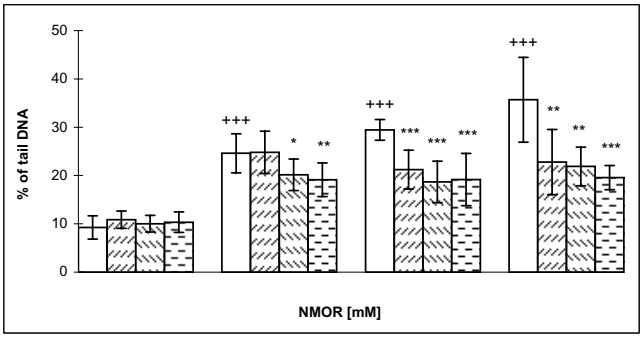
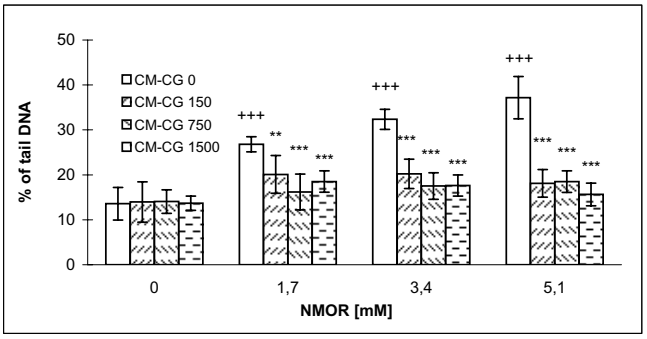


Fig. 1b
79 cells



Levels of DNA strand breaks in HepG2 (Fig. 1a) and V79 cells (Fig. 1b) treated with 0, 1.7, 3.4, and 5.1 mM NMOR (white columns) and in those pre-incubated with 150, 750, and 1500 μg/ml of CM-CG for 24 h (hatched columns).
+++ refers to differences between control (0) and NMOR 1.7, 3.4, and 5.1 mM
***, **, and *** refer to differences between samples with and without CM-CG pre-incubation. All values are expressed as mean ± SD of three independent experiments**

Table 1. Chromosomal aberrations induced by NMOR in HepG2 cells pre-incubated with CM-CG [ab]

Sample	Aberrant metaphases (%)	Number of chromosomal aberrations								Total number of CA
		Chromatid		Isochromatid		Exchange				
		g	b/f	g	b/f	ring	dic	qr	tr	
IC	7	4	5	-	-	-	2	-	-	7
SC	8	3	8	1	-	-	-	-	-	8
A	8	6	8	-	-	-	-	-	-	8
B	5	4	5	-	-	-	-	-	-	5
C	7	3	6	-	-	-	1	-	-	7
NMOR 1	31***	2	32	-	1	-	2	-	-	35***
NMOR 1 / A	25***	2	21	-	2	-	3	-	-	26***
NMOR 1 / B	12/+++	2	10	-	-	-	2	-	-	12/+++
NMOR 1 / C	8/+++	4	6	-	1	-	1	-	-	8/+++
NMOR 2	35***	5	43	-	4	-	1	-	-	48***
NMOR 2 / A	24**	5	23	1	2	-	1	-	-	26***/++
NMOR 2 / B	22**/+	1	29	2	2	-	-	-	-	31***/+
NMOR 2 / C	21**/+	7	21	-	1	-	2	-	-	24**/+++

[a] A number of 100 metaphases were scored in each sample. The number of chromatid and isochromatid gaps were recorded for each treatment group; however, since their genetic significance is not clearly understood, they are not included in our assessment of chromosomal damage.

[b] IC: intact control; SC: solvent control (PBS); NMOR 1: NMOR 0.5 mM; NMOR 2: NMOR 1.0 mM; A: CM CG 150 μg/ml; B: CM-CG 750 μg/ml; C: CM-CG 1500 μg/ml; CA: chromosomal aberrations; g: gap; b/f: break and/or fragment; dic: dicentric; qr: quadriradial; tr: triradial.

** Significantly different from the SC ($0.001 < P < 0.01$).

*** Significantly different from the SC ($P < 0.001$).

+ Significantly decreased from the NMOR-treated sample ($0.01 < P < 0.05$).

+ + Significantly decreased from the NMOR-treated sample ($0.001 < P < 0.01$).

+ + + Significantly decreased from the NMOR-treated sample ($P < 0.001$).

Giemsa solution. The classification of aberration was carried out as described by Venitt and Parry [37] and in the International System for Human Cytogenetic Nomenclature [10]. The metaphases were analyzed for the following structural aberrations: chromatid gaps and breaks, isochromatid gaps and breaks, and exchanges (dicentrics, double minutes, quadriradials, triradials, and rings). The results were statistically evaluated using the test of difference of two relative values. Since the genetic significance of gaps is not clearly understood, they were not included in the assessment of the chromosomal damage and thus were not evaluated statistically.

Results

Induction of DNA lesions and chromosomal aberrations in NMOR-treated human hepatoma cells HepG2 and hamster lung cells V79 have already been investigated and reported [28]. Pre-incubation of cells with CM-CG (24 h; concentrations 0, 150, 750, and 1500 mg/ml) manifested a preventive effect against oxidative DNA lesions induced in hamster lung cells V79 by H_2O_2 and visible light-excited Methylene Blue [32]. We used the same concentrations of NMOR and CM-CG and the same times of treatment as described in these papers.

Fig.1a demonstrates the DNA-protective effect of CM-CG on NMOR-treated HepG2 cells and Fig. 1b on NMOR-treated V79 cells. After 30 min treatment, NMOR in the used concentrations (1.7, 3.4, and 5.1 mM) increased the level of single strand (ss) DNA breaks in cellular DNA 2.66, 3.18, and 3.85 times in HepG2 cells (Fig.1a) and 1.97, 2.38, and 2.73 times in V79 cells (Fig. 1b) in comparison with untreated control. As evident from Fig. 1a, CM-CG reduced significantly the level of NMOR-induced ss DNA breaks mainly in HepG2 cells treated with higher concentrations of NMOR (3.4 and 5.1 mM). This effect was less pronounced in cells treated with the lowest NMOR concentration (1.7 mM). Fig 1b shows results obtained with V79 cells. Pre-incubation of cells with CM-CG (150, 750, and 1500 μg/ml) for 24 h led to a statistically significant reduction of NMOR-induced ss DNA breaks. Generally, the reduction of NMOR-induced ss DNA breaks by CM-CG was in both cell lines independent of the CM-CG concentration used (with the exception of HepG2 cells pre-incubated with 150 mg/ml of CM-CG and treated with 1.7 mM NMOR where no DNA-protective effect was observed).

Induction of chromosomal aberrations by NMOR and their reduction by three different concentrations of CM-CG is summarized in Table 1 (HepG2 cells; NMOR 0.5 and 1 mM) and Table 2 (V79 cells; NMOR 0.5 and 1mM). Continuous treatment, which equaled approximately 1.5 of the generation time of the respective cell line, was applied according to the recommendation of OECD [24]. This time was approximately 37 h for HepG2 and 22 h for V79 cells. The percentage of aberrant metaphases and total number of aberrations induced by two different concentrations of NMOR were compared with those observed in the intact control cells (IC), control cells treated with solvent (SC), cells pre-incubated with three different concentrations of CM-CG (CM-CG 150, CM-CG 750, CM-CG 1500), as

Table 2. Chromosomal aberrations induced by NMOR in V79 cells pre-incubated with CM-CG [ab]

Sample	Aberrant metaphases (%)	Number of chromosomal aberrations								Total number of CA
		Chromatid		Isochromatid		Exchange				
		g	b/f	g	b/f	ring	dic	qr	tr	
IC	5	2	3	-	1	-	1	-	-	5
SC	5	2	4	1	-	-	1	-	-	5
A	5	-	5	4	-	-	-	-	-	5
B	4	4	3	-	1	-	-	-	-	4
C	6	5	5	5	1	-	-	-	-	6
NMOR 1	34***	8	21	11	19	-	-	-	-	40***
NMOR 1 / A	17**/++	10	13	11	5	-	-	-	-	18**/+++
NMOR 1 / B	17**/++	14	15	30	4	-	-	-	-	19**/++
NMOR 1 / C	21***/+	3	11	35	10	-	-	-	1	22***/++
NMOR 2	45***	7	34	27	19	-	-	2	4	59***
NMOR 2 / A	13*/+++	4	8	19	5	-	-	-	-	13*/+++
NMOR 2 / B	18**/+++	13	11	37	8	-	1	1	-	21***/+++
NMOR 2 / C	19**/+++	19	5	52	17	-	-	-	-	22***/+++

[a] A number of 100 metaphases were scored in each sample. The number of chromatid and isochromatid gaps were recorded for each treatment group; however, since their genetic significance is not clearly understood, they are not included in our assessment of chromosomal damage.

[b] IC: intact control; SC: solvent control (PBS); NMOR 1: NMOR 0.5 mM; NMOR 2: NMOR 1.0 mM; A: CM CG 150 μg/ml; B: CM-CG 750 μg/ml; C: CM-CG 1500 μg/ml; CA: chromosomal aberrations; g: gap; b/f: break and/or fragment; dic: dicentric; qr: quadriradial; tr: triradial.

* Significantly different from the SC ($0.01 < P < 0.05$).

** Significantly different from the SC ($0.001 < P < 0.01$).

*** Significantly different from the SC ($P < 0.001$).

\+ Significantly decreased from the NMOR-treated sample ($0.01<P < 0.05$).

\+ + Significantly decreased from the NMOR-treated sample ($0.001<P < 0.01$).

\+ + + Significantly decreased from the NMOR-treated sample ($P < 0.001$).

well as with samples treated with NMOR after pre-incubation with three different concentrations of CM-CG (CM-CG 150/NMOR, CM-CG 750/NMOR, CM-CG 1500/NMOR). The number of chromosomal aberrations and aberrant metaphases induced by NMOR (0.5 and 1 mM) revealed a significant decline in HepG2 cells and V79 cells pre-incubated with CM-CG (150, 750, and 1500 mg/ml). Reduction of the clastogenic effect of NMOR was enhanced in HepG2 cells gradually with increasing concentrations of CM-CG; with the concentration of 1500 μg/ml being the most effective. In V79 cells, a similar dependence was not observed as all CM-CG concentrations tested manifested comparable anticlastogenic effects.

Discussion

(1→3)-β-D-glucan derivatives are known to be equipped with various properties important from the pharmacological point of view (e.g. ability to activate the host immune system, accelerate tissue repair and regeneration, tissues synergistic effect with antibiotics, antifungals and antiparasitics at combined administration) [35, 12]. As early as in 1995, Chorvatovičová and Šandula [8], using female ICR mice, demonstrated that both intraperitoneal and intravenous administration of CM-CG decreased the clastogenic effect of cyclophosphamide (CP) in peripheral blood, however oral pretreatment of mice with CM-CG prior to administration of CP did not reduce the level of micronucleated reticulocytes in peripheral blood. It was therefore conceivable that CM-CG failed to pass through the gastrointestinal tract. Later, Chorvatovičová et al. [9] and Machová et al. [21] found that ultrasonicated CM-CG with lower molecular mass administered either intraperitoneally or orally prior to CP injection significantly decreased the clastogenic effect of CP not only on intraperitoneal but also on oral administration. Under *in vitro* conditions, several authors [15, 32, 25; 1, 39, 26] investigated the antioxidative activity and antimutagenic effects of different water-soluble (1→3)-β-D-glucan derivatives (carboxymethyl glucan and sulfoethyl glucan prepared from the cell wall glucan of the baker's yeast *Saccharomyces cerevisiae;* CM-CG prepared from the CG complex isolated from filamentous fungus *Aspergillus niger;* as well as β-D-glucan extracted from barley). CM-CG exhibited a significant antimutagenic effect against the damage of chloroplast DNA of the flagellate *Euglena gracilis* induced by ofloxacin and acridine orange [15]. It revealed a very high antioxidative activity in the luminol-dependent photochemical method using trolox as a standard [15] as well as a DNA protective effect against oxidative DNA damage induced by H_2O_2 and visible light-excited Methylene Blue in V79 hamster lung cells cultured *in vitro* [32]. Using the single cell gel electrophoresis technique, Slameňová et al. [32] demonstrated that CM-CG exhibited protective effects against oxidative damage to DNA as a consequence of scavenging both •OH radicals and singlet oxygen. Significant results were obtained in the *ex vivo* studies aimed at induction of DNA lesions in freshly isolated Sprague-Dawley rat cells by different genotoxins and their

reduction by CM-CG applied as a dietary component [16, 17, 18]. It was demonstrated that compared to DNA of cells isolated from control rats, DNA of lymphocytes, hepatocytes, testicular cells, alveolar macrophages, and epithelial II cells of rats fed a CM-CG containing diet was significantly more resistant towards the oxidative agents (H_2O_2 and visible light-excited Methylene Blue), multiorgan carcinogen (benzo[a]pyrene), hepatocarcinogens (dimethyldibenzocarbazole and N-nitrosomorpholine) as well as to a complex mixture of organic compounds adsorbed on ambient air particles (TP-S). The results also indicated that diet enriched with CM-CG did not induce any negative effect on DNA nor did the mitotic indexes and the frequencies of necrotic and apoptotic cells differ statistically from the controls.

This study was focused on the ability of CM-CG to reduce the level of DNA lesions and chromosomal aberrations induced in human hepatoma cells HepG2 and hamster lung cells V79 cultured *in vitro* by a well-known hepatocarcinogen NMOR. This compound belongs to the group of carcinogenic nitrosamines, which are indirect-acting and require metabolic activation by nitroreductase. The activity of nitroreductase was detected in different mammalian cell lines including HepG2 and V79 cells, though the level of this enzyme was higher in hepatocytes than in V79 cells [22]. The activity of antioxidative enzymes such as superoxide dismutase, catalase and peroxidase, and the presence of glutathione-S-transferase was detected in both hepatocytes and V79 cells [22]. According to Appel and Graf [2] and Djuric [4] indirect-acting N-nitrosamines are activated by reduction of nitro groups to N-hydroxylamines. On the other, hand Fujita and Kamataki [6], using a Salmonella mutation test with genetically engineered *Salmonella typhimurium* YG7108 cells, suggested that cytochrome 2A6 (CYP 2A6) was responsible for the mutagenic activation of different N-nitrosamines, such as NMOR. CYP2A6 mRNA can be induced in estrogen receptor (ER)-positive HepG2 cells by treatment with estradiol [7]. As in our experiments no pre-treatment of HepG2 cells with estradiol was included and the native, non-transfected hamster cells V79 did not express any form of the human cytochrome P450 (CYP), we assume that reduction of NMOR by nitroreductase to DNA-reactive N-hydroxylamines may be responsible for both DNA-damaging (Figs.1a and 1b) and clastogenic (Tables 1 and 2) effects of NMOR on the cells studied. Results of fluorescent measurement of NMOR-treated colonic cells Caco-2 [29] indicated that NMOR might induce DNA damage not only indirectly, when activated by drug-metabolizing enzymes, but also via direct formation of reactive oxygen/nitrogen species (ROS/RNS), which could be responsible for a certain part of DNA lesions. By comparison of NMOR-induced DNA lesions in HepG2 cells (Fig. 1a) with induction of DNA lesions in V79 cells (Fig. 1b), it is evident that the level of NMOR-induced DNA strand breaks was rather similar in both cell types, and so was the total number of chromosomal aberrations per 100 scored metaphases, i.e. 35 (0.5 mM NMOR) and 48 (1 mM NMOR) in HepG2 cells (Table 1) and 40 (0.5 mM NMOR) and 59 (1 mM NMOR) in V79 cells (Table 2). The main aim of this work was to assess the protective activity of CM-CG against DNA lesions and chromosomal aberrations induced by NMOR. As follows from the results presented in the Figures and Tables, pre-treatment of cells with CM-CG significantly reduced both the level of NMOR-induced DNA strand breaks and chromosomal aberrations. With respect to the above-mentioned miscellaneous nature of DNA lesions induced in mammalian cells by NMOR (single and double nuclear and mitochondrial DNA strand breaks induced predominantly by alkylation and partially by induction of ROS/RNS species) a precise mechanism of the observed DNA-protective and anticlastogenic effect of CM-CG remains still unclear. It is however plausible to presume a combined effect of a free radical scavenging activity, adsorptive effect, and/or modulation of metabolism of N-nitrosomorpholine in NMOR-treated cells HepG2 and V79. Yet in any case it can be concluded that carboxymethyl chitin-glucan is able to protect DNA of human hepatoma cells HepG2 and hamster lung cells V79 against genotoxic effects of N-nitrosomorpholine.

The excellent technical assistance of Lívia Šebová and Alžbeta Vokáliková is appreciated. The experiments were supported by VEGA grants No. 2/3093/23, 2/7137/7 and 2/7033/7.

References

[1] ANGELI JP, RIBEIRO LR, GONZAGA ML et al.:Protective effects of beta-glucan extracted from *Agaricus brasiliensis* against chemically induced DNA damage in human lymphocytes. Cell Biol Toxicol 2006; 22: 285–291.

[2] APPEL KE, GRAF H. Metabolic nitrite formation from N-nitrosamines: evidence for a cytochrome P-450 dependent reaction. Carcinogenesis 1982; 3:293–296.

[3] BRAMBILLA G, CAJELLIE, FINOLLO R et al.: Formation of DNA-damaging nitroso compounds by interaction of drugs with nitrite, a preliminary screening for detecting potentially hazardous drugs. J Tox Environ Health 1985;15: 1–24.

[4] DJURIC Z. Comparative reduction of 1-nitro-3-nitrosopyrene and 1-nitro-6 nitrosopyrene: implications for the tumorigenicity of dinitropyrenes. Cancer Lett. 1992; 65:73–78.

[5] FERGUSON LR. Review: antimutagens as cancer chemopreventive agents in the diet. Mutat Res 1994; 307: 395–410.

[6] FUJITA K, KAMATAKI T. Predicting the mutagenicity of tobacco-related N-nitrosamines in humans using 11 strains of *Salmonella typhimurium YG7108*, each coexpressing a form of human cytochrome P450 along with NADPH-cytochrome P450 reductase. Environ Mol Mutagen. 2001; 38: 339–346.

[7] HIGASHI E, FUKAMI T, ITOH M et al. Human CYP2A6 Is Induced by Estrogen via Estrogen Receptor. Drug Metab Dispos 2007; 35: 1935–1941.

[8] CHORVATOVIČOVÁ D, ŠANDULA J. Effect of carboxymethyl-chitin-glucan on cyclophosphamide induced mutagenicity. Mutat Res 1995; 346: 43–48.

[9] CHORVATOVIČOVÁ D, MACHOVÁ E, ŠANDULA J. Ultrasonication: the way to achieve antimutagenic effect of

carboxymethyl-chitin-glucan by oral administration. Mutat Res 1998; 13: 83–89.

[10] ISCN An International System for Human Cytognetic Nomenclature. Cytogenet. Cell Genet 1978; 21: 309–404

[11] KITANO M, TAKADA N, CHEN T et al.:Carcinogenicity of methylurea or morpholine in combination with sodium nitrite in a rat multi-organ carcinogenesis bioassay. Jpn J Cancer Res 1997; 88: 797–806.

[12] KOGAN G. (1→3, 1→6)-β-D-glucans of yeast and fungi and their biological activity. In: Atta-ur-Rahman editor. Studies in Natural Products Chemistry. Bioactive Natural Products (Part DF). Elsevier, Amsterdam 2000; 23:107–152.

[13] KOGAN G, RAUKO P, MACHOVÁ E. Fungal chitin-glucan derivatives exert protective or damaging activity on plasmid DNA. Carbohydr Res 2003; 338:931–935.

[14] KORR H, BOTZEM B, SCHMITZ C et al.: N-Nitrosomorpholine induced alterations of unscheduled DNA synthesis, mitochondrial DNA synthesis and cell proliferation in different cell types of liver, kidney, and urogenital organs in the rat. Chem Biol Interact 2001;134: 217–233.

[15] KRIŽKOVÁ L, ĎURAČKOVÁ Z, ŠANDULA J et al. Fungal beta-(1-3)-D-glucan derivatives exhibit high antioxidative and antimutagenic activity in vitro. Anticancer Res 2003; 23: 2751–2756.

[16] LAZAROVÁ M, LÁBAJ J, KOVÁČIKOVÁ Z egt al.: Diet containing fungal (1→3)-beta-D-glucan derivative exhibits protective effects against DNA lesions induced in freshly isolated rat cells. Neoplasma 2004; 51: 431–435.

[17] LAZAROVÁ M, LÁBAJ J, KOGAN G,et al.:Carboxymethyl chitin-glucan enriched diet exhibits protective effects against oxidative DNA damage induced in freshly isolated rat cells. Neoplasma 2006; 53: 434–439.

[18] LAZAROVÁ M, LÁBAJ J, ECKL P et al.: Effects of dietary intake of a fungal beta-D-glucan derivative on the level of DNA damage induced in primary rat hepatocytes by various carcinogens. Nutr Cancer 2006; 56: 113–122.

[19] MACHOVÁ E, KOGAN G, ŠOLTÉS L et al.: Ultrasonic depolymerization of the chitin-glucan isolated from *Aspergillus niger*. React Funct Polym 1999; 42: 265–271.

[20] MACHOVÁ E, KVAPILOVÁ K, KOGAN G et al.: Effect of ultrasonic treatment on the molecular weight of carboxymethylated chitin-glucan complex from *Aspergillus niger*. Ultrason Sonochem 1999; 5: 169–172.

[21] MACHOVÁ E, KOGAN G, CHORVATOVIČOVÁ D et al.:Ultrasonic depolymerization of the chitin-glucan complex from *Aspergillus niger* and antimutagenic activity of its product. Ultrason Sonochem 1999; 6: 111–114.

[22] Mc GREGOR DB, EDWARDS I, WOLF CR et al.: Endogenous xenobiotic enzyme levels in mammalian cells. Mutat Res 1991; 26: 29–39.

[23] MIRVISH SS, WALLCAVE L, EAGEN M. et al.: Ascorbate-nitrite reaction: possible means of blocking the formation of carcinogenic N-nigtroso compounds, Science 1972; 177: 65–68.

[24] OECD, Guidelines for Testing of Chemicals. Genetic Toxicology 473, Organisation for Economic Co-operation and Development, Paris, 1983

[25] OLIVEIRA RJ, RIBEIRO LR, da SILVA Afet al.: Evaluation of antimutagenic activity and mechanisms of action of beta-glucan from barley, in CHO-k1 and HTC cell lines using the micronucleus test. Toxicol In Vitro 2006; 20:1225–1233.

[26] OLIVEIRA RJ, MATUO R, da SILVA AF et al.: Protective effect of beta-glucan extracted from *Saccharomyces cerevisiae,* against DNA damage and cytotoxicity in wild-type (k1) and repair-deficient (xrs5) CHO cells. Toxicol In Vitro 2007; 21:41–52.

[27] ROBICHOVÁ S, SLAMEŇOVÁ D. Study of N-nitrosomorpholine-induced DNA strand breaks in Caco-2 cells by the classical and modified comet assay: influence of vitamins E and C. Nutr Cancer 2001; 39: 267–272.

[28] ROBICHOVÁ S, SLAMEŇOVÁ D, CHALUPA et al.:DNA lesions and cytogenetic changes induced by N-nitrosomorpholine in HepG2, V79 and VH10 cells: the protective effects of Vitamins A, C and E. Mutat Res 2004; 560: 91–99.

[29] ROBICHOVÁ S, SLAMEŇOVÁ D, GÁBELOVÁ A et al.: An investigation of the genotoxic effects of N-nitrosomorpholine in mammalian cells. Chem Biol Interact 2004; 148: 163–171.

[30] SASAKI T, ABIKO N, NITTA K et al.:Antitumor activity of carboxymethylglucans obtained by carboxymethylation of (1 leads to 3)-beta-D-glucan from *Alcaligenes faecalis var*. myxogenes IFO 13140. Eur J Cancer 1979; 15: 211–215.

[31] SINGH NP, McCOY MT, TICE RR et al.: A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175: 184–191.

[32] SLAMEŇOVÁ D, LÁBAJ J, KRIŽKOVÁ L et al.: Protective effects of fungal (1—>3)-beta-D-glucan derivatives against oxidative DNA lesions in V79 hamster lung cells. Cancer Lett. 2003; 198:153-160.

[33] SLAMEŇOVÁ D, HORVÁTHOVÁ E, ŠRAMKOVÁ al.: DNA-protective effect of two components of essential plant oils carvacrol and trhymol on mammalian cells cultured in vitro. Neoplasma 2007; 54: 108–112.

[34] STAGG CM, FEATHER MS. The characterization of a chitin-associated D-glucan from the cell walls of *Aspergillus niger.* Biochim Biophys Acta 1973; 17: 64–72.

[35] STONE BA, CLARKE AE. Chemistry and biology of (1-3)-β-glucans. In: La Trobe University Press, Bundoora, Australia, 1992; 808 pp.

[36] ŠANDULA J, KOGAN G, KAČURÁKOVÁ M et al.: Microbial (1→3)-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity. Carbohydrate Polymers 1999; 38: 247–253

[37] VENITT S, PARRY JM. Mutagenicity testing "A Practical Approach". In: IRL Press, Oxford, Washington, DC, 1984; pp. 353.

[38] VERMEER ITM, MOONEN EJC, DALLINGA JW et al.: Effect of ascorbic acid and green tea on endogenous formation of N-nitrosodimethylamine and N-nitrosopiperidine in humans. Mutat. Res 1999; 428: 353–361.

[39] VLČKOVÁ V, NAĎOVÁ S, DÚHOVÁ V et al.: Natural microbial polysaccharide sulphoethyl glucan as antigenotoxic and cancer preventing agent. Neoplasma 2006; 53: 524–529.