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Anticancer and Antioxidative Effects of Micronized Zeolite Clinoptilolite

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Abstract. *Background: Treatment of cancer-bearing mice and dogs with micronized zeolite clinoptilolite (MZ) led to improvement of the overall health status, prolongation of life span and decrease of tumor size in some cases. It also reduced lipid peroxidation in the liver of mice. Materials and Methods: The experiments were performed on various tumor cell cultures and tumor-bearing animals. Immunohistochemistry was used to analyze if MZ could interfere with Doxorubicin-induced lipid peroxidation and consequential production of 4-hydroxynonenal (HNE). Results: MZ reduced the metabolic rate of cancer cells and increased binding of HNE to albumin in vitro. It selectively reduced generation of HNE in vivo in tumor stroma after Doxorubicin treatment leaving onset of lipid peroxidation intact in malignant cells. Combined treatment with Doxorubicin and MZ resulted in strong reduction of the pulmonary metastasis count increasing anticancer effects of Doxorubicin. Conclusion: Interference of MZ with lipid peroxidation might explain some of the beneficial effects of this particular zeolite in combined cancer therapy.*

Natural zeolites are volcanic minerals with unique characteristics. Their chemical structure classifies them as hydrated aluminosilicates, comprised of hydrogen, oxygen, aluminum and silicon, arranged in an interconnecting lattice structure. Their unique structure makes zeolites different from other aluminosilicates, due to their ability of gas and water absorption and ion exchange (1).

Since many biochemical processes are closely related to ion exchange, absorption and catalysis, it is believed that natural and synthetic zeolites could make a significant contribution to the pharmaceutical industry and medicine in the near future. For instance, some zeolites are already used as contrast medium in diagnostic methods (2), as antidiarrheal drugs (3), as antibacterial and antifungal drugs (4), as support for enzymes and antibodies (5) *etc.* They are also used in the healing of cuts and wounds and are very effective as a glucose absorbent, so diabetes mellitus patients could use them (6). They reversibly bind small molecules such as oxygen or nitric oxide, possess size and shape selectivity, the possibility of metalloenzyme mimicry (7) and have immunomodulatory activity (8, 9).

Our 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. *In vitro* tissue culture studies showed that finely ground clinoptilolite inhibited protein kinase B (c-Act), induced expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ tumor suppressor proteins and blocked cell proliferation in several cancer cell lines (10).

Accumulating evidence has indicated that zeolites play an important role in modulation of the immune system. Lymphocytes from lymph nodes of healthy mice fed on MZ for 28 days provoked significantly higher alogeneic graft-versus-host (GVH) reaction than cells of control mice. After *i.p.* application of MZ, translocation of p65 (NFkB subunit) to the nucleus of spleen cells was observed (9). Similarly, it was shown that exposure of alveolar macrophages to silicate particles leads to activation of mitogen-activated protein kinases (SAPK) (11), while transcription factors such as AP-1 and NFkB are also activated and expression of pro-inflammatory cytokines such as IL-1α, IL-6 or TNF-α is enhanced (12, 9).

In healthy mice fed on MZ for 28 days, the concentration of lipid-bound sialic acid (LSA) in serum was increased, but lipid peroxidation in the liver was decreased (9), indicating the possible systemic antioxidative effects of MZ. Oxidative

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stress in cancer cells may constantly activate transcription factors, such as NFkB, through intracellular signal transduction (13) and induce expression of proto-oncogenes such as c-fos, c-jun and c-myc (14). Besides, oxidative stress induces DNA damage such as modified base products and strand breaks, that may lead to further mutation and chromosomal aberration of cancer (genomic instability) (15). The carcinogenic effects of reactive oxygen species (ROS) are well-known as well as their cytotoxic effects, when induced by radio- and/or chemotherapy. In spite of the evidence that cancer cells are oxidatively stressed (16), the stress is usually insufficient to cause cell death. Moreover, some tumor cells are resistant to oxidative cytolysis (17), while oxidative stress can also activate antioxidant systems in cancer cells that could make cancer cells resistant to chemotherapy.

Lipid peroxidation is an integral part of oxidative stress, resulting in the production of reactive, toxic aldehydes, the end product of lipid peroxidation denoted also a "second messengers of free radicals", among which the best known are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (18). HNE is also known as a growth modifying factor and proapoptotic agent, while its effects are dependent on its high affinity to bind to cellular proteins (19, 20). Since MZ is able to reduce lipid peroxidation *in vivo* (9), it seems likely that it could interfere with the HNE effects, too.

Here we present new evidence for the antitumor and antioxidative activity of MZ as well as its possible mechanism of *in vivo* action.

Materials and Methods

Micronized zeolite (MZ). Tribomechanically micronized natural zeolite clinoptilolite (MZ) from Slovakia, with described characteristics (9), was used. Additionally, the nano- and crystal structure of micronized and non-micronized zeolite was measured using small angle X-ray scattering (SAXS) and wide angle X-ray scattering (WAXS) spectroscopy at the Austrian high-flux SAXS beamline of the 2GeV electron storage ring ELETTRA (Trieste, Italy) (21). There was no pronounced distinction in nonstructural features (void structure) between micronized and non-micronized samples.

Measurement of cell proliferation (MTT-assay). The effect of MZ on cell proliferation *in vitro* was studied on several human cell lines: HeLa (cervical carcinoma), MiaPaCa-2 (pancreatic carcinoma), CaCo-2 (colon carcinoma), MCF-7 (breast cancer) and Hep-2 (laryngeal carcinoma). The cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS, Sigma, USA) is standard conditions. For the purpose of proliferation assay experiments, the cells were plated at a density of 3×10^4/ml onto 96-microwell plates (200 µl/well) (Greiner, Germany). After overnight incubation, the medium was replaced with fresh DMEM containing different concentrations (0.05-0.5 mg/ml) of MZ. After 72 hours of incubation the cells were gently washed with PBS in order to remove MZ. The cell viability was determined using the MTT assay (Sigma), which detects dehydrogenase activity in viable cells (10). Statistical analysis was performed using the One Way ANOVA test. A level of $p < 0.01$ was accepted as statistically significant.

Animals and treatments. Mice were bred in the Animal Facility of the Ruder Boskovic Institute and rats were obtained from the Institute of Medical Research in Zagreb, Croatia. Food (Domzale, Slovenia) and tap water were given *ad libitum*. The animals were kept in conventional circumstances: light/dark rhythm 12/12 hours, temperature 22°C and humidity 55%.

Antimetastatic effects of MZ and Doxorubicin. Mammary carcinoma-bearing 3.5-month-old female CBA/HZgr mice were used to study the effects of Doxorubicin and MZ on the development of artificial lung metastasis. The spontaneous mammary carcinoma developed on CBA/HZgr strain mice was used in the experiment (22). Tumor cells were obtained from the sacrificed tumor-bearing mice and 5×10^4 live cells (according to Trypan blue exclusion giving <35% of the Trypan blue-positive cells) were injected *i.v.* into the tail vein of each mouse. Doxorubicin was given by a single *i.p.* injection one day after injection of the tumor cells at 10 mg/kg dose.

MZ was applied in the tap water given *ad libitum* (0.5 g/kg average daily dose). This route of application and dose was chosen because it resembles the protocols used for the therapy of cancer patients (Dr. D. Zapčić, personal communication). The consumption of tap water by each animal was determined during two weeks before the beginning of the experiment to determine the concentration of the substance needed to achieve the average daily dose of approximately 0.5 g/kg. For the daily MZ application, a fresh solution of the substance was prepared every second day. Control animals were treated equally with saline *i.p.* and plain tap water only.

The mice were kept alive until the 18th day when they were sacrificed by ether and the autopsy was perfomed to verify development of the tumor metastases and count the tumor nodules in the lungs. The lungs were afterwards fixed in 10% formalin for pathohistological evaluation, while the differences in the number of pulmonary tumor nodules were evaluated by the Student's *t*-test.

Effects of MZ on Doxorubicin-induced oxidative stress in W256 rat carcinoma. To study the effects of MZ on chemically-induced oxidative stress we used six-month-old male Wistar rats bearing Walker 256 carcinoma (W256), weighing 308 ± 25 g treated with antracycline (Doxorubicin, Sigma, USA), which achieves anticancer activity by induction of lipid peroxidation in malignant cells. W256 carcinoma cells (10^7 cells/rat) were injected *i.m.* in the hind limb. Six days later, when the tumors were 3-4 cm in diameter, the rats were divided into four groups of 4-6 animals: 1) controls, 2) rats treated with MZ, 3) rats treated with Doxorubicin *i.p.*, 4) rats treated with MZ and Doxorubicin *i.p.* MZ was given *per os* by sonde in a single dose of 2 g/kg one hour before *i.p.* injection of Doxorubicin (10 mg/kg). After three hours, the animals were killed, the tumors were removed and stored in 10% buffered formalin to be used for immunohistochemistry of HNE-protein conjugates in tumor tissue.

Immunohistochemistry of HNE-protein conjugates in W256 carcinoma tissue. Tissue sections (5 µm) of the formalin-fixed paraffin-embedded tumors were used. For immunohistochemical detection (using peroxidase-antiperoxidase method) of HNE-protein conjugates, as in the case of dot-blot, the same specific monoclonal antibodies raised against HNE-histidine conjugate were used, as described before (23). Monoclonal antibodies for detection of HNE-protein conjugates were obtained from the culture medium of the clone derived from a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALBc mouse immunized with HNE modified keyhole limpet hemocyanine (Institute of Biochemistry, Graz, Austria). The antibody is specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates (23).

To analyze possible differences in the intensity of HNE-staining between the tumors of differently-treated animals, semiquantitave evaluation was done by a pathologist and defined in staining intensity grades as follow: (-) negative; (+) weakly positive staining (<5% of the cells); (++) moderate staining (approximately 25% of the cells) and (+++) strong staining (≥50-75% of the cells). Afterwards, the grades of immunopositivity staining were assigned to respective numerical values

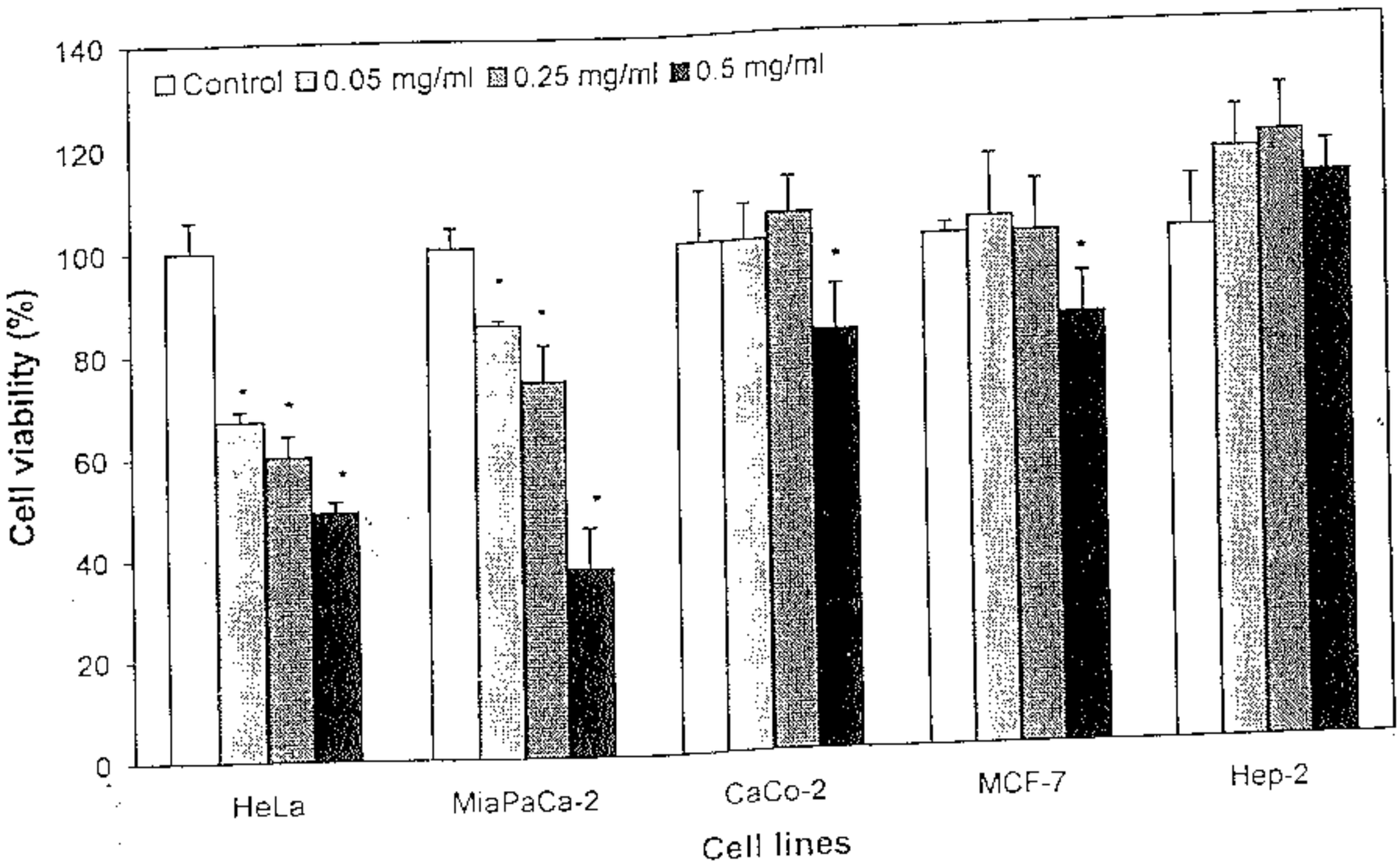


Figure 1. *The effect of MZ treatment on growth of different human cell lines: 0.05 mg/ml, 0.25 mg/ml and 0.5 mg/ml. The results are represented as a percentage of the growth of control cells. Statistically significant results ($p < 0.01$) are represented with asterisks.*

from 0 to 3 and evaluated by two tailed non-parametric Mann Whitney *U*-test, considering $p < 0.05$ as an indicator of difference.

Influence of MZ on HNE binding to serum albumin. The semi-quantitative dot-blot method using specific monoclonal antibodies raised against HNE-histidine conjugate was applied to determine interference of MZ with the development of HNE-protein conjugates. MZ solution in saline (concentration of 400 mg/ml) was prepared by using an ultrasound water bath (Transsonic 310, Elma) for 30 minutes to achieve dispersion of the hardly soluble powder. The solution was than centrifuged at 500 g for 10 minutes (Heraeus, Labofuge 400). For the following experiment we used the thus obtained supernatant. Bovine serum albumin (BSA, Haemosan, Austria) at a concentration of 10 mg/ml was mixed at an equal volume ratio with the supernatant of the MZ 200 mg/ml solution and HNE (100 μM). After a 30-minute incubation we applied 50 μl solution samples as the original sample, and saline dilutions of 1:10 and 1:100 (all in duplicates) on nitrocellulose membranes (0.2 μ, Bio-Rad, Germany) using the dot-blot wash system (Bio-Dot, Bio-Rad, Germany).

The same monoclonal antibodies used for detection of HNE-protein conjugates in tumor tissue were also used in 1:10 dilution to detect HNE-BSA conjugates. The peroxidase activity of sample enzymes was blocked with a solution consisting of 1.5% H_2O_2, 0.1% Na-azide and 2% BSA before adding secondary antibody. The immunoperoxidase technique was used, with secondary rabbit anti-mouse antibodies (DAKO), applying PAP-DAB staining as described before (2). Secondary antibody (rabbit anti-mouse, DAKO, Denmark) was added in dilution 1:500. Binding of the antibodies was determined by the peroxidase activity of the enzyme-marked tertiary antibody complex (PAP-mouse, DAKO, Denmark), diluted 1:1300 and applying diaminobenzydine (DAB, DAKO, Denmark) staining. Densistometry was done by the image analysis system and a comparison of spot intensity was done with a densitometry computer program (Zero-D scan, Scanalytics, USA).

Results

The effect of MZ on proliferation of cell lines in vitro (MTT-assay). The effects of MZ on the growth of different human cancer cell lines are presented in Figure 1. The growth of all cell lines, except Hep-2, was significantly inhibited, in a dose-dependent manner. The strongest inhibition was observed with HeLa and MiaPaCa-2 cells. MZ in the concentration of 0.05 mg/ml significantly ($p < 0.01$) inhibited the viability of these two cell lines (for 20-30%). At a concentration of 0.5 mg/ml the viability of HeLa and MiaPaCa-2 cell lines was inhibited by about 50 to 60%, respectively. The viability of other cell lines was significantly inhibited only at the concentration of 0.5 mg/ml.

Effects of MZ and Doxorubicin on development of artificial lung metastasis of mammary carcinoma. The effects of Doxorubicin and MZ, given alone or in combined treatment, on the formation of artificial lung metastasis of murine mammary carcinoma are presented in Table I.

Doxorubicin, given as a single 10 mg/kg dose, did not reduce the metastases count ($p > 0.1$). Similarly, MZ given daily in tap water did not influence the development of the lung metastases ($p > 0.1$), but combining daily MZ application with Doxorubicin resulted in reduction of the metastases count that was significant both if compared with the control ($p < 0.0001$) or with the group treated by Doxorubicin only ($p = 0.001$).

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

Treatment	Animal No.	HNE positivity in: tumor cells apart from necrosis or infiltration	tumor cells near necrosis	tumor cells within zone of tumor infiltration of normal tissues (muscle, skin)	stroma
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, no positive cells; +, weak positivity (<5% of the cells); ++, moderate staining (approximately 25% of the cells); +++, strong staining (≥50-75% of the cells).

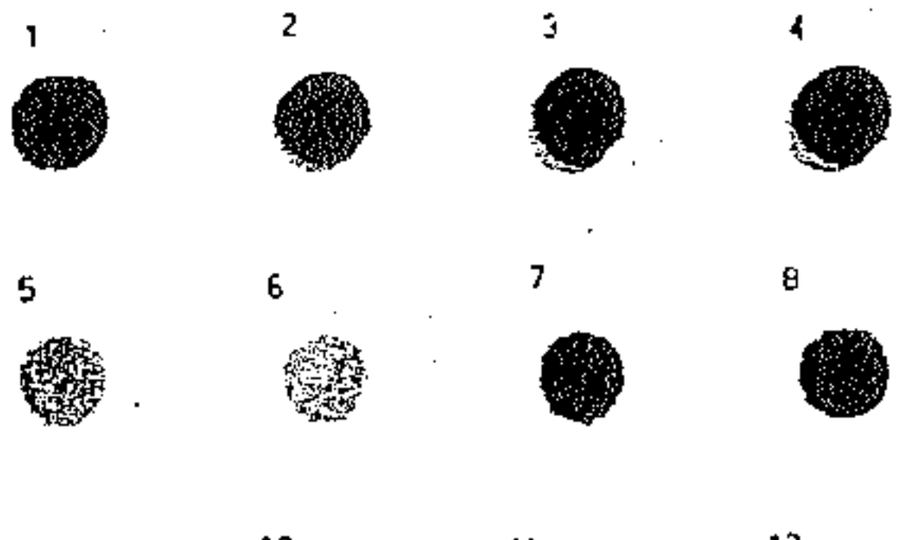
Effects of MZ on chemically-induced oxidative stress of W256 carcinoma in vivo. The results of the pathohistological evaluation of W256 carcinoma treated either with Doxorubicin or MZ or combined treatment are summarized in Table II. Although there were no general differences in the pathohistological appearance of differently treated tumors. Doxorubicin induced prominent lipid peroxidation (generation of HNE-protein conjugates), both in malignant cells ($p < 0.01$) as well as in normal stromal components of tumorous tissue ($p = 0.02$). Most intensive HNE-positivity was noticed in tumor cells near necrosis, both for the control and for Doxorubicin-treated tumors. MZ itself did not change the immunohistochemical appearance of HNE-protein conjugates compared to untreated tumor ($p > 0.05$). However, it had selective, tissue-specific effects on Doxorubicin-induced oxidative stress (lipid peroxidation within tumor). Namely, when MZ was applied, it did not influence Doxorubicin-induced oxidative stress (HNE-protein conjugates) in malignant cells ($p > 0.1$). while it completely prevented the formation of HNE-protein conjugates in tumor stroma ($p = 0.005$).

Effects of MZ on HNE-protein binding affinity in vitro. The immunoblotting (dot-blot) findings of HNE-BSA conjugates

Table II. *Number of artificial lung metastases of the murine mammary carcinoma of the mice.*
The animals received i.v. $5x10^4$ live tumor cells and were treated one day after by a single i.p. dose of 10 mg/kg Doxorubicin and/or MZ in tap water (0.5 g/kg average daily dose until the end of experiment). The animals were sacrificed by ether on the 18^{th} day after tumor cells injection.

	Control	Doxorubicin	MZ	Doxorubicin + MZ
1	37	31	3	15
2	41	43	43	11
3	38	33	44	33
4	28	27	4	16
5	52	35	29	7
6	49	20	61	8
7	45	68	26	2
8	56	12	55	15
9	32	39	24	17
10		31	2	4
Mean	42.00	34.22	32.00	12.55
SD	7.55	10.69	16.66	6.83
p (*t*-test) *vs.* Control		0.1801	0.1157	<0.0001
p (*t*-test) *vs.* Doxorubicin				0.001

formed *in vitro* in the presence of MZ are presented in Figure 2. The affinity of HNE to form conjugates with proteins, as evaluated in this experiment using BSA, was increased 30-40% if the HNE was mixed with BSA in the presence of MZ. That was most obvious when the protein / aldehyde / zeolite solution was diluted ten-fold before applying to the nitrocellulose membrane (samples 5 and 6 *vs* samples 7 and 8, *i.e.* middle dots).



Sample No.	Type of sample/dilution	Absorbance
1	BSA-HNE/1:1	111.64
2	BSA-HNE/1:1	108.81
3	BSA-HNE+MZ/1:1	137.02
4	BSA-HNE+MZ/1:1	117.73
5	BSA-HNE/1:10	57.94
6	BSA-HNE/1:10	44.62
7	BSA-HNE+MZ/1:10	80.39
8	BSA-HNE+MZ/1:10	95.92
9	BSA-HNE/1:100	8.66
10	BSA-HNE/1:100	8.91
11	BSA-HNE+MZ/1:100	13.95
12	BSA-HNE+MZ/1:100	12.56

Figure 2. *Immunodetection of HNE-BSA conjugates formed in vitro in the presence of MZ or without it.*

Discussion

Our previous *in vitro* experiments (10) showed inhibition of the proliferation of several cell lines after incubation with MZ (0.5-50 mg/ml) pre-treated medium. Now we added MZ directly to the culture medium, and the cell proliferation was also inhibited even with the much lower concentration used. The best and the most consistent results were obtained with the HeLa and MiaPaCa-2 cell lines. However, the Hep-2 cell line appeared to be resistant to the inhibitory effect of MZ (at least for the concentrations of the zeolite used). According to these experiments, our conclusion is that MZ response was cell line-dependent. We previously analyzed the effects of MZ *in vitro* on mitogenic and survival-signaling pathways in tumor cells (10). The most significant results were observed in the activity of Akt protein that was strongly inhibited after MZ treatment of cancer cells. That resulted in growth inhibition and an increase in apoptosis of cancer cells, but only in the presence of serum. Absorption of serum components, at least *in vitro*, could be one of the mechanisms of MZ action. In favor of this assumption are findings of an increased HNE-protein conjugate formation in the presence of MZ. HNE has high affinity of binding to proteins, so it can bind serum albumin, which can thus attenuate its toxicity. However, binding to proteins, like BSA, stabilizes highly reactive HNE that could still achieve its cytotoxic effects (18). We assume that the cytotoxic effects of MZ could be modified by its physical or biological interference with lipid peroxidation and in particular HNE. MZ might act as an HNE-scavenger but HNE binding to MZ might also affect the activity of MZ in particular in the presence of proteins that could bind to both substances as well as to the cells. This might also be relevant for the *in vivo* effects of MZ, in particular in conditions of systemic oxidative stress, such as chemotherapy of cancer.

Since orally-administered MZ is not absorbed into blood, its effects *in vivo* cannot be due to direct biochemical interactions. We speculate that MZ may induce certain immunological responses or general stress response triggered by the gastrointestinal system, in particular considering the gut as a stress organ (24). If so, MZ should also influence inflammatory processes as well as sytemic oxidative stress.

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 tumor stroma. TNF-α and TGF-β are among the cytokines that are likely to be mediators of this complex network system, that interfere with lipid peoxidation and consequently with biological effects of HNE (known as a second messenger of free radicals). On the other hand, HNE can also act as a bifunctional growth regulating factor (cytotoxic or growth stimulatory), interfering with the activity of serum growth factors (25). The growth modulating effects of HNE involve signaling pathways affecting c-fos expression and might interfere with the activity of EGF and PDGF (26). HNE as second messenger of ROS activates AP-1, followed by further TGF-β 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 with further lipid peroxidation and production of HNE (27). 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* as soon as half an hour after administration (28). Thus, we assume that applying MZ in water by gastric intubation could, in a relatively short period of time (one hour as done in the experiment presented), provoke response of the gastrointestinal system, which might further induce a systemic response. Regarding the specificity of the MZ antioxidant effect on stromal cells in Doxorubicin-treated W256 carcinoma, we assume that differential responses of malignant and normal cells to the antioxidative effects of MZ could be due to: 1) different pattern of oxygen metabolism between these cells (internal oxidative stress); 2) the difference in their response to cytokines involved in the determination of the 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 lipid peroxidation and thus may cause alterations in HNE and MDA levels (29). In our previous study, lipid peroxidation was reduced in animals that were fed a diet supplemented with MZ (9). According to Gonzales (30), levels of oxyradical scavenging enzymes are reduced in tumor cells. Thus, a reduced level of lipid peroxidation in MZ-treated tumor-bearing mice might be the result of the MZ antioxidant potential. A significantly increased SOD content in the liver of mice fed for 3 weeks with MZ supplemented to standard food also confirmed this data (9).

Our earlier results (9, 10) have shown that some antitumor and antioxidative effects of MZ *in vivo* antimetastatic are associated with its immunomodulating effects, in particular related to macrophage activity triggered by phagocytosis of MZ microparticles. It is known that phagocytosis and/or ROS can stimulate macrophages to secrete TNF-α and other cytokines that normally stimulate immunological responses (31). TNF-α also enhances the expression of SOD (32). In our experiments, the SOD content in the liver of mice fed MZ was increased. In our current study we found that MZ might have an antioxidative effect on stromal, but not on tumor cells, in Doxorubicin-treated W256 carcinoma. These data confirm that malignant and normal cells respond differently to the antioxidative effects of MZ, which means that MZ could have antioxidative and antitumor efects at the same time, which was further confirmed with the findings of the increased antimetastatic effects of Doxorubicin on mammary carcinoma (artificial lung metastasis model) if the animals were also treated with MZ. Thus, MZ could be used as a nutriceutical (food suplement) during chemotherapy of cancer.

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