

## **Antioxidative and immunostimulatory effect of natural clinoptilolite *in vivo***

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## Abstract

Many biochemical processes are closely related to ion exchange, adsorption and catalysis. Zeolites reversibly bind small molecules such as oxygen or nitric oxide; they possess size and shape selectivity, the possibility of metalloenzyme mimicry, and immunomodulatory activity. These properties make them interesting for pharmaceutical industry and medicine. Our *in vitro* experiments showed inhibition of tumor cell proliferation as well as micronized zeolite (MZ) to be the possible scavenger of 4-hydroxynonenal (HNE). MZ administered by gastric intubation to rats protected specifically stroma, but not tumor cells from oxidative stress caused by doxorubicin. It also significantly reduced the number of melanoma metastases in mice. In mice fed MZ lipid peroxidation in liver was decreased. The cells of lymph node of these mice provoked significantly higher allogeneic graft-*versus*-host (GVH) reaction than cells of control mice. After i.p. application of MZ, the number of peritoneal macrophages, as well as their production of oxide anion, was increased. However, NO generation was totally abolished. At the same time, translocation of NF $\kappa$ B p65 subunit in splenic cells was observed. Thus, here we report anticancer, antioxidative and immunostimulatory effect of MZ and we propose a possible mechanism of *in vivo* zeolite action.

**Key words:** micronized zeolite, clinoptilolite, scavenger, oxidative stress, antioxidative effect, immunostimulation, T-lymphocyte, NF $\kappa$ B

## Introduction

Zeolites are hydrated natural and synthetic microporous crystals with well-defined structures containing  $AlO_4$  and  $SiO_4$  tetrahedra linked through the common oxygen atoms [1]. Zeolites have properties to act as catalysts, ion-exchangers, adsorbents and detergent builders [2, 3, 4, 5, 6]. Except for being extensively used in different industrial applications it is known that silicates and aluminosilicates possess also either positive or negative biological activity. Well-defined structures and catalytic activity make aluminosilicates an attractive model system for protein and enzyme mimetics [7]. Recent results have demonstrated that it was very effective as a glucose adsorbent [8] as well as a potential adjuvant in anticancer therapy [9]. Zeolites reversibly bind small molecules such as oxygen or nitric oxide, they possess size and shape selectivity, the possibility of metalloenzyme mimicry, and immunomodulatory activity [10].

Accumulating evidence has indicated that zeolites play an important role in modulation of

the immune system. It was reported that silica, silicates and aluminosilicates act as non-specific immunostimulators similarly to superantigens [11]. Superantigens (SAG) are a class of immunostimulatory and disease-consisting proteins of bacterial and viral origin with the ability to activate relatively large fractions (5-20%) of the T cell population. Activation requires simultaneous interaction of the SAG with V $\beta$  domain of T cell complex (MHC) class II molecules on the surface of antigen presenting cells [11]. Pro-inflammatory macrophages, that belong to the class II MHC antigen presenting cells, are activated by fibrinogen silicate particulate [12, 13].

It was shown that exposure of some cells to silicate particles leads to activation of mitogen activated protein kinases (MAPK), protein kinase C and stress activated protein kinases (SAPK) [14]. Important transcription factors such as AP-1 and NF $\kappa$ B are also activated and expression of pro-inflammatory cytokines such as IL-1 $\alpha$ , IL-6 or TNF- $\alpha$  was enhanced [15]. Modifications of receptor activation kinetics or activity of integrins may be responsible for the observed behavior. Alternatively, particles engulfed by phagocytosis were shown to stimulate production of reactive oxygen species (ROIs) that have been found to be important second messengers for signal transduction in general [16]. Alterations in the redox homeostasis of cells may play an important role in modulating immune functions. For example, transmembrane redox signaling activates NF $\kappa$ B in macrophages and T lymphocytes [17, 18]. Nuclear factor kappa B (NF $\kappa$ B)/Rel proteins are dimeric, sequence-specific transcription factors involved in the activation of an exceptionally large number of genes in response to inflammation, viral and bacterial infections and other stressful situations requiring rapid reprogramming of gene expression.

Previous results showed that clinoptilolite treatment of mice and dogs suffering from various tumor types led to improvement of the overall health status, prolongation of life span, and decrease of tumor size in some cases. In addition, toxicology studies on mice and rats demonstrated that the same treatment did not have any negative effect [9]. *In vitro* tissue culture studies showed that finely ground clinoptilolite inhibited protein kinase B (c-Akt), induced expression of *p21<sup>WAF1/CIP1</sup>* and *p27<sup>KIP1</sup>* tumour suppressor proteins, and blocked cell proliferation in several cancer cell lines. Furthermore, tribomechanically activated clinoptilolite increased total antioxidant status of patients with malignant diseases as well as healthy individuals (Ivković S, Žabčić D, personal communication). Here we present some new *in vitro* evidence for antitumour activity of clinoptilolite as well as possible mechanism of *in vivo* zeolite action. Also, we report a novel use of clinoptilolite as a potential antioxidant and stimulant of immunological response.

## Materials and Methods

*Natural clinoptilolite.* A fine powder of natural clinoptilolites (MZ; micronized zeolite) from Slovakia was obtained by tribomechanical micronization. Particle size distribution curves of the MZ were taken by a Mastersize XLB (Malvern) laser light-scattering particle size analyzer. Tribomechanically treated natural clinoptilolite contained approximately 80 wt % clinoptilolite. The remaining 20% consisted of silica, montmorillonite and mordenite zeolites. Chemical composition of clinoptilolite was SiO<sub>2</sub> 70.06%, Al<sub>2</sub>O<sub>3</sub> 12.32%, Fe<sub>2</sub>O<sub>3</sub> 1.48%, CaO 3.42% MgO 0.96%, TiO<sub>2</sub> 0.71%, P<sub>2</sub>O<sub>5</sub> 0.05%, MnO 0.02%, Na<sub>2</sub>O 0.68%, K<sub>2</sub>O 2.38%, SO<sub>3</sub> 0.17%, H<sub>2</sub>O 7.3%. Humidity at 105°C was max. 6%, pH 6.9-7.1, specific mass 2.39 g/cm<sup>3</sup>, specific area 360-390 m<sup>2</sup>/g and NH<sub>4</sub><sup>+</sup> substitution capacity 8500 mg NH<sub>4</sub><sup>+</sup>/kg. Particle size analysis of the clinoptilolite showed that maximum frequency of particles appeared at 1 µm.

*Animals and treatment.* The following animals were used: 6-month-old male Wistar rats bearing Walker carcinoma W256, weighting 308 ± 25 g, were treated with MZ (in a single dose of 2g/kg, by gastric intubation). C57Bl/6 4-month-old male mice bearing lung metastases were used for antimetastatic effect of MZ. Healthy CBA mice, 3.5-month-old females, were fed (12.5% or 25% MZ) over 28 days and used for oxidative stress parameters measurement. These mice were afterwards donors of lymph node cells that were tested on RFM mice for cellular immune response. CBA mice bearing mammary carcinoma were fed 14 days before tumor cell (1x10<sup>5</sup> cells/mouse) injections into the right hinder leg and were fed for additional 28 days. Three-month-old male RFM mice were used for i.p. administration of MZ (3 mg/0.3 ml/mouse). Control mice were injected with Hank's solution.

Mice were bred in the Animal Facility of the Ruđer Bošković Institute. Food (Domžale, Slovenia) and tap water were given *ad libitum*. Animals were kept in conventional circumstances: slight dark rhythms 12/12 hours, temperature 22°C and humidity 55%.

*In vitro assay for tumor growth.* Effect of MZ on *in vitro* cell proliferation was studied on several human cell lines: MiaPaCa-2 (pancreatic carcinoma), HeLa (cervical carcinoma), CaCo-2 (colon carcinoma), MCF-7 (breast cancer) and WI 38 (diploid fibroblasts). The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma, USA) in standard conditions. Thirty thousand cells/ml were plated in standard medium onto 96 microwell plates (200 µl/well) (Greiner, Germany). After overnight incubation the medium was replaced with the fresh DMEM containing different concentrations (0.05-0.5 mg/ml) of MZ. After 72 hours of incubation the cells were washed with PBS in order to remove MZ. The cell viability was determined using MTT assay, which detects dehydrogenase activity in viable cell [9].

Statistical analysis was performed using One-way ANOVA test ( $p < 0.05$ ).

*MTT Assay for interference with 4-hydroxynonenal (HNE).* HeLa cells were cultured under standard conditions in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; Sigma). The cells were seeded at  $2 \times 10^4$ /well density into the plastic 96-microwell plates (Greiner). They were incubated for 2 hours before treatment with either plain medium, MZ-pre-treated medium (0.5  $\mu\text{g/ml}$ ), medium with plasma at final concentration of 1% and 10% (in saline) and HNE (10  $\mu\text{M}$ ) that was added 30 minutes after pre-treatment of medium, or different combinations of already mentioned components. Concentration of HNE (10  $\mu\text{M}$ ) corresponds to the aldehyde values generated during oxidative stress *in vivo*. The experiment was performed in triplicate. After further 24h incubation the viability of cells (metabolic activity) was measured using MTT assay. Substrate (10  $\mu\text{l}$ ) was added per well and the cells were further incubated for 2 hours when intensity of colored reaction was determined with plate reader (Anthos, Germany) at 450 nm wavelength (with 620 nm as reference).

*Chemical induction of oxidative stress by doxorubicin in vivo.* Rats were divided into four groups, each comprising 4-6 animals: 1) control tumor-bearing rats; 2) tumor-bearing rats treated with MZ p.o.; 3) tumor-bearing rats treated with doxorubicin i.p. 4) tumor-bearing rats treated with MZ p.o. and doxorubicin i.p. Walker carcinoma tumor cells ( $10^7$  live cells/rat) were injected i.m. in the hind limb of male Wistar rats. MZ was applied six days later, when tumors developed to 3-4 cm diameter on the average. One-hour later rats were injected i.p. with doxorubicin (Sigma) at 10 mg/kg dose to induce oxidative stress. After three hours, animals were killed, tumors were removed and stored in 10% buffered formalin to be used for immunohistochemistry of HNE-protein adducts in tumour tissue.

*Immunohistochemistry of HNE-protein adducts in W256 carcinoma tissue.* Tissue sections (5  $\mu$ ) of the formalin-fixed paraffin-embedded tumors were used. For immunohistochemical detection (with peroxidase-antiperoxidase method) of HNE-protein adducts, we used specific monoclonal antibodies raised against HNE-histidine conjugate, as described before [19]. Sections were pre-treated with normal rabbit serum and  $\text{H}_2\text{O}_2$  using 1% BSA solution as scavenger for slide washing (three times). Secondary rabbit-anti-mouse antibodies were obtained from Dako, USA.

*Evaluation of antimetastatic effect of MZ.* Ten mice (C57Bl/6) were injected i.v. with  $7.5 \times 10^4$  melanoma B16 cells. For the next 16 days, they were treated daily with MZ (100 mg/ml distilled  $\text{H}_2\text{O}$  per mouse) by gastric intubation. Controls (6 mice) were intubated daily

with distilled H<sub>2</sub>O. Mice were killed and lungs were removed and fixed in Bouen. Metastases were counted and statistical analysis was performed by Student's *t*-test.

*Isolation of peritoneal macrophages.* Peritoneal macrophages were aseptically collected from the peritoneal cavities of mice 24 hours after i.p, or 7, 14, 21 and 28 days after *per os* administration of MZ. Macrophages were resuspended in RPMI 1640 (without phenol red; Sigma) and erythrocytes were removed by NH<sub>4</sub>Cl lysis. The remaining cells were washed three times, suspended in RPMI 1640 supplemented with antibiotics and 10% fetal calf serum (FCS; Sigma) and adjusted to 2 x 10<sup>6</sup> cells/ml.

*Assay for superoxide anion (O<sub>2</sub><sup>-</sup>) release.* In macrophages, superoxide release was measured as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C using a modification of the method of Johnston et al [20]. Samples contained 1 ml of cytochrome C (1 mg/ml) in phenol-free Hank's balanced salt solution and 2 x 10<sup>6</sup> cells in 100 µl of medium. The specificity of the reaction was tested by the addition of 60 IU SOD per ml of the reaction mixture. The reactivity of the cells was tested by the addition of cytochrome C in phenol-free Hank's solution for 30 min at 37°C. After incubation, the reaction mixture was centrifuged for 5 min at 800xg, and the absorbance of the supernatant was determined spectrophotometrically at 550 nm. The concentration of reduced cytochrome C was calculated using the formula  $E_{550\text{nm}} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . Experiments were performed in duplicate and the results were expressed as nmol O<sub>2</sub><sup>-</sup> (10<sup>6</sup> cells)<sup>-1</sup> (30 min)<sup>-1</sup>.

*Measurement of nitrite production.* The measurement of nitric oxide (NO) from macrophages was assayed according to Naslund et al [21]. Briefly, cultures of isolated peritoneal macrophages were incubated in plastic 24-well flat-bottom microplates (Falcon, USA) for 48 hours at 37°C and 5% CO<sub>2</sub>. Aliquots (800 µl) of each supernatant were placed in tubes and mixed with 800 µl of GRIESS reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.5% naphthylethylenediamine in 2.5% phosphoric acid; 1:1). The resulting colorimetric reaction was measured spectrophotometrically at 540 nm. Nitrite concentration was calculated from a standard curve using sodium nitrite (0-100 µM) as standard.

*Measurement of lipid-bound sialic acid (LSA) in serum, total sialic acid (TSA) in spleen and assay for lipid peroxidation (LPO) in liver.* After exanguination, sera from fed mice were collected and prepared for LSA measurement according to Katopodis et al [22]. The spleen and liver were removed from i.p. and *per os* treated mice. Concentration of TSA in the spleen was determined according to Hadžija et al [23] and expressed as mg/10<sup>6</sup> spleen cells. Lipid peroxidation (LPO) was estimated according to the presence of thiobarbituric acid reactive

substances (TBARS) in the liver as reported by Ohkawa et al [24]. Protein concentration was measured by the method of Lowry, using bovine serum albumin (BSA; Sigma) as standard.

*Local allogeneic graft versus host reaction.* A modified version of LXGVHR described by Shohat and Trainin [25] was used. In our experiment, LAGVHR was done on allegoric mice instead of rats. For each experiment, 10 control mice (fed conventional food) and 10 mice in each experimental group (mice fed 12.5% or 25% MZ, during period of 21 or 28 days) were used. Mice were anesthetized and killed by exsanguination. Pooled lymphocytes from lymph nodes of 3 to 5 treated or control mice were washed two times with Hank's by centrifugation. Cells of lymph nodes ( $2 \times 10^7$ ) were injected intradermally into the shaved abdominal skin of RFM mice (irradiated with 7Gy, 24 hours before) where they provoked the GVH reaction and damage of skin. On day 5, the treated mice were injected intravenously with 0.4 ml of 0.5 % Evans blue. Five hours later the entire abdominal skin was excised and two perpendicular diameters of the blue stained area were measured with a caliper. A mean diameter of each area was calculated. Statistical analysis was performed by the Student's *t*-test.

*NFκB activation in spleen.* Twenty-four hours after i.p. injection of MZ into experimental, and Hank's solution into control mice, animals were killed. For preparation of cytoplasmic and nuclear fractions, spleens were isolated and crude splenic extracts prepared. Erythrocytes were removed by ammonium chloride lysis. The nuclear and cytosolic fractionation procedure was a modification of the protocol of Lernbecher et al [26]. Cells were washed twice with phosphate-buffered saline without calcium and magnesium and resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM PMSF). After lysis on ice for 60 minutes, nuclei were spun down, and the supernatant, after additional centrifugation at 17500 g, was stored as the cytoplasmic fraction. The nuclear pellet was suspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol), vortexed, and incubated on ice for 45 minutes. Centrifugation at 17500 g was performed to remove insoluble debris. The supernatant was used as nuclear extract.

*Western blot (immunoblot) analysis.* Concentrations of proteins in nuclear and cytoplasm fractions were determined by Bradford assay. Equal amounts of nuclear and cytoplasmic protein lysates (20 μg and 80 μg respectively) were separated by 9% - SDS PAGE. Proteins were transferred onto PVDF membrane (Immobilon-P, Millipore). Comparable loading of each protein sample was checked by Ponceau S and Commassie blue staining. Membranes were blocked overnight with TBS/2.5% BSA at 4°C. After that, they were incubated for 90 minutes with primary antibodies (anti-p50, anti-RelB, and anti-p65), washed in TBS/0.05% Triton X-100, and

then incubated for 1 hour with appropriate secondary antibody. Following further washes, immunoblots were visualized using enhanced chemiluminescence reagent (POD; Boehringer-Mannheim, Germany). For immunoblots, polyclonal antibodies against p50 and RelB (Santa Cruz<sup>TM</sup>, USA) and monoclonal antibody against p65 (Transduction Laboratories, USA) were used. Secondary antibodies were peroxide-conjugated rabbit anti-mouse immunoglobulin (Amersham/Pharmacia, Sweden) and peroxide-conjugated protein A from Kierkegaard and Perry Laboratories.

## Results

*Effect of MZ on tumor cell growth in vitro.* The results, showing cell growth inhibition of HeLa, MiaPaCa-2, CaCo-2, MCF-7 and WI 38 cells, are presented in Figure 1. The growth of all tumor cell lines was significantly inhibited. The strongest and dose-dependent inhibition was observed on HeLa and MiaPaCa-2 cells (30-50% and 15-65%, respectively). CaCo-2 and MCF-7 cells were inhibited only at the highest MZ concentration. The growth of WI 38 normal (diploid) fibroblast cell line was even stimulated (about 20%).

*Effect of MZ on 4-hydroxynonenal.* The results of the experiment evaluating influence of MZ on the biological effects of HNE *in vitro* are presented in Table 1. MZ increased MTT values for the cells cultured in the absence of plasma ( $p < 0.05$ ), but not if the cells were cultured also with HNE.

MZ did not influence stimulation of the cell growth caused by 1% plasma addition. On the other hand, in the presence of 1% plasma it abolished inhibiting effects of HNE ( $p < 0.05$ ). In the presence of 10% plasma neither MZ nor HNE showed any effects.

*Effects of MZ on chemically induced oxidative stress of W256 carcinoma in vivo.* Results of pathohistological evaluation of W256 carcinoma treated either with doxorubicin or MZ, or combined treatment are summarized in Table 2 and presented in Figure 2.

Although there were no particular differences in general appearance of differently treated tumors, doxorubicin induced prominent lipid peroxidation (generation of HNE-protein conjugates), and both in malignant cells as well as in normal stromal components of tumor tissue. MZ itself did not change immunohistochemical appearance of 4-hydroxynonenal (HNE-protein conjugates) compared to untreated tumour-bearing rats. However, it had selective, tissue specific effects on doxorubicin-induced oxidative stress (lipid peroxidation within tumour). Namely, when MZ was applied, it did not influence doxorubicin-induced oxidative stress (HNE-protein conjugates) in malignant cells, while it completely prevented formation of HNE-protein



conjugates in tumor stroma. Specificity of the antioxidant effects of MZ for tumor stroma was also supported by the finding that MZ did not influence the presence of HNE-protein conjugates in necrotic tumor tissue.

*Antimetastatic effect of MZ.* MZ strongly reduced the number of lung metastasis in treated mice. While control mice had  $5.2 \pm 1.64$ , treated mice had  $0.7 \pm 1.06$  metastasis. Statistical significance was  $p < 0.001$ .

*Influence of MZ on macrophage  $O_2^-$  production, lipid peroxidation (LPO) in liver and lipid-bound sialic acid (LSA) in serum.* The results are shown in Table 3. Concentration of  $O_2^-$  (in peritoneal macrophages) started to change slightly 14 days after administration of 12.5% MZ. However, TBARS concentration (in liver) started to change significantly after 21 days regardless of MZ concentration. Significant differences regarding concentration of LSA (in serum) was obtained with 12.5% MZ on day 21 and 25% MZ on day 28.

In the experiment with mammary carcinoma, CBA mice were divided into four groups: 1) control; 2) tumor-bearing mice (T); 3) tumor-bearing mice fed 12.5% MZ and 4) tumor-bearing mice fed 25% MZ. Each group consisted of 16 mice. Neither 12.5 nor 25% MZ reduced tumor volume during 28 days after tumour injection. Concentration of TBARS (measured in 5 of survived tumor-bearing mice) was significantly increased compared to healthy control. However, administration of 25% MZ for 28 days decreased TBARS to the control value (Figure 3A). LSA concentration was significantly increased in tumor-bearing mice compared to controls and MZ did not influence it additionally (Figure 3B).

*Effect of MZ on local allogeneic graft versus host reaction.* Results of two separately prepared experiments with healthy mice 21 and 28 days after administration are shown in Figure 4. The cells of lymph node of mice fed 28 days with 25% MZ provoked significantly higher GVH reaction than cells of control group mice. A treatment with lower dose (12,5%) of MZ for 21 or 28 days showed also the higher reaction than control group, but was not significant.

*Effect of i.p. administration of MZ on peritoneal macrophages, ROIs generation and oxidative stress (OS) parameters.* Intraperitoneal administration of MZ at doses higher than 3 mg was lethal for mice and dose of 3 mg was sublethal but proinflammatory (data not shown). In our experiments, dose of 3 mg was used and the number of macrophages, production of  $O_2^-$  and NO as well as measurement TSA in spleen and TBARS concentration in liver was performed 24 hours after intraperitoneal injection of MZ.

MZ provoked accumulation of macrophages in peritoneum. The number of peritoneal macrophages (PM) after treatment was 7 times higher than in control mice (Figure 5A). The

concentration of  $O_2^-$  was 10 times higher in macrophages of treated mice than in controls (Figure 5B). Since  $O_2^-$  release was calculated to  $10^6$  cells, the increased release was not the result of increased number of macrophages, but represents truly increased activity. Production of NO by peritoneal macrophages isolated from treated mice, and cultivated for another 24 hours *ex vivo*, was strongly decreased (Figure 5C). There was no change in liver TBARS concentrations (expressed in nmol/mg protein) between control ( $0.907 \pm 0.17$ ) and treated ( $0.886 \pm 0.16$ ) group. Also, TSA concentration (expressed as  $\mu\text{g}/10^6$  splenocytes) was not changed after treatment with MZ. The value of control group was  $5.25 \pm 0.73$ , and  $5.58 \pm 0.78$  for treated group.

*Effect of MZ on NF $\kappa$ B/Rel proteins.* The spleens of MZ treated animals were slightly (11%) heavier than spleens of control animals (data not shown). Effect of MZ on NF $\kappa$ B/Rel proteins could be seen after preparation of cytoplasm and nuclear fractions, electrophoresis and Western blotting. MZ treatment increased translocation of p65 subunit into nucleus (Figure 6) suggesting that NF $\kappa$ B containing p65 subunit had been activated. However, MZ treatment did not have any effect on RelB or p50 subunit concerning translocation into the nucleus. However, total amount of RelB subunit was increased by about 40% and total amount of p50 was slightly decreased compared to controls.

## Discussion

*Antitumour effect of MZ in vitro.* Previous *in vitro* and *in vivo* animal studies showed that MZ was a non-toxic compound that acted as an adjuvant in anticancer treatment [9]. Previous *in vivo* results showed diverse effects, ranging from no antitumour response, to normalization of biochemical parameters and prolongation of life span.

Our previous *in vitro* experiments [9] showed 30-50% inhibition of proliferation (by MTT as well as  $^3\text{H}$ -thymidine test) of several cell lines after incubation with MZ-pre-treated medium. Now we used another approach: instead of MZ-pre-treated medium, we added MZ directly to the culture medium. The cell proliferation was also inhibited in a dose-dependent manner. The best and the most consistent results were obtained with HeLa and MiaPaCa-2. However, the proliferation of WI 38 cell line was even slightly stimulated by MZ. According to these experiments, our conclusion is that MZ response was cell line dependent. We previously analyzed mitogenic and survival signaling pathways in tumor cells [9]. The most significant results were observed in the activity of Akt protein that was highly inhibited after MZ treatment of cancer cells. That resulted in growth inhibition and increase in apoptosis of cancer cells, but only in the presence of serum. We suppose that the same mechanism could be responsible for

results obtained with direct application of MZ. However, since MZ was constantly present in the medium, it probably enabled lower concentrations of MZ to have the same (or even better) inhibitory effects on tumor cells.

Absorption of serum components, at least *in vitro*, could be one of the mechanisms of MZ action. In favor of this assumption are also preliminary findings of an increased HNE-protein conjugate formation in the presence of MZ (data not presented). MZ might further act as HNE-scavenger but HNE binding to MZ might also affect activity of MZ. HNE has high affinity of binding to proteins so plasma (serum) or albumin can attenuate its toxicity. HNE can also act as a bifunctional growth regulating factor, probably by physical or biological interference of the aldehyde and MZ as well as interfering with the activity of humoral growth factors [27]. This might be also relevant for its *in vivo* effects.

Since our *in vitro* results referred to indirect effect on tumor cell lines, orally administrated MZ is not absorbed into blood, and that which does get into the body will be phagocytized by macrophages, the MZ effect *in vivo* cannot be due to a direct biochemical interaction. We speculate that MZ may induce certain immunological responses. To try to reveal a possible mechanism of MZ *in vivo* effect, we used different ways of application of MZ either to healthy or tumor-bearing mice as well as to tumor-bearing rats.

*Antioxidative effect of MZ in vivo.* Cancer and inflammation can be considered as interfering processes that share two common pathophysiological mechanisms: cytokine network and oxidative stress. Both of these processes involve lipid peroxidation and are linked by tumour stroma. TNF- $\alpha$  and TGF- $\beta$  are among cytokines that are likely to be mediators of this complex network system, those interfering with lipid peroxidation and consequently biological effects of HNE (known as a second messenger of free radicals). Growth modulating effects of HNE involve signaling pathways affecting *c-fos* expression and might interfere with activity of EGF and PDGF [28]. HNE as second messenger of radical oxygen intermediates (ROIs) activates AP-1, followed by further TGF- $\beta$  synthesis and fibrogenesis (spread of connective tissue). Moreover, low-density lipoprotein modified by lipid peroxidation (oxLDL) is known as a potent cytotoxic, immunogenic and pro-inflammatory factor, which activates production of cytokines by stromal cells (fibroblasts, endothelium, and macrophages). This leads to further oxidative stress and spread of inflammation associated by further lipid peroxidation and production of HNE [29]. Doxorubicin is a very potent inducer of hydroxyl radical and consequential lipid peroxidation (resulting in HNE production), the effects of which could be demonstrated *in vivo* already half an hour after administration [30, 31]. Thus, we assume that applying MZ in water by gastric

intubation could in a relatively short period of time (several hours as done in experiment presented) provoke response of the gastrointestinal system, which might further induce a systemic response. Regarding specificity of MZ antioxidant effect on stromal cells in doxorubicin-treated W256 carcinoma, we assume that differential response of malignant and normal cells to antioxidative effects of MZ could be due to: 1) different patterns of oxygen metabolism between these cells (internal oxidative stress), 2) the difference in their response to cytokines involved in determination of tumor host relationship, 3) systemic response to MZ application and doxorubicin-induced oxidative stress.

Tumor cells have an abnormal lipid composition and level of enzymes of the cytochrome P450 system which can initiate and propagate LPO and thus may cause alterations in LPO level [32]. In our experiment, the statistically increased LPO in mice with mammary carcinoma was abolished in animals that were fed a diet supplemented with 25% of MZ. According to some data, the level of oxyradical scavenging enzymes is reduced in tumor cells [33]. Thus, the reduced LPO level in MZ treated tumor-bearing mice might be the result of the MZ antioxidant potential. These data show, to the best of our knowledge, the first time this effect on lipid peroxidation.

*Immunostimulatory effect of MZ in vivo.* Immunostimulators are associated with an increase of serum sialic acid level [34]. Sialic acid also may be a useful indicator for the diagnosis and staging of malignancy, and it may have a regulatory role in immunological processes, particularly masking the antigen site and being a marker for inflammation. The data presented here demonstrated that application of MZ increased the serum LSA concentration in healthy mice, which is probably associated with inflammatory process, i.e. activation of macrophages. This was confirmed by our data of elevated  $O_2^-$  in MZ fed mice. We suspect that factors activating and influencing the proliferation or increasing the synthetic capacity of the phagocyte system might cause a change in the serum LSA level. It is also possible that macrophages participate in this process indirectly by releasing  $TNF-\alpha$  and interleukin-1 or is connected with an elevation of other acute phase proteins.

Although parenteral route of application is not suitable, to confirm immunomodulatory effect of MZ we examined the processes that followed MZ intraperitoneal application. For that purpose, different amounts of MZ were injected in normal, healthy mice. It was shown that effect of MZ was dose dependent. Doses higher than 3mg/mouse were lethal. However, since lower doses (3mg/mouse) had non-toxic, but proinflammatory effect, some immunological parameters were measured. In the acute phase of an inflammatory process, large number of

polymorphonuclear leukocytes (PMNs) migrates from the blood and accumulates in the exudate [35]. In our experiments, 24 hours after MZ administration, high accumulation of macrophages was found in the peritoneum of treated animals. Our results confirmed that macrophages of treated mice were activated since they generated 9 times higher amount of  $O_2^-$  than those of control mice.

Normally, NO react rapidly with  $O_2^-$  and the reaction is completed in less than 1  $\mu$ sec [36]. Therefore, any NO produced under aerobic conditions is converted rapidly to peroxynitrite anion. Peroxynitrite anion is a strong oxidant with bactericidal activity. At physiological pH it is protonated to form peroxinitrous acid, a relatively long-lived strong oxidant that could initiate oxidation of lipids. This could explain the toxic (at higher doses) and inflammatory (at lower doses) effect of MZ administrated in peritoneum. Also, the observed depletion of NO from peritoneal macrophages might have significantly increased superoxide generation and in this way could have intensified the effect of MZ. Since concentration of TBARS in liver and TSA in spleen of treated mice remained the same; this is obviously a local reaction.

Phagocytosis per se or reactive oxygen species (ROS) can stimulate macrophages to secrete TNF- $\alpha$  and other cytokines that normally stimulate immunological response [37]. One ubiquitous transcription factor of particular importance in immune and inflammatory responses is nuclear factor kappa B (NF $\kappa$ B) [38]. Therefore, we wanted to examine the activation of NF $\kappa$ B in splenocytes of MZ treated mice. Our results showed MZ induced translocation of p65 to the nucleus of RFM mice spleen cells. This finding suggests that MZ acts as immunoactivator, activating NF $\kappa$ B, and therefore inducing transcription of genes regulated with NF $\kappa$ B.

Decreased amount of p50 and increased amount of RelB proteins in treated compared to control mice could be due to changed number and/or ratio of B- and T-lymphocytes. The fact that spleens of treated mice were 11% heavier contributes to that assumption. B-lymphocytes have a basal level of p50 homodimer that is not inducible upon stimulation [39] and/or serve as regulators of NF $\kappa$ B activity [40]. Decreased total amount of p50 protein, as well as it not being translocated into the nucleus, could imply that B-lymphocytes were not stimulated by MZ. Also, RelB/p50 heterodimer is constitutively active in primary lymphoid cells and its presence correlates with constitutive lymphoid-specific transgene expression of genes involved in B- and T-cell development [26, 41]. This explains our finding that MZ treatment did not have any (or had only a slight) effect on translocation of RelB subunit into the nucleus of RFM mice spleen cells. These facts also explain a relatively high basal amount of p50 and RelB proteins in nuclei of control splenocytes.

While B-lymphocytes and several other cells exhibit both constitutive and stimulated NFκB activation, only inducible NFκB activity has been described in T-cells or T cell lines [42]. However, as complete T-cell activation requires at least two signals provided by the T-cell receptor (TCR) complex and another stimulatory molecule, optimal NFκB activation in the T-cell is also dependent on dual signaling mechanisms [43]. Many agents have been shown to promote activation of NFκB in T-cells including TNF-α [44], calcium ionophores [18] and H<sub>2</sub>O<sub>2</sub> [42]. However, maximal NFκB activation has been observed in response to combinations of stimulants which fulfil the dual signaling requirements of T-cells [43, 45]. ROS have been found to act as second messengers in activation of NFκB/Rel proteins [42] and oxidative stress can modulate the activity of NFκB in T-cells [18]. In addition, previous results have shown that NFκB is activated in inflammatory diseases. Therefore, according to all these as well as to our results, we concluded that MZ, in our experiments, activated T-cell immunological (cellular) response, that could be involved in anticancer effect of MZ *in vivo*.

*Possible mechanism of MZ in vivo action.* We propose a mechanism for MZ *in vivo* action (Figure 7). MZ causes local inflammation at the place of application, e.g. peritoneum. Macrophages will be attracted and activated, which has been shown with increased O<sub>2</sub><sup>-</sup> production. We suggest that activated macrophages produce TNF-α that, together with some other stimulants (e.g. other cytokines, ROS or changed calcium concentration), stimulate splenic T-cells. Since products of the genes that are regulated by NFκB also cause its activation, this type of positive regulatory loop may amplify and perpetuate the local inflammatory response. Our hypothesis is that MZ will act the same way after *per os* administration, affecting intestinal macrophages. Results of experiments with local allogeneic graft *versus* host reaction, as well as strong reduction of immunogenic melanoma B16 lung metastasis, support this hypothesis. This is in agreement with accumulating evidence that zeolites could play an important role in modifying the immune system as well as with the report that silica, silicates and aluminosilicates act as non-specific immunomodulators similarly to superantigens.

To additionally confirm the hypothesis, TNF-α in serum should be measured, as well as activation of NFκB in macrophages, and B- and T-lymphocytes separately.

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Dr. Georg Waeg, Institute of Biochemistry, Graz, Austria, kindly provided monoclonal antibodies for HNE-immunohistochemistry.

## Figure legend

**Figure 1.** The effect of MZ treatment on growth of different human cell lines: 0.05 mg/ml, 0.25 mg/ml and 0.5 mg/ml. The results are presented as percentage of the growth of control cells.

\* indicates significant difference compared to control cells (ANOVA,  $p < 0.05$ ).

**Figure 2.** Influence of MZ on oxidative stress *in vivo*. Immunohistological distribution of HNE-protein conjugates in W256 carcinoma. Control tumor (upper left panel): anaplastic tumor infiltrates skeletal muscle; only occasionally, near muscle infiltration, malignant cells show mild (yellow) HNE presence in cytoplasm, while stroma is negative. MZ treated tumour (upper right panel): while necrosis of tumor tissue (right and left top side) contains several slightly HNE-positive cells, stromal as well as tumor cells remote from necrosis are HNE-negative. Doxorubicin treated tumour (lower left panel): almost all tumour as well as stromal cells show very strong membrane and cytoplasmic HNE immunopositivity. Doxorubicin and MZ treated tumour (lower right panel): while all tumor cells show diffuse HNE immunopositivity, stroma is entirely negatively stained. Magnification 400 x.

**Figure 3.** Concentration of TBARS in liver and LSA in serum of control and tumour-bearing mice as well as tumor-bearing mice fed with either 12.5% or 25% MZ.

**Figure 4.** Effect of MZ on allogeneic graft *versus* host reaction. The GVH reaction of lymph node cells of control (empty column), mice fed with 12.5% (gray column) and 25% (black column) MZ were tested.

**Figure 5.** Number of peritoneal macrophages per mouse (A), superoxide generation in macrophages (B) and nitric oxide concentration (C) in peritoneal macrophages *ex vivo* 24 hours after i.p. treatment with 3 mg MZ/mouse. There were 18 control and 21 mice treated with MZ in two experiments.

**Figure 6.** Effect of MZ on level and distribution of p65 (I), p50 (II) and RelB (III) subunits of NF $\kappa$ B. (a) Western blot analysis of NF $\kappa$ B subunits in nuclear and cytoplasm fractions. C indicates control groups of animals, whereas MZ indicates treated animals. (b) Densitometric quantification of signal intensity. Total level indicates the sum of signal intensities of cytoplasm and nuclear fraction in each group (C and MZ). Results are expressed as percentage of each subunit compared to their total level in controls.

**Figure 7.** Clinoptilolite-induced stimulation of cellular immune response; proposed mechanism of MZ action *in vivo*.

Table 1.  
Influence of MZ on the biological effects of HNE *in vitro*.

HNE	Without plasma		1% plasma		10% plasma	
	- MZ	+ MZ	- MZ	+ MZ	- MZ	+ MZ
-	24	51 <sup>a</sup>	90	81	100	108
+	42 <sup>a</sup>	38 <sup>a,b</sup>	70 <sup>a</sup>	91 <sup>c</sup>	98	105

*The results are expressed as percentages of the viability of the control group (with 10% plasma, without MZ and HNE). Significant (Student's t-test,  $p < 0.05$ ) if compared (absolute values) with the cells cultured: <sup>a</sup>without HNE and without MZ, <sup>b</sup>without HNE, but with MZ, <sup>c</sup>without MZ but with HNE, in experiment where plasma was absent as well as in experiments with 1% or 10% plasma.*

Table 2. Immunohistochemical findings of HNE distribution in Walker 256 carcinoma tissue.

Treatment	Animal No.	HNE positivity in:				
		tumor cells	tumor cells near necrosis	tumor cells within zone of tumor spreading	stroma	necrosis
Saline control	1	+	++	-	++	+
	2	-	+	-	-	+
	3	-	+	-	-	+
	4	-	+	-	++	-
	5	-	-	-	-	-
	6	-	-	+	-	++
Doxorubicin	7	+	++++	+	+	++
	8	+++	++++	+++	+++	++
	9	+++	++++	+++	+++	+++
	10	++	+++	++	++	++
	11	-	+++	++	-	++
MZ	12	-	++	-	-	+
	13	+	+	+++	-	++
	14	-	-	++	-	++
	15	-	-	-	++	-
MZ + Doxorubicin	16	+	+	+	-	+++
	17	++	++	+++	-	+++
	18	+++	+++	++++	-	++
	19	++	++	+++	-	++

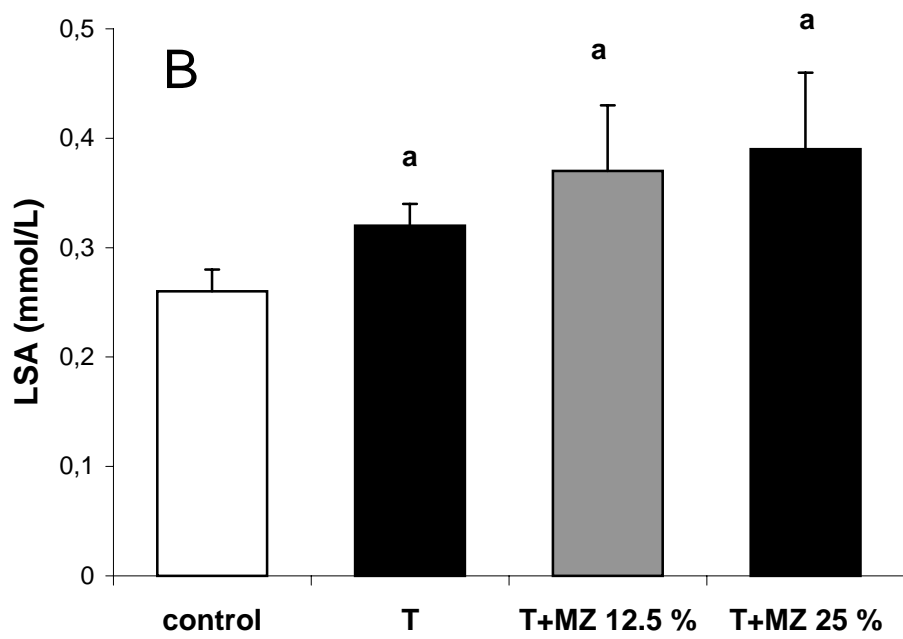
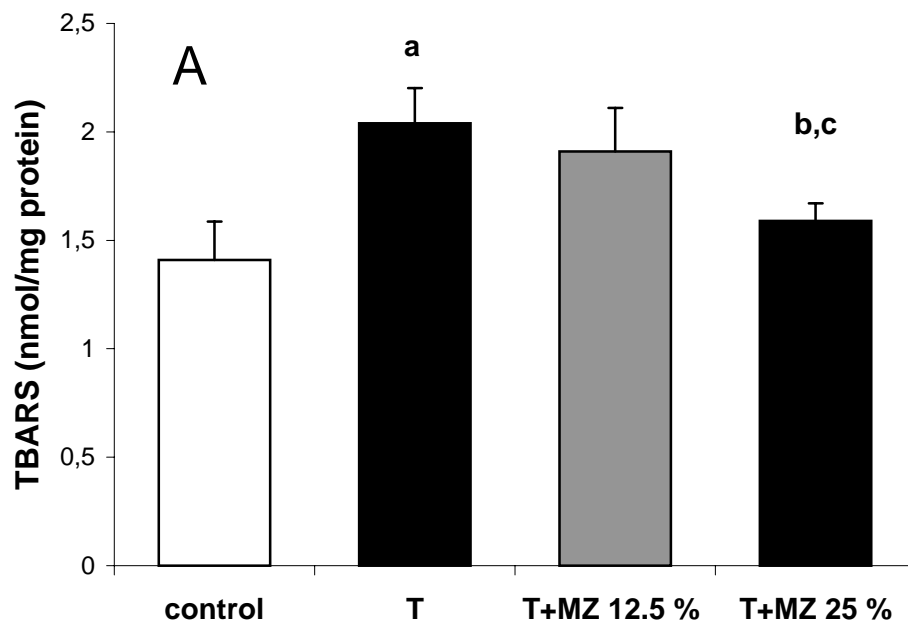
(-) negative; (+) weak positivity (<5 % of the cells); (++) moderate staining approximately 25 % of the cells; (+++) strong staining ( $\geq$ 50-75 % of the cells)

Table 3.

Influence of MZ on  $O_2^-$  production, lipid peroxidation (TBARS) and lipid bound sialic acid (LSA) in healthy mice

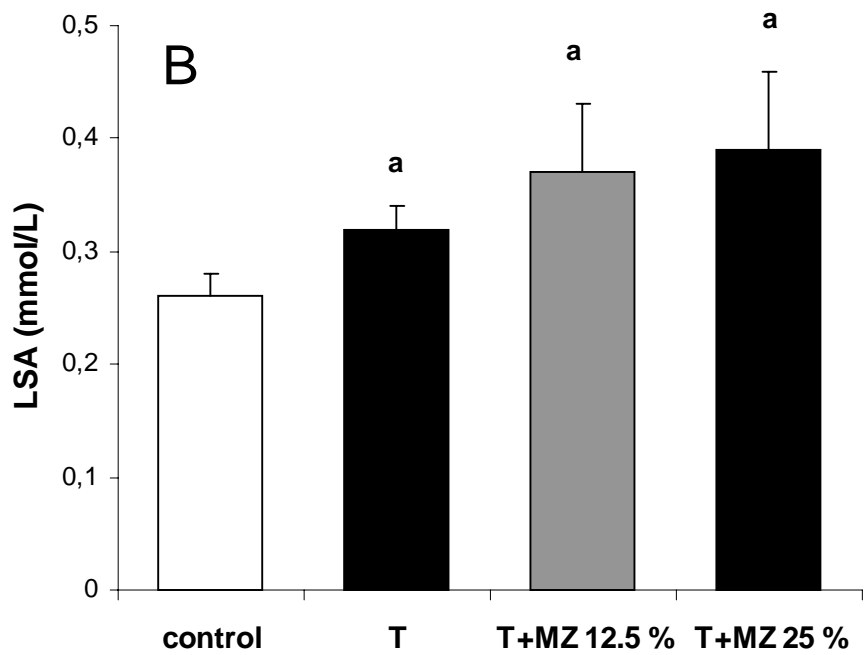
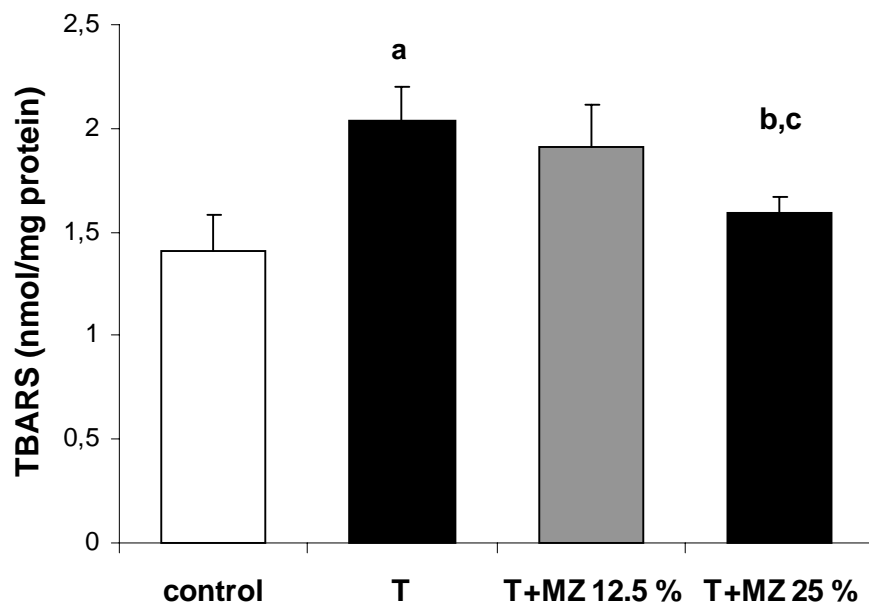
	Days	7	14	21	28
$O_2^-$ nmol/ $2 \times 10^6$ macrophages	Control	$3.10 \pm 0.14$	$1.50 \pm 0.23$	$0.45 \pm 0.07$	$1.20 \pm 0.9$
	12.5% MZ	$3.95 \pm 0.8$	$0.68 \pm 0.25^*$	$2.55 \pm 0.21^*$	$0.55 \pm 0.07^*$
	25% MZ	$3.20 \pm 0.56$	$0.95 \pm 0.35$	$0.57 \pm 0.39$	$1.10 \pm 0.14$
TBARS nmol/mg liver proteins	Control	$0.97 \pm 0.11$	$0.94 \pm 0.22$	$1.89 \pm 0.18$	$1.78 \pm 0.09$
	12.5% MZ	$0.95 \pm 0.11$	$0.72 \pm 0.04$	$1.20 \pm 0.29^*$	$1.16 \pm 0.08^*$
	25% MZ	$0.94 \pm 0.13$	$0.61 \pm 0.09$	$0.85 \pm 0.11^*$	$0.85 \pm 0.12^*$
LSA nmol/l	Control	$0.18 \pm 0.07$	$0.26 \pm 0.11$	$0.29 \pm 0.03$	$0.27 \pm 0.03$
	12.5% MZ	$0.29 \pm 0.13$	$0.25 \pm 0.04$	$0.42 \pm 0.06^*$	$0.46 \pm 0.20$
	25% MZ	$0.28 \pm 0.06$	$0.28 \pm 0.08$	$0.34 \pm 0.16$	$0.49 \pm 0.15^*$

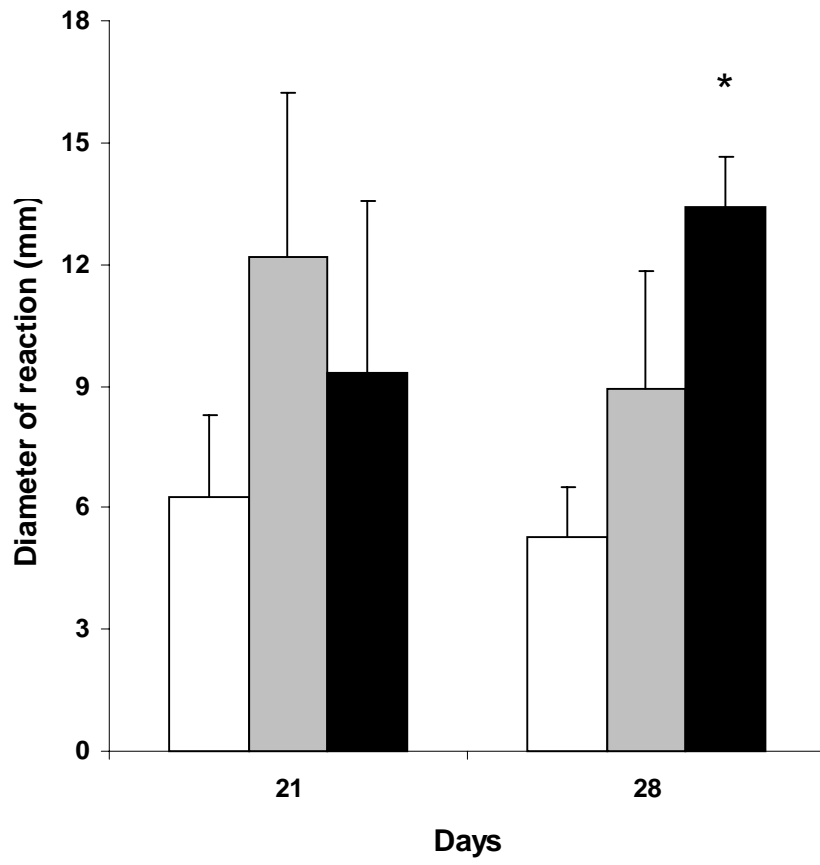
The values are  $\bar{x} \pm SD$ ; \* indicates significant (*t*-test,  $p < 0.05$ ) result compared to relevant control

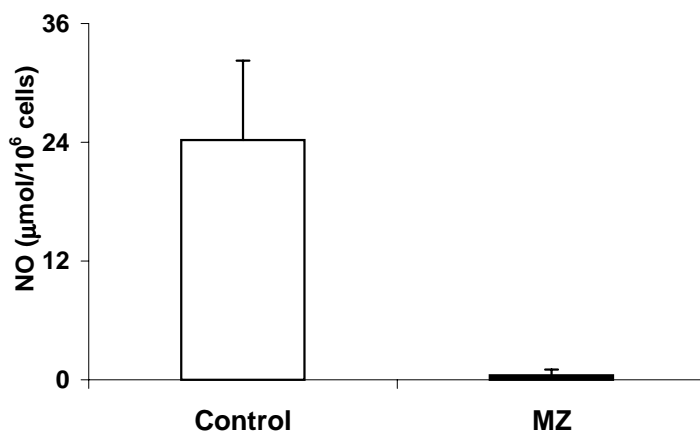
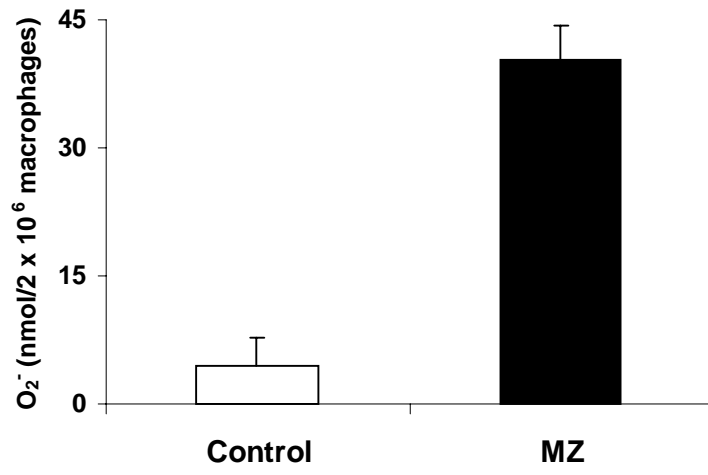
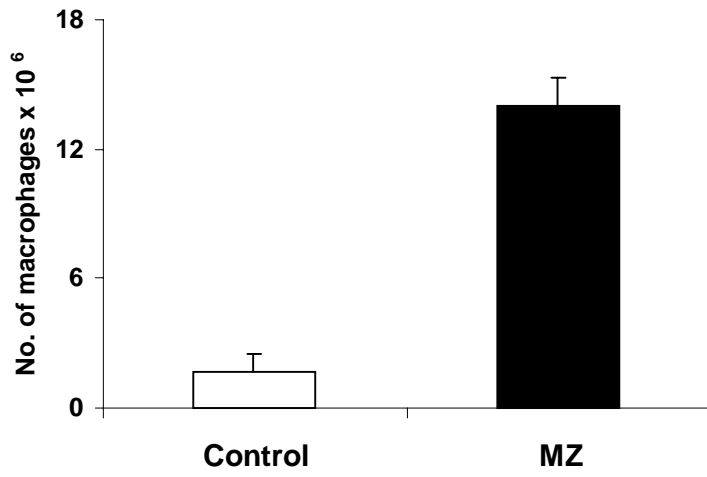


MZ jedan reprezentativni pokus								
	control	0.05 mg/ml	0.25 mg/ml	0.5 mg/ml	devijacije			
HeLa	100	67	60	49	6	2	4	2
MiaPaCa-2	100	85	74	37	4	1	7	8
CaCo-2	100	100	115	82	4	7	7	9
MCF-7	100	103	100	84	2	12	10	8
WI 38	100	125	121	106	11	7	7	12

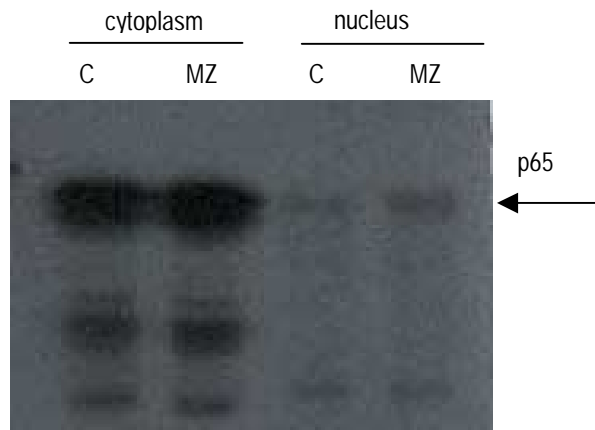






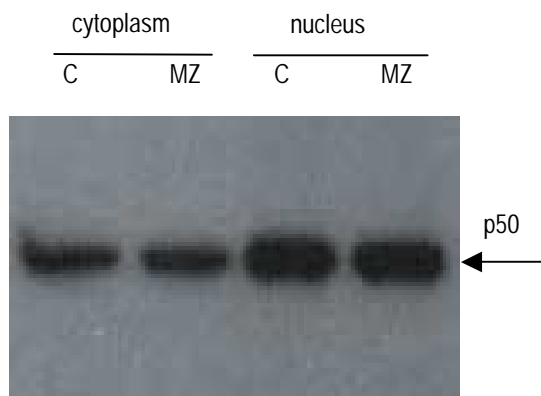


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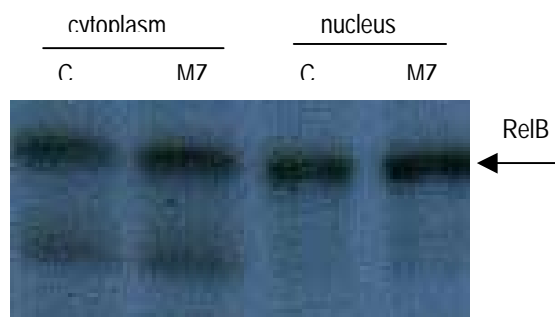
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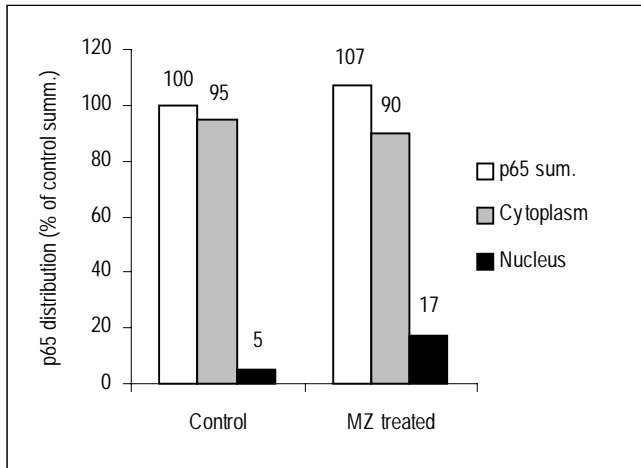
WB: anti-p50

III a

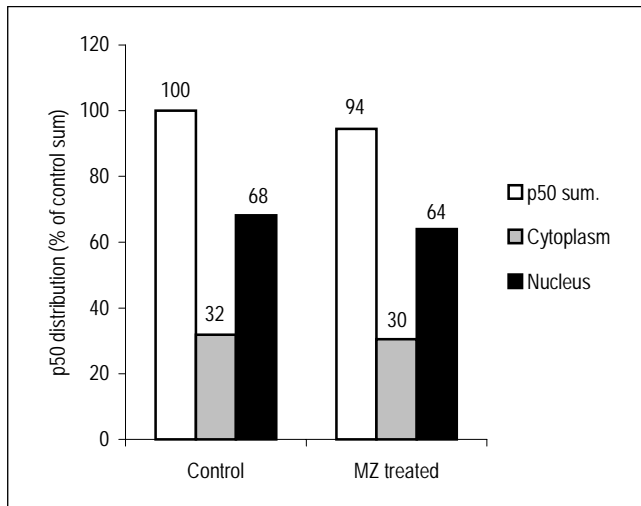


WB: anti-RelB

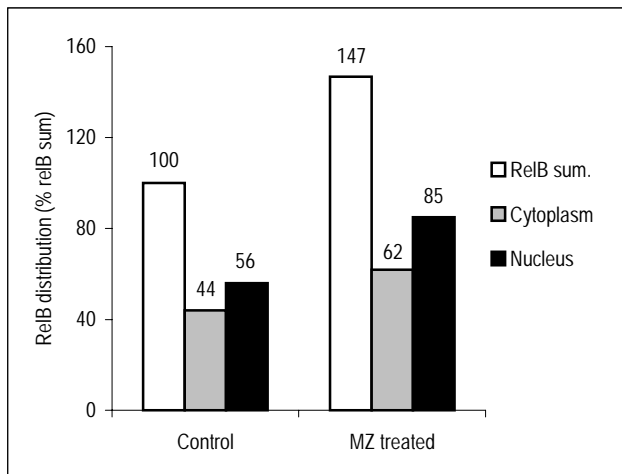
### III b



### II b



### III b



# Clinoptilolite-induced stimulation of cellular immune response

