



## Scientific Studies concerning activated zeolite.



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Active Mineral  
**PANACEO**



**SPORT**

# INFORMATION



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## Use the elementary power of the volcano for your maximum benefit!

Considerable reduction of free radicals, reduction of lactate levels, faster regeneration, thus more energy, better performance values, increased stamina, increased transport of oxygen into muscles and tissue, vitality and well being: High High Potency Mineral Panaceo Sport gives everything active people need. Many famous and successful top athletes already tested and experienced the positive effects of Panaceo Sport.

Panaceo Sport is a dietary supplement, containing purely natural ingredients. One of the chief ingredients is zeolite, a volcanic mineral mainly consisting of silicon, which is one of the most abundant and important elements of the earth.

### Unique production



Using a unique, **brand-new nanotechnology procedure**, the raw zeolite is micronized at very high kinetic energies to the size of a few nanometres. This procedure ideally prepares the mineral providing it with properties important for the human body.

The special treatment gives the zeolite the unique capacity to **neutralize free radicals** and **considerably reduce lactate levels**. This refinement creates a zeolite performance impossible to obtain with any other production process.

The processed zeolite furthermore intensifies the effects of the substances being added. Panaceo Sport is optimized by adding three pure natural products - **Dolomite** (calcium and magnesium), **Royal Jelly** (the nourishment of queen bees, a valuable protein source) and **Maca** (an Indian medical plant, thanks to its properties also called "Peruvian ginseng").

### Guaranteed Quality



A **certificate of the doping laboratory at Seibersdorf** (Austria) is drawn up for each charge to provide comprehensible proof that Panaceo Sport does not contain any possible doping substances.

#### Volcanic mineral zeolite as raw material

The main ingredient used for producing Panaceo Sport is the natural crystalline mineral clinoptilolite of the zeolite family.

The knowledge about the **positive effects** of zeolites is nothing new: ancient civilizations ground zeolite and used it for healing wounds, and South American Indians have been using zeolite for many centuries.

Scientific research proved that these minerals are **strong ion exchangers and adsorbents**, which absorb and neutralise toxins, heavy metals and free radicals.

For Panaceo Sport a clinoptilolite is used, which is well known for its adsorption capacity and selectivity, as well as its high potential as an ion exchanger.



## Activation

The zeolites used for producing Panaceo Sport are crushed finely using a brand-new method, which is protected by international patent.

This **unique process**, activation at very high kinetic energies, brings zeolite crystals into a form which can be best absorbed by human organism. The volcanic mineral is micronised to the size of a few nanometres, i.e. one million times smaller than one millimetre. Through the collision of the particles, the original substance is transformed considerably in its size and shape – it is activated.

The process of activation enlarges the surface of this substance and **intensifies its effects**. Nanoparticles have the right size and shape, and with their free charge and their extremely large specific surface they easily bind to harmful metabolism products and **cleanse the cells**.

The **specific surface of 5 grams** of zeolite powder corresponds to 5,000 square metres (approximately the **size of football pitch**). If all the **cavity channels in 1 gram of Panaceo powder** were lined up, they would stretch over **200 million kilometres**. Just as a reference point: the average distance of Earth and Sun is approx. 150 million kilometres.

In the human organism, zeolite functions like a sponge or filter, ready to absorb and neutralise harmful substances, excess acids, ammonia and free radicals. The body's own enzymes are stimulated, immune system and metabolism are boosted.

## Fighting free radicals



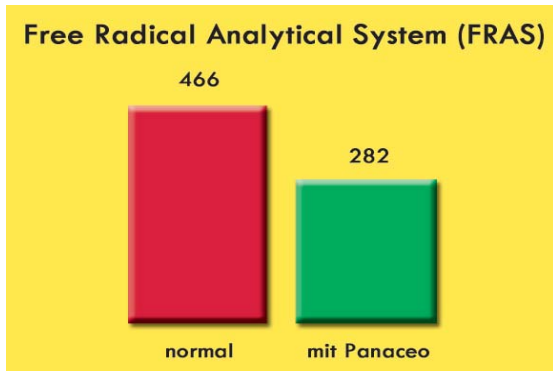
The volcanic mineral zeolite used in Panaceo Sport has been treated in a special process and has the unique capacity of **neutralising free radicals**. In the body, free radicals are built during metabolism processes. But more frequently they enter the body from outside – they exist in environmental toxins produced by air pollution or ozone damage. Free radicals are atoms or molecules with one or more unpaired electrons (oxygen molecules with a negative electric charge), which search for electrons to combine with. They **“rummage around” in the cells so aggressively** that they cause damage to important structures.

When the number of free radical molecules exceeds a certain amount, **oxidative stress** which can be detected in blood, sets in. The process caused by free radicals in the body is called oxidation; it is basically quite similar to the process we know as rusting. The consequences for the body become visible only gradually, but the impact is always present and can be felt. The attacks by free radicals must not be underestimated: Every single one of our 70 billion cells is attacked by free radicals an average of approx. 10,000 times a day.

Oxidative stress affects physical performance activity and concentration powers; it also leads to premature aging and various illnesses. It has been scientifically proved that free radicals can be linked to almost 90 per cent of illnesses and to premature aging. How fast our organism ages, depends, on the one hand, on the quality of our genetic material (body's own enzymes), and on the other, on our way of life, above all our nutrition.

## Significant reduction of free radicals!

The zeolite used for Panaceo Sport is considered one of the strongest natural free-radical fighters. The agents of the volcanic mineral very quickly counteract the aggressive oxygen compounds which attack our cells.



The study showed an average reduction of free radicals in the blood by 20.1 % if Panaceo Sport was taken.

All figures expressed in U.Carr., measured with Free Radical Analytic System (FRAS).

200-300 U.Carr = healthy  
over 300 U.Carr = oxidative stress

## Zeolite as an antioxidant

Nature has developed a self-defence system against free radicals, the **antioxidants** or free-radical scavengers. Important antioxidants are certain vitamins, minerals and enzymes. These substances quickly counteract aggressive oxygen compounds binding them before they can cause damage. The mineral used for Panaceo Sport the tribomechanically activated zeolite is one of the strongest known natural antioxidants.

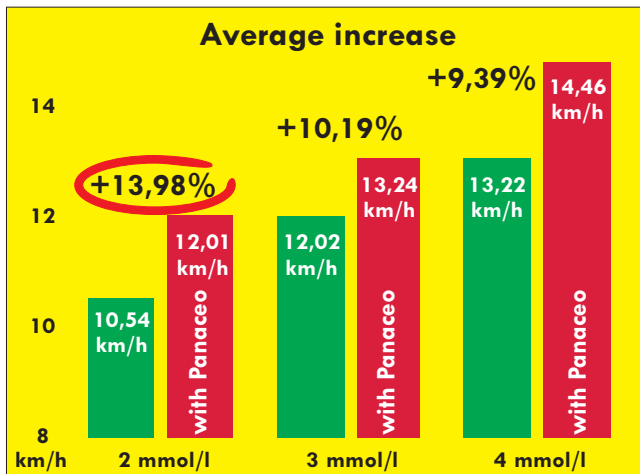
Panaceo Sport has the unique capacity of **neutralizing free radicals and filtering the body's own harmful substances**. With the ability to adsorb and absorb body toxins and the exceptional antioxidant properties, Panaceo Sport boosts the body's defence and detoxification systems, supporting self-cleansing processes.

## Reduction of lactate levels

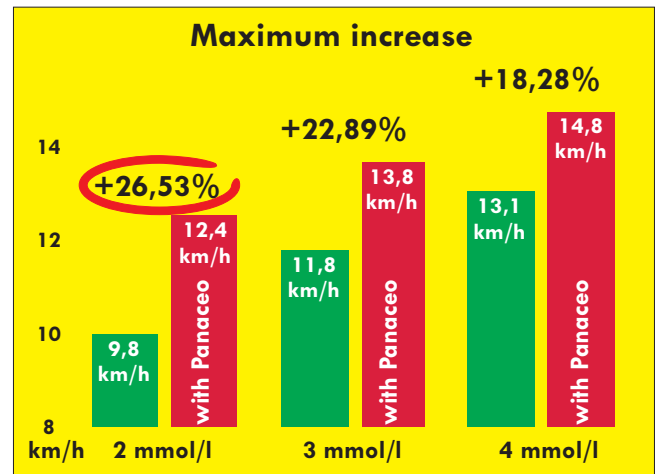
In cooperation with the Carinthian Athletics Club (Kaerntner Leichtathletik Club or KLC) and 24 athletes a randomised, double-blind, placebo-controlled trial was carried out. This trial proved another remarkable feature of Panaceo Sport: Those athletes who were given Panaceo Sport showed an average **increase in performance** by 13.98 % (which corresponds to approx. 1.6 km/h) at 2 mmol/l lactate.

Lactate is a by-product of the anaerobic metabolism. This substance is produced during heavy exercise while the muscles cannot be supplied with sufficient oxygen by the lungs and the blood circulation in order to produce energy. The concentration of lactic acid in blood is directly related to the physical capacity and performance of an athlete. In order to achieve the best training results and to avoid over-training, excessive stress and injuries lactate values should not exceed certain levels.

During normal activity, lactate concentration in blood is at about 1 mmol/l. During training this value can climb up to 20 mmol/l. The aerobic threshold is at about 2 mmol/l and the anaerobic threshold at approx. 4 mmol/l. The range between these two thresholds is the optimum range. By taking Panaceo Sport athletes will be able to increase their physical capacity without exceeding the aerobic threshold.



The group which was given Panaceo Sport showed an average increase in performance of 13.98 % at 2 mmol lactate.



Without exceeding the lactate threshold of 2 mmol, maximum increase was 2.6 km/h

## Calcium und Magnesium in their purest form

Dolomite is a mineral frequently found in central and southern Europe. It consists of magnesium and calcium in their pure natural forms. Dolomite which is one of the ingredients of Panaceo Sport contains the right ratio of magnesium and calcium in their pure natural form. Therefore, the dolomite does not have to be processed technologically or chemically.

Due to their physical activity, athletes have an increased need for calcium and magnesium which cannot be covered by food only. Mineral deficiency manifests itself in fatigue, slow reaction and cramps. Numerous studies have proven the eminent importance of an adequate calcium and magnesium supply of the human body.

Furthermore, a healthy magnesium-calcium balance is increasingly considered an important prerequisite for a fully functional brain and optimal mental performance.

### Calcium and magnesium

- stimulate physical fitness
- are essential for metabolism
- strengthen body tissue
- boost muscular performance
- optimise mental performance

## Maca the Peruvian Ginseng

Maca, or Peruvian Ginseng, is an energy food frequently used by the Incas. Maca root contains many valuable proteins, all the essential amino acids and significant amounts of iron, zinc, magnesium, calcium, carbohydrates, phosphorus, sugar, starches, important minerals and almost all vitamins. Maca has traditionally been used as an aphrodisiac, therefore in some regions of the Andes its consumption is still restricted to married men and women only. Maca root is said to help against insomnia and menopause syndrome. It also boots physical and mental performance, lowers blood pressure, and helps against anxiety and stress.

### Maca

- gives power and energy
- revives
- stimulates stamina

## Royal Jelly - the nourishment of queen bees

Royal Jelly is the only food given to bee queen larvae. While worker bees have a six-week life span, queens live three to five years and grow to enormous sizes. The queen can lay as many as two thousand eggs a day which is more than her own weight. Royal Jelly is considered one of the richest foods containing a high concentration of all vital substances.

Studies have proven its life prolonging, cholesterol-lowering, blood pressure regulating and invigorating effect. Royal Jelly also exhibits bactericidal and viricidal activities. The queen's sustenance improves the resistance of the organism, supports oxygen intake of the tissue and has a balancing effect.

### Royal Jelly

- increases the body's resistance
- supports oxygen intake of the tissue
- has a balancing effect

## New Energies

Due to its ingredients and to how they are processed, Panaceo Sport adsorbs and binds toxin substances. This way energies which otherwise would have been used for detoxication and regeneration, are now set free for a better performance. The result is **improvement in performance** which is achieved in a natural and healthy way. Clinical trials and sport science tests have shown that the regular use of Panaceo Sport results in:

increased stamina

better performance

faster regeneration

vitality and well being

strengthening the immune system

reduction of lactate levels

considerable reduction of free radicals

creased transport of oxygen into muscles and tissue





***"I take Panaceo Sport in capsules during training, while I also mix Panaceo Basic powder into my isotonic drink. At the first competition I clearly felt the positive effect."***

Markus Kröll, the world's number three in WMRA, six-time Dolomitenmann winner



***"I have started taking Panaceo Sport regularly this year. I can only confirm the positive effect, which in the meantime has been backed by a scientific study."***

H. Totschnig, professional bicyclist



***"Before taking Panaceo Sport I lagged more than 4 minutes behind my strongest competitor. For the next race I started taking Panaceo Sport and was able to get an advantage of 2 minutes over her."***

Karoline Käfer, several-time 400-meter Austrian champion



***"During a 1200 km long-distance bicycle race in Canada I took Panaceo Sport every half hour. I won the race with an advantage of more than 4 hours. The regenerating effects were convincing. The following day I was able to run at a reduced pace for one hour."***

Othmar Altmann, extreme bicyclist



***"I learned about Panaceo Sport from my tennis double partner Andrea Fendrich. We have both experienced that our concentration increased. Regeneration and performance have improved noticeably."***

Ingrid Resch, women's tennis world champion +40



***"I took Panaceo Sport during my world record race as well as during training. Lactate tests, carried out by my sports physician Dr. Markus Wenger-Öhn during training sessions, showed a reduction of lactate levels of up to 30%."***

Hubert Gantioler, world record holder in 24-hour mountain running (2003)

**Private Hospital Villach**

## **CLINICAL TRIAL**

FRAS Test for proving the antioxidative effect of Panaceo

## 1. Partners

The partners involved in this clinical trial are represented by the following:

Dr Wolfgang Thoma, head of the Private Hospital Villach  
Dr Claudia Gunzer, certified expert, head of the laboratory for genetic engineering,  
Private Hospital Villach  
the managing partner of Panaceo International Active Mineral Production GmbH

## 2. Objectives

In the course of this trial we intend to prove the antioxidative effect of the active volcanic mineral zeolite (tribomechanically micronised clinoptilolite) in the human organism, and its significance for the immune system.

The influences of activated zeolites on the concentration of free radicals in human body are to be analysed.

## 3. What are free radicals?

**Free radicals** are instable and highly reactive, they may attack cellular components (mainly cell membranes and DNA), and cause damage as well as transformations. They are created within the body, e.g. during breakdown of amino acid and fats, or during oxidation reactions (metabolism). They can also enter the body from outside and have diverse sources: **unhealthy diet, environmental pollution, cigarette smoke, waste gases, radiation, heavy metals, cytostatic drugs, pesticides, solvents, ozone, petrol fumes, etc.** Physical stress also leads to an increased production of free radicals in the organism (e.g. athletes).

Free radicals are very aggressive and highly reactive molecules, which react quickly with other compounds and start a chain reaction. Once the process is started, more and more free radicals are formed. Free radicals attack the body's own cells, damage them and might even affect their function.

An excessive number of free radicals destroys the natural balance between free radicals and antioxidants, and will finally lead to "oxidative stress". Today, most of the endemic diseases such as cancer, arteriosclerosis, coronary heart conditions and even rheumatism are considered to be directly linked to "oxidative stress". 1)

Antioxidants protect cells against free radicals by acting as scavengers and by stopping the chain reaction. They neutralise free radicals.

The number of free radicals in the body has to be reduced to a healthy level. The human organism has already a clever system to handle free radicals and to neutralise them. Certain enzymes like catalysis, superoxide dismutase and GSH-Px, as well as external scavengers like vitamin C, vitamin E, beta-carotene, vitamin A, etc. fulfil this task. Still, the body's own defence mechanism cannot cope with the free radicals and therefore antioxidants have to be taken. According to latest research, dietary supplements do make sense, since vitamins and trace elements cannot be stored by the body.

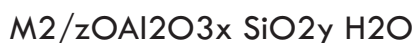
Activated volcanic minerals can help to protect the organism against harmful effects of free radicals.

Professor Umberto Cornelli from Loyola University in Chicago found out that oxidative stress is an independent risk factor and therefore it can intensify a number of pathological conditions. Controlling the oxidative status by dieting or by taking antioxidants improves pathological conditions.

1 See also: „Spektrum der Gesundheit 3/2000 “: Antioxidantien und freie Radikale - ein Balanceakt (Antioxidants and free radicals balancing act).

## 4. About the products

Zeolites are an abundant group of crystalline silicates. They are alkaline aluminium silicates containing water molecules and alkaline earth metal aluminium silicates, with the general formula:



**M** can stand for a monovalent or polyvalent metal, hydrogen, ammonium ion etc., **z** is for the value, = 1.8 to approx. 12 and = 0 to approx. 8.

The water can be forced from most zeolites by heat, without changing the structure of the crystal, and other solutions can be forced through the zeolite instead of the water. Therefore zeolites can be used as ion exchangers, especially for ammonium ions and catalysts.

The crystal lattice structure of zeolites consists of silicon and aluminium oxide tetrahedrons, which are bound together by oxygen bridges. Thus a well-defined structure is formed with void space identical cavities connected to each other by channels. Small molecules can enter these cavities and be adsorbed. There are over 30 different types of natural zeolites, which can be divided in three main groups, according to their crystal lattice structure: chain-like structures (e.g. Laumontite, Mordenite), sheet-like structures (e.g. Heulandite, Phillipsite) and framework zeolites (e.g. Faujasite, Chabazite) (MUMPTON and FISHMAN 1977).

For tribomechanical processing the crystalline zeolite, clinoptilolite (also known as heulandite) is used, due to its absorption capacity and selectivity, as well as its high potential as an ion exchanger. This mineral poses no risk to human health a fact that has been proved by analysis of its chemical composition and by toxicological tests carried out by expert scientists.

Average chemical composition of clinoptilolites

<b>Components from (%) to (%)</b>		
SiO <sub>2</sub>	61.96	67.17
TiO <sub>2</sub>	0.15	0.32
Al <sub>2</sub> O <sub>3</sub>	12.46	15.12
Fe <sub>2</sub> O	0.98	2.05
MnO	traces	0.05
MgO	1.30	1.96
CaO	3.03	4.35
Na <sub>2</sub> O	0.70	1.11
K <sub>2</sub> O	0.78	1.32
H <sub>2</sub> O at 100 °C	4.05	4.74
H <sub>2</sub> O at 1000 °C	7.56	9.56

## **Description:**

Tribological engineering:

Field of mechanical engineering dealing with physical and mechanical changes of materials under the influence of mechanical forces.

Tribomechanical micronisation:

A process for increasing reactivity and enlarging physical surface of materials by using mechanical forces (shock, friction).

Activity:

Degree of reactivity of a material.

## **5. Form and dosage:**

The subjects of the clinical trial took 3 tablets 3 times daily (600 mg active agent zeolite/tablet) or 2 teaspoons powder 3 times daily.

## 6. Method: F.R.A.S. (Free Radical Analytical System)

Free radicals in blood may damage cells. Lipids, proteins, amino acids etc. oxidise. This oxidation process leaves behind by-products (hydroperoxides), which can be detected by the F.R.A.S. The number of radicals corresponds to the number of hydroperoxides.

A drop of the subject's capillary blood is mixed with the reagent. The colour will change depending on the number of oxidised lipids. A photometer will analyse colour intensity and will calculate the value of the oxidative stress results are expressed in U.CARR (Carratelli units).

## 7. Results of the clinical trial

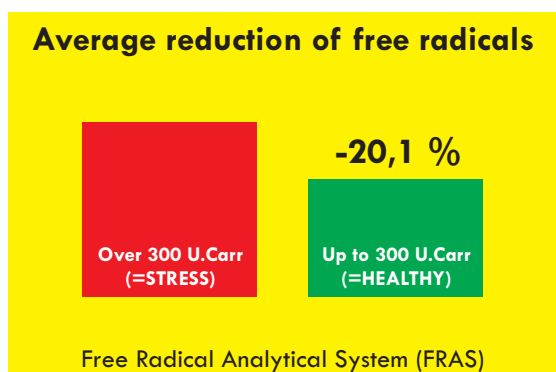
33 subjects took part in the clinical trial.

The average change per unit was **63 U.CARR** in case of persons with illnesses, and **83 U.CARR** in case of healthy persons (see also attached chart).

## 8. Conclusion

None of the tests carried out so far by the producer of FRAS equipment involving antioxidants such as vitamin C or vitamin E did show an improvement of U.CARR values. All values were within the measurement tolerance of 5%. The results of this trial show an average improvement of approx. 20 % - therefore the zeolites used in Panaceo can be regarded as very effective free-radical scavengers.

### Diagram showing the results:



The study showed an average reduction of free radicals in the blood by 20.1 % if Panaceo Sport was taken.

All figures expressed in U.Carr., measured with Free Radical Analytic System (FRAS).

up to 300 U.Carr = healthy  
over 300 U.Carr = oxidative stress

**In all subjects, a reduction of free radicals could be detected.**

**In 49% of the cases the stress of 300 U.CARR (=stressed) could be reduced to a value between 200 and 299 U.CARR (=healthy).**

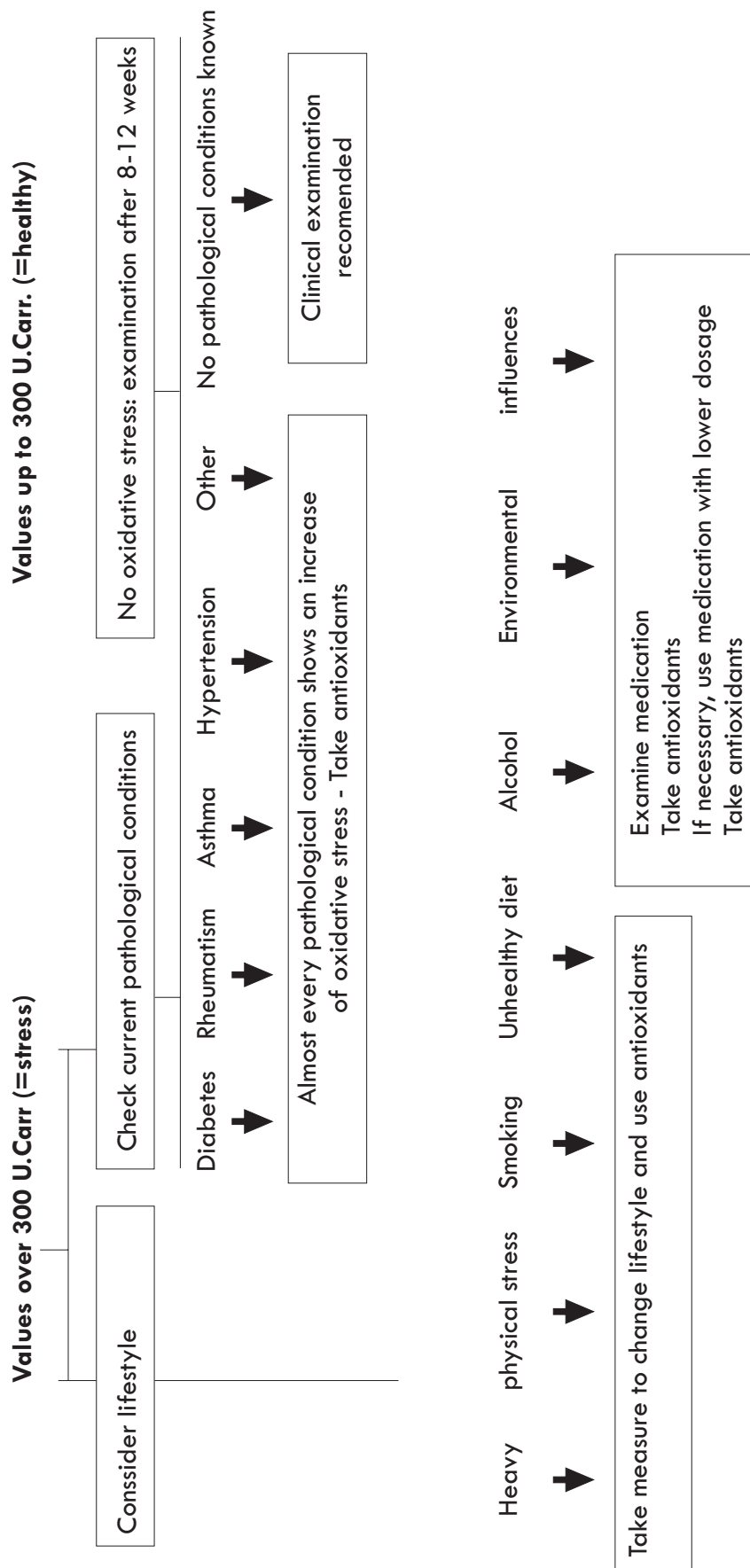
Date of birth	Weight kg	Size cm	1st check	Value	2nd check	Value	Improvement	%
2.6.1960	76	179	27.3.2002	325	8.5.2002	282	43	13,23%
3.3.1962	51	160	11.1.2002	395	13.2.2002	289	106	26,84%
16.4.1952	80	178	27.3.2002	404	2.5.2002	277	127	31,44%
24.5.1943	75	174	27.3.2002	383	24.4.2002	329	54	14,10%
			27.3.2002	327	24.4.2002	234	93	28,44%
			17.1.2002	498	31.1.2002	400	98	19,68%
20.2.1980	60	165	27.3.2002	580	8.5.2002	446	134	23,10%
21.8.1948	86	185	29.11.2001	395	20.12.2001	334	61	15,44%
6.12.1965	62	174	17.4.2002	382	21.5.2002	298	84	21,99%
			21.2.2002	450	25.3.2002	389	61	13,56%
			30.11.2001	408	17.12.2001	329	79	19,36%
10.8.1945	83	172	27.3.2002	324	24.4.2002	249	75	23,15%
4.10.1972	63	172	10.4.2002	527	16.5.2002	423	104	19,73%
			30.11.2001	359	20.12.2001	347	12	3,34%
			4.12.2001	525	20.12.2001	427	98	18,67%
15.9.1940	92	183	11.4.2002	342	13.5.2002	286	56	16,37%
27.12.1960	69	161	27.3.2002	410	8.5.2002	310	100	24,39%
18.6.1949	74	178	27.3.2002	364	24.4.2002	279	85	23,35%
			6.2.2002	430	21.5.2002	298	132	30,70%
14.7.1948	60	168	4.4.2002	386	14.5.2002	376	10	2,59%
1.12.1966	80	172	27.3.2002	323	24.4.2002	262	61	18,89%
22.1.1964	51	164	10.4.2002	466	14.5.2002	308	158	33,91%
							<b>83</b>	<b>20,10%</b>

Date of birth	Weight kg	Size cm	Comments	1st check	Value	2nd check	Value	Improvement	%
29.1.1948			HepC, smoker Birth control pill, acne	23.11.2001	377	30.11.2001	302	75	19,89%
14.12.1930	90	186	Vaskulitis	21.1.2002	657	15.2.2002	463	194	29,53%
27.10.1943	61	156	Mamma Ca	18.4.2002	335	14.5.2002	250	85	25,37%
				7.12.2001	402	21.12.2001	364	38	9,45%
				27.2.2002	407	12.3.2002	391	16	3,93%
				15.4.2002	450	7.5.2002	400	50	11,11%
13.9.1963	54	166	Asthma Migraine	11.4.2002	454	26.4.2002	403	51	11,23%
			rad.prost.ekt	17.1.2002	378	14.2.2002	328	50	13,23%
24.3.1956	60	165	Ca, Chemotherapy	29.11.2001	480	4.4.2002	418	62	12,92%
			Bypass	5.4.2002	471	8.5.2002	452	19	4,03%
11.3.1942			MammaCa	20.12.2001	394	2.1.2002	339	55	13,96%
								63	14,06%

Method to be applied: FRAS (Free Radical Analytical System) for monitoring changes, expressed in U.CARR.

## Guidelines for monitoring oxidative stress

Oxidative Stress = cell destruction. Mehtod to be applied: FRAS  
(Free Radical Analytical System) for monitoring changes, expressed inU.Carr.





**An investigation of the antioxidant activities of PANACEO on the basis of a study conducted by Dr. Peter M. Abuja, university lecturer at the “Institut für Biophysik und Röntgenstrukturforschung”, Schmiedlstrasse 6, A-8042 Graz, Austria**

The purpose of the study was to investigate the antioxidant activities and the effect mechanisms of activated Zeolith of the pharmaceutical company PANACO International GmbH. The test samples comprised pure activated Zeolith in the standard trade packaging (capsules of pure activated Zeolith). The capsules were opened for the purpose of the investigation and the powder contained was used without further preparation.

Use was made on the one hand of the DPPH test in which the oxido-reduction of an antioxidant with the stable DPPH radical is used, measuring the characteristic as a direct anti-oxidant, while on the other hand various emulsion oxidation approaches imitating the biologically important lipid-peroxidation processes (the oxidation of lipid proteins, lipid emulsions, tissue homogenates) thus measuring the effect on the development of free radicals, and thus to what extent Panaceo can already have an effect in this approach.

## Interpretation of the results:

**An antioxidant effect is observed in the peroxy-radicals developed from AAPH that is probably derived from the ability of the Zeolith to bind cations** (the relevant peroxide radicals are cationic). **PANACEO results in an extension of the lag-phase per mg by some 120%.** The results for  $\text{Cu}^{2+}$  catalysed lipid peroxidation are even more significant. Since  $\text{Cu}^{2+}$  **is already effective in low concentrations**, the concentrations used lead to an approximately equal **delay in the oxidation of around 100**. Here too the cation binding capacity is most likely decisive, whereby **even small quantities of PANACEO probably binds all the accessible  $\text{Cu}^{2+}$  in the system.** The observed lipid peroxidation is thus the result of transitional metal ions for which PANACEO is not accessible. The binding capacity for transition metals should be determined separately by another method as a result of the difficulties involved in diluting adequately.

**The oxidation of the muscle tissue is also delayed by some 50% by PANACEO**, here too a real concentration dependence is not given, this having similar reasons to those for  $\text{Cu}^{2+}$ -catalysed lipid peroxidation. In the case of Met-Hb the embedding of the oxidant in the protein lipid matrix certainly leads to a still further significant reduction in the oxidation speed.

In those cases where emulsions (soya lipids or muscle tissue) where are oxidised by metal ions or metmyoglobin, the accessibility of the oxidant for the insoluble Zeolith clearly plays a special role.  **$\text{Cu}^{2+}$  that is freely soluble in water or met-Mb is inactivated by PANACEO**, while the oxidant deposited or stored in/on the emulsion droplet is not captured. The DPPH test showed that the PANACEO Zeolith did not react directly with the stable radical.

**Since gastric lipid peroxidation is a probable significant factor of influence for oxidative stress as a result of nutrition, substances that check this process are to be evaluated as interesting.** As a result of the insolubility of PANACEO a direct systemic effect does not occur as a result of absorption into the blood circulation, but a delayed/reduced development of lipid peroxidation products. Panaceo Zeolith thus already intervenes during the development of free radicals. It is to be assumed that a capsule taken after a meat meal will slow down the gastric lipid peroxidation by around a half. **This effect is to be greatly valued since PANACEO not only delays the formation of lipid hydroperoxides, but can then**

**subsequently also bind the transition metals that are released (in the intestinal tract). The antioxidant effect is thus of longer duration than that achieved with vitamins C and E, that have a more short-term direct effect in the blood.**

A direct comparison of PANACEO with the “classical” anti-oxidants is not practicable on account of the insolubility of the Zeoliths. The cation binding capacity of Panaceo Zeolith for transition metal ions and Met-Hb is so high that a very substantial effect could be established even with the smallest laboratory quantities. No further concentration dependence could be established in the technically possible concentrations made in experiment.

**In summary it can be established that PANACEO is effective through its ion exchanging capability, and this even with very high ion concentration strengths and low pH values. PANACEO is an anti-oxidant that reduces the catalytic formation of radicals by transition metals and this both in pure lipid emulsions as also in more complex systems. As a result Panaceo has an indirect effect that leads to a decrease in the formation of free radicals thus retarding the development of oxidative stress at an early phase. It is to be assumed that Panaceo in contrast to vitamins C and E does not have a short-term effect on free radicals direct in the blood, but has an indirect effect in that it halves the development of free radicals in the gastrointestinal tract.**

### ***Lipid peroxidation***

#### **Principle of measurement**

Polyunsaturated fatty acids, PUFAs, in natural lipids are oxidation sensitive. Some specific lipophile antioxidants (e. g.  $\alpha$ -tocopherol = vitamin E, VE) have an effect as antioxidants and prevent lipid peroxidation (LPO), which was measured in the present case by means of the oxygen consumption of an oxidising oil-in-water emulsion. Emulsions of PUFAS and VE under continuous antioxidant stress show a so-called lag-phase of low oxygen absorption speed in which the antioxidants are first used up and only after this is the lipid matrix oxidised, which is shown in the significant increase in the oxygen absorption. The duration of the lag-phase is dependent on the antioxidant concentration and the intensity of the oxidative stress. Additional antioxidants extend the lag-phase, or reduce the oxidation speed generally, depending on the mechanism involved.

In the present investigation the oxygen concentration in a watery solution (stirred, 37°C) was determined by the fading of the fluorescence of a dye material by a sensor measurement. This

method has an advantage over electro-chemical methods in that it is entirely insensitive to oxido-reduction systems in the test sample. Moreover the visual characteristics of the test sample are of no significance for the measured result as a result of the specific configuration.

**Experimental procedure:**

The relevant quantity of Zeolith (0, 0.625, 1.25, 2.5, 5 mg/ml) was suspended in a PBS emulsion (20 mM phosphate buffer, pH = 7.4, 130 mM NaCl, 1 mg/mL soya lipids, 7.5  $\mu$ M  $\alpha$ -tocopherol). The suspension was saturated with air oxygen at 37°C, the oxidant (10 mM AAPH, 50  $\mu$ M Cu<sup>2+</sup>) was added, the reactor vessel was closed and the oxygen consumption observed and timed.

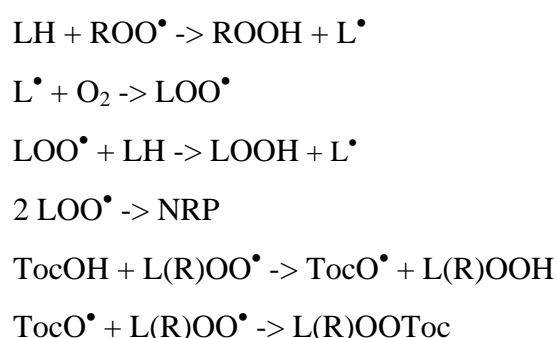
A problem arose as a result of the rapid sedimentation that prevented a reliable dilution series from being made – all concentrations were made by direct weighted sample and concentrations under 0.5 mg/mL are not possible by this means.

## Excursus peroxidation

The fatty acids can be subject to chemical reactions in the presence of oxygen when subject to the effects of certain hormones or in the absence of certain vitamins or trace elements or in the process of aging that can transform their unsaturated compound bonds into unstable elements termed peroxides. These unstable fats are particularly poisonous for the cells and can lead to cell death. This cell death process can in itself lead to other oxidation reactions and bring about chain reactions, the avoidance of which is of very particular importance. Most edible oils and unsaturated fats (e.g. butter) are transformed into peroxidised fats as a result of frying and cooking processes. Lipid peroxidation is for example the combustion of fats to unstable elements.

### *Lipid peroxidation with AAPH*

AAPH (2,2 azobis (dimethylaminopropane hydrochloride) is a thermo-labile radical generator that releases peroxy radicals in accordance with a kinetic 1. order ( $v = k * [AAPH]$ ). During the time period of the experiment this represented a constant radical flow and thus a constant oxidative stress. The water soluble peroxy radicals reacted with the emulsion particles and also with any other components present in the solution/emulsion/suspension:



(LH: PUFA,  $ROO^{\bullet}$ ,  $LOO^{\bullet}$ : peroxy radical (L: from PUFA, R from AAPH),  $L^{\bullet}$ : lipid radical, NRP: non-radical product, TocOH:  $\alpha$ -tocopherol (vitamin E),  $TocO^{\bullet}$ : tocopheroxyl radical;  $L(R)OOToc$ : peroxytocopheron)

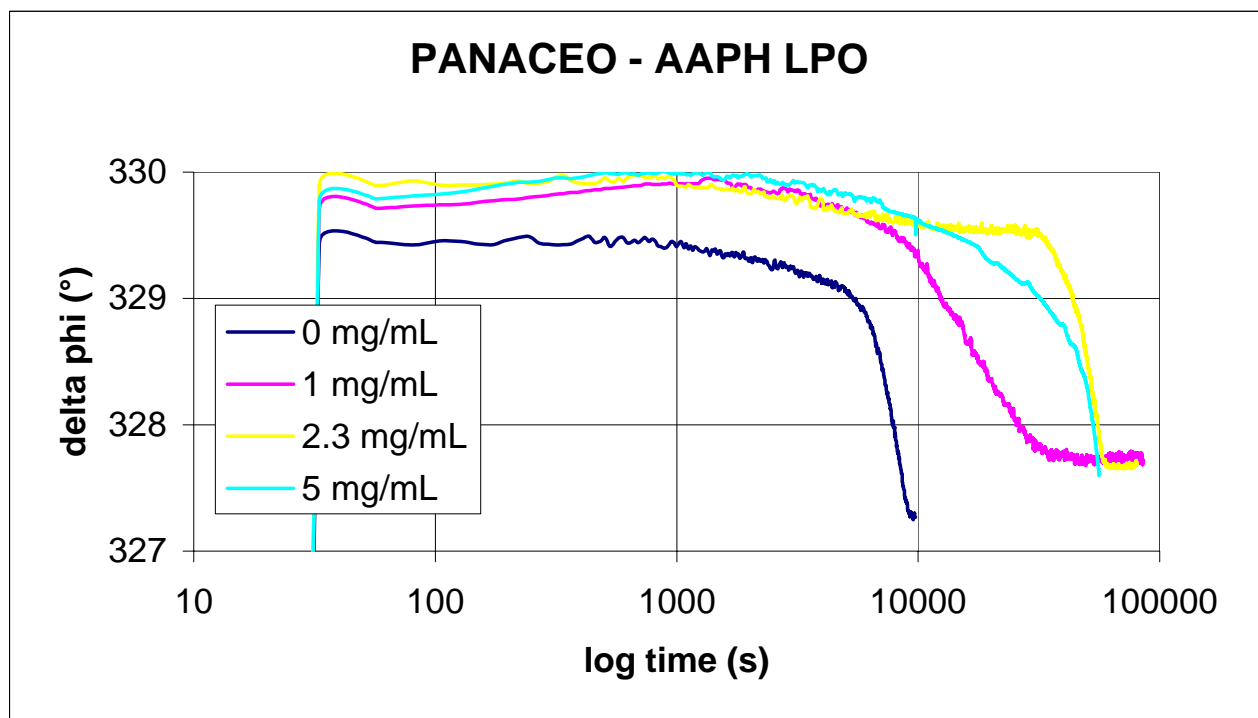
A considerable extension of the lag-phase was observed in dependence on the quantity of PANACEO in application (s. Table 1, Fig. 1), and PANACEO can thus delay lipid peroxidation by water-soluble peroxy radicals.

Table 1:

[PANACEO] mg/mL	t-lag [min]	Vmax [hPa O <sub>2</sub> / min]	% increase of lag	% increase / mg
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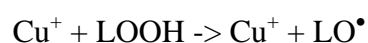
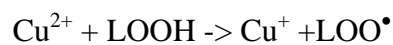
0	104	2.13	0	
1	160	0.38	53.8461538	53.8461538
2.3	529	0.35	408.653846	177.675585
5	818 --		686.538462	137.307692
				122.943144

Fig. 1:



### *Lipid peroxidation by means of Cu catalysis:*

Cu<sup>2+</sup> ions can lead to the catalytic formation of radicals from lipid hydroperoxides (Haber-Weiss reaction)(1)



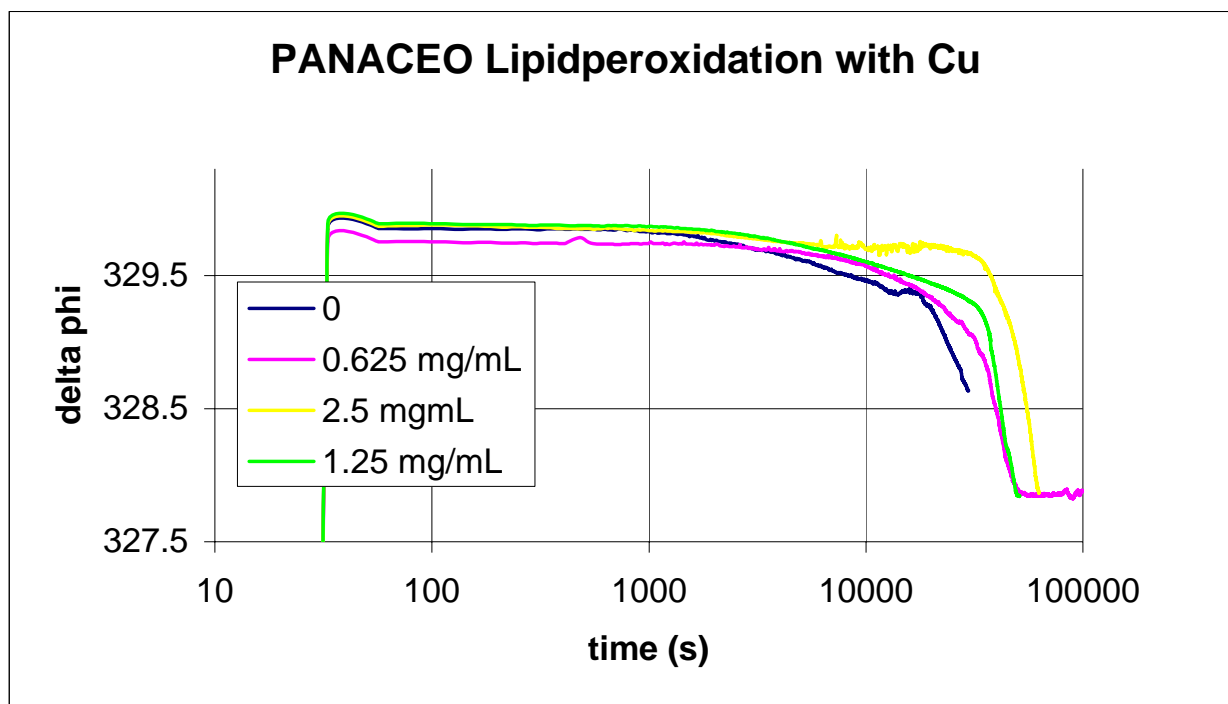
whereby both of the radicals formed (LO<sup>•</sup>: lipidalkoxyl radicals) are able to induce lipid peroxidation.

The addition of PANACEO to the reaction emulsion in the concentrations as given (0, 0.625, 1.25, 2.5 mg/mL) resulted in a significant extension of the lag-phase.

Table 2:

[PANACEO]	lag-time (min)	% increase lag	%/mg
0	299	0	
0.625	583	94.9832776	151.973244
1.25	588	96.6555184	77.3244147
2.5	678	126.755853	50.7023411

Fig. 2:



### ***Muscle tissue oxidation:***

#### **Principle of measurement**

The gastrointestinal tract is probably a major site of antioxidant stress (2), and the oxidation of muscle tissue by metmyoglobin in the acid milieu of the stomach can result in the production of significant quantities of oxidised lipids, which in turn can lead to the formation of radicals through the Haber-Weiss reaction. Gastric lipid peroxidation is simulated in this test and the oxygen requirement measured.

#### **Experimental procedure:**

Minced beef (approx. 30g) was placed in a thin layer (approx. 5mm) without the addition of extra fat in a Teflon-coated pan, fried until medium brown on both sides and then homogenised in distilled water (100 mg meat/mL water) and brought to pH = 4.0 with the addition of HCl. The samples were frozen in test portions (-80°C) and thawed before use. The metmyoglobin that is formed under these conditions (3) forms lipid peroxide with the PUFAs

in the muscle tissue under the conditions existing in the stomach. This reaction can be neutralised by antioxidants (polyphenol in particular). The count evaluated is the initial oxidation speed, since a lag-phase does not necessarily occur.

**Table 3:**

[Panaceo] mg/mL	Vox (hPa/min)
0	0.588
0.625	0.274
2.5	0.387
5	0.28

### ***DPPH test***

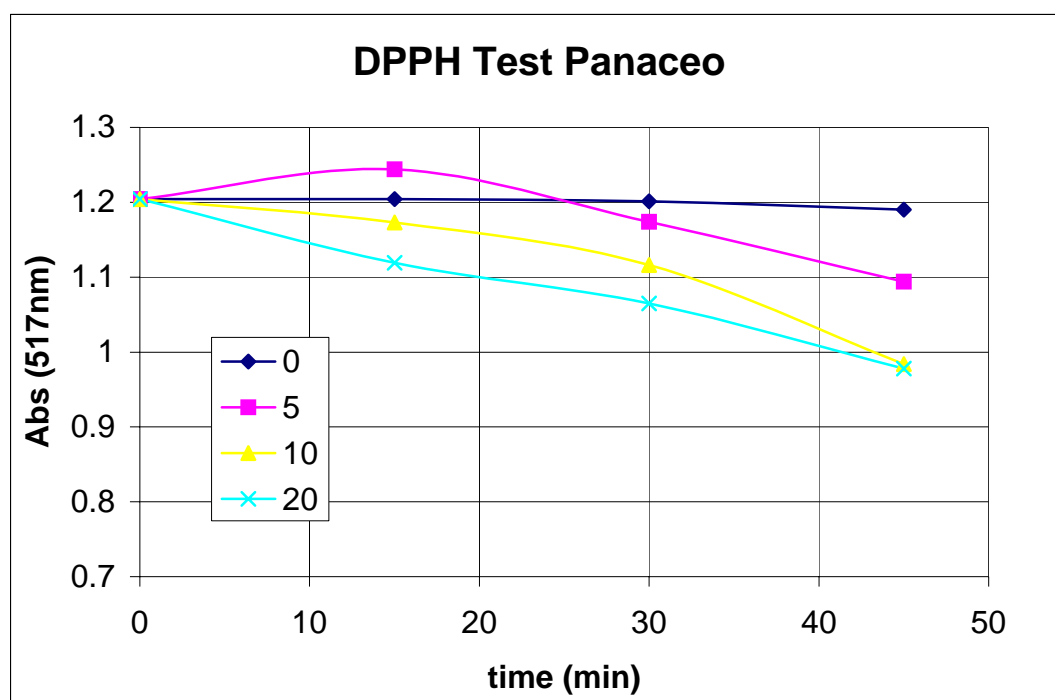
#### **Principle of measurement**

DPPH (1,1-diphenyl-2-picryl-hydrazyl) is a stable radical that is slightly soluble in methanol. It has a strong blue-violet colouration as a result of the unpaired resonance-stabilised electrons ( $\lambda_{\text{max}} = 517 \text{ nm}$ ). Antioxidants that can react with radicals (radical catchers) colour DPPH which is considered to be a proof of radical catcher characteristics.

#### **Experimental procedure:**

The quantity of Zeolith (0, 5, 10, 20 mg/ml) was suspended in a DPPH solution (20  $\mu\text{L}$  saturated methanol solution in double-distilled water) and incubated for 10 minutes at room temperature. Following this the Zeolith was centrifuged for 5 minutes and the supernatant was then photometer measured. After this it was re-suspended and incubated for a further 10 minutes etc.





In the given conditions radical catchers lead to a very rapid colour fading of the DPPH solution (e.g. in a matter of seconds with vitamin C, the same too for vitamin E in ethanol), while the Zeolith was discoloured only by about 15% in 45 minutes. A direct link between the concentration and the colour fading speed could be observed. The changes observed are possibly the result of an easing of the combination of DPPH with itself or of other spontaneously formed radicals. Adsorption is also improbable, because here too a dependence on the Zeolith concentration must be shown.

#### References

1. Abuja, P. M., Albertini, R., and Esterbauer, H. (1997) *Chem. Res. Toxicol.* 10, 644-651.
2. Halliwell, B., Zhao, K., and Whiteman, M. (2000) *Free Radic. Res.* 33, 819-830.
3. Kanner, J. and Lapidot, T. (2001) *Free Radic. Biol. Med.* 31, 1388-1395.



Active Mineral  
**PANACEO**



**SPORT**

# LACTATE STUDY



INTERNATIONAL ACTIVE MINERAL PRODUCTION GMBH • 9585 Villach-Gödersdorf, Austria  
Order-Hotline: +43 4257-29064 • [www.panaceo.com](http://www.panaceo.com)  
Contact: [office@panaceo.com](mailto:office@panaceo.com)

Randomised, placebo-controlled,  
double-blind trial  
of the dietary supplement  
**PANACEO SPORT**  
for lactate levels and  
improvement of performance

## **Trial on how PANACEO SPORT influences lactate concentration in athletes**

### Details of the institution

Doctors: Dr Knapitsch, Prof Schmölzer  
Department: Dr Knapitsch and Prof Schmölzer's institute for sports medicine and sports  
sciences  
Location: St. Veiter Str. 180, A-9020 Klagenfurt, Austria

### Initiator of trial

Panaceo International Active Mineral Production GmbH  
Finkensteinerstrasse 5, A-9585 Gödersdorf  
phone: 04257 / 29064  
fax: 04257/ 29064-19  
e-mail: [office@panaceo.com](mailto:office@panaceo.com)

### Title of trial

Randomised, placebo-controlled, double-blind trial of the effect of PANACEO SPORT on  
lactate values in subjects during physical stress.

### Internal title and/or code number

### Lactate test

## **1 Notes on the necessity of the trial**

### **1.1 Objectives, trial design**

This trial proves that lactate values during physical stress can be reduced by taking PANACEO SPORT; and subjects who take PANACEO SPORT will be able to improve their performance without exceeding the lactate threshold.

### **1.2 Hypothesis**

Our hypothesis is that PANACEO SPORT reduces lactate concentration in the body, and prevents the production of more lactate thus increasing the performance considerably.

### **1.3 Endpoints of the trial**

The trial shows, that Panaceo Sport

- reduces lactate;
- boosts power and energy;
- revives;
- helps to regenerate faster;
- increases the body's resistance;
- stimulates stamina.

The objective of this trial is to confirm the above hypotheses, so that they can be used as the statement of positive effects of PANACEO SPORT.

### **1.4 Present scientific findings regarding the subject matter of the trial**

All tests carried out so far involving top athletes, produced excellent results. With PANACEO SPORT lactate concentration could be reduced by almost 30%. All subjects produced considerably better performances, had more stamina and needed less time for regeneration. Generally, the athletes felt better subjectively. Furthermore, they seemed less prone to illnesses and injuries.

### **1.5 Significance of the trial**

This trial will prove that for the first time a purely natural mineral can act as a very strong lactate buffer, thus leading to excellent performances.

## **2. Notes on the overall organisation of the trial**

### **2.1 General information**

#### **2.1.1 Type of trial**

A randomised, placebo-controlled, double-blind trial. That means subjects randomly receive placebo or active agent. Neither the subjects, nor those in charge of the tests know which group is receiving placebo and which active agent.

### **2.1.2 Schedule of the trial**

The trial was carried out in August 2004.  
Length of trial: 2 weeks

## **2.2 Subjects**

### **2.2.1 Inclusion/Exclusion criteria**

Inclusion criteria:

Male and female competitive athletes, who have some years of experience, and who are highly health-conscious.

Exclusion criteria:

Men and women who do not do sports on a regular basis.  
Ill or injured persons.

### **2.2.2 Number of subjects**

The total number of subjects is 24 persons, half of them receiving PANACEO SPORT and the other half placebo.

### **2.2.3 Biostatistical analysis**

In the field of sports athletes' bodies are more or less at the same level of performance. Considering the positive effects we have already obtained in the past, this number of subjects will certainly be sufficient for solid results.

## **2.3 The method specified**

Small blood samples will be taken from subjects in order to measure lactate concentration.

## **2.4 Implementation of the trial**

### **2.4.1 Description of the methods and the implementation of the trial**

- Athletes who will be our subjects receive randomly placebo/active agent (everyone takes a box from a case, where boxes containing placebo as well as boxes containing Panaceo Sport are kept).
- Neither the athletes themselves, nor the doctors in charge of the tests know who received placebo and who active agent. The only identification is a consecutive numbering on each box. Only after the end of tests will participants learn about the contents of the boxes.
- Before the first test, a thorough medical checkup will be made on each athlete by Dr Knapitsch to make sure that the subjects are able to do the tests. With the first test the actual condition of each subject is determined by measuring lactate concentration in blood. After this test the subjects take the boxes containing placebo/active agent.
- After the first test, subjects take 3 capsules Panaceo Sport 3 times daily with meals.

- After 7 days lactate concentration is measured again. On this day 12 capsules Panaceo Sport are taken 30 minutes prior to the test instead of the usual dosage of 3 times 3 capsules.
- After the test, subjects continue with 3 capsules Panaceo Sport 3 times daily with meals.
- After another 7 days lactate concentration is measured one last time. On this day 12 capsules Panaceo Sport are taken 30 minutes prior to the test instead of the usual dosage of 3 times 3 capsules.

## 2.5. Risk estimate

These tests pose no risk to participants.

## 4 Further information

Product information: PANACEO SPORT

PANACEO SPORT consists of activated zeolite, dolomite (calcium and magnesium), Maca and Royal Jelly.

### • Zeolite

Zeolites are natural microporous silicate minerals; they can be colourless, white or pale red. Zeolite consists mainly of silicon, one of the most abundant and important elements of the earth.

The crystal lattice structure of zeolites consists of silicon and aluminium oxide tetrahedrons, which are bound together by oxygen bridges. Thus a well-defined structure is formed with void space identical cavities connected to each other by channels. Small molecules can enter these cavities and be adsorbed (e.g. ammonium). Zeolites are very strong ion exchangers.

### • Maca

Maca, a plant from the Andes, was used by the Incas; it is considered one of the richest plants and therefore it is also called “Peruvian ginseng”.

### • Royal Jelly

Royal Jelly is the only food given to bee queen larvae. While worker bees have a six-week life span, queens live three to five years and grow to enormous sizes. The queen can lay as many as two thousand eggs a day which is more than her own weight.

### • Magnesium and Calcium

Absolutely essential for every athlete, and of eminent importance for the nervous system, the bone structure as well as the musculoskeletal system.

**ORIGINAL**

Dr. Christian Knapitsch FA für physikalische Medizin  
9020 Klagenfurt St. Veiter Strasse 180 Tel.: 0463/ 416 16

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**Evaluation of the double-blind trial of the dietary supplement  
PANACEO SPORT versus placebo for lactate levels**

Description:

From August 2, 2004 to August 17, 2004 a double-blind trial on measures for enhancing physical performance was carried out in Dr Knapitsch and Mr Schmölzer's institute for sports medicine and sports sciences (St. Veiterstrasse 180, A-9020 Klagenfurt).

A total of 24 subjects took part in this trial.

12 subjects received the dietary supplement PANACEO. Six of them were female and the other six male.

The other 12 subjects received placebos which have no effect. In this group four subjects were female and eight were male.

Subjects themselves took random boxes containing either placebo or Panaceo Sport (without knowing the content). This was supervised by Mr Poosch who also managed the course of the tests.

Test configuration:

August 2nd and 3rd, 2004 the first tests involving all subjects before taking Panaceo/placebo. The results obtained for lactate concentrations at the 2, 3 and 4 mmol/l thresholds are considered as the 100% reference value.

August 9th and 10th, 2004 second series of tests involving all subjects after one week of taking Panaceo/placebo.

The results at the 2, 3 and 4 mmol/l thresholds were compared with the 100% reference value.

August 16th and 17th, 2004 the third series of tests involving all subjects.

The results at the 2, 3 and 4 mmol/l thresholds were compared with the 100% reference value obtained after the first test.

The subjects were recruited from a athletic group; they have different performance levels. They did not change the length and intensity of their training sessions in the course of the trial

All tests on the treadmill were taken under the same conditions.

Blood samples were analysed using the BIOSEN5030 lactate analyser.

Female subjects started their training sessions with 6 km/h, after 3 minutes 20 seconds they had a break (blood sample from the ear lobe). After each break the speed was increased by 2 km/h. Male subjects started their training sessions with 8 km/h.



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For one of the female subjects, a top athlete, the speed was increased by 2 km/h from the start. For one of the male subjects, the speed had to be reduced by 2 km/h to the same level as the female group.

#### Form and dosage:

3 capsules 3 times daily, after the first test  
12 capsules, at once - 30 minutes prior to the test

#### Zeolite as raw material:

PANACEO Sport is activated zeolite, a powder made of volcanic ash and a silicon which has been activated by a special tribomechanical process. Research results obtained by Professor Pavelic (Rudjer Boskovic Institute for Molecular Biology Marburg) and Professor Colic (University of Santa Barbara) show that it is a strong antioxidant, immune modulator as well as an antiviral agent.

In the course of this trial we as sports scientists were mainly interested in the antioxidative effect of zeolite and in how it improves the performance.

Free radicals are highly aggressive; they attack and destroy cellular compounds. They are highly reactive molecules or atoms, with one or more unpaired electrons in their outer shell. Free radicals are formed by exposure to stress situations (radiation exposure, chemo therapy, electromagnetic pollution, excessive alcohol consumption, viral or bacterial infections, environmental pollution, as well as excessive physical stress etc.)

The body's own immune system often cannot cope with free radicals and so the organism is prone to their attacks. Antioxidants help to maintain our health.

An oxidation process, where oxygen is used, helps to buffer the negative effects of free radicals. PANACEO Sport replaces this process and helps to save oxygen, which means that

- 1.) ... you can exercise within your fat burning zone for a longer period, thus extending the 2 mmol threshold (aerobe / anaerobe).
- 2.) ... due to a better oxygen supply, heart rate will remain at a low level for a longer period and your training will be more efficient.
- 3.) ... taking PANACEO Sport continuously reduces the risk of rupture of a muscle fibre, since it enhances oxygen supply. It also prevents colds which would have negative effects on your training.

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Statistical evaluation:

### **PANACEO**

The average changes at 2 mmol/l of the initial value from the 1st test to 3rd test were 13.98 %.  
At the 3mmol/l threshold there was a difference of 10.19 % between the 1st and 3rd test.  
At the 4mmol/l threshold there was a difference of 9.39% between the 1st and 3rd test.  
The maximum improvement was 26.53 % at the 2 mmol/l threshold; 22.89% at the 3 mmol/l threshold and 18.28% at the 4 mmol/l threshold.

### **Placebo**

In the group who were given placebo, the values changed as follows:  
2.53% at the 2 mmol/l threshold;  
1.77% at the 3 mmol/l threshold, and  
2.30% at the 4 mmol/l threshold.

### **Interpretation:**

After revealing which group had taken PANACEO and which placebo, we found out that all subjects who had taken PANACEO showed a considerable increase in performance in lactate tests.

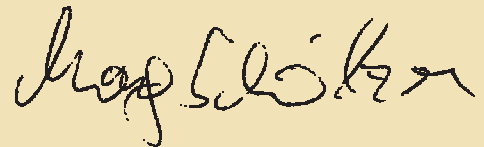
We were quite surprised by the results of this trial, since we usually expect such an increase in performance only after weeks of training. So it seems that taking PANACEO continuously will lead to better performance, as it has been proved by this trial.

By taking PANACEO free radicals are neutralised faster due to the oxidation process. Usually muscle metabolism consumes a lot of oxygen with PANACEO more oxygen is left for the muscle to use.

Dr Christian Knapitsch



Prof Siegfried Schmölder



## ACTIVE AGENT

Name	Test 1	Test 2	Test 3	Average change	Maximum increase
Average values	Test 1	Test 2	Test 3	Test 1-3	Test 1-3
2 mmol/l	10.54 km/h	11.65 km/h	12.01 km/h	13.98%	26.53%
3 mmol/l	12.02 km/h	13.02 km/h	13.24 km/h	10.19%	22.89%
4 mmol/l	13.22 km/h	14.14 km/h	14.46 km/h	9.39%	18.28%

Köck Barbara	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	7.00 km/h	7.90 km/h	8.60 km/h	22.86%
3 mmol/l	8.30 km/h	10.00 km/h	10.20 km/h	22.89%
4 mmol/l	9.30 km/h	11.00 km/h	11.00 km/h	18.28%

Kozak Erwin	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	15.50 km/h	17.60 km/h	17.60 km/h	13.55%
3 mmol/l	17.60 km/h	19.10 km/h	19.30 km/h	9.66%
4 mmol/l	19.10 km/h	20.20 km/h	20.40 km/h	6.81%

Daberer Maximilian	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	11.20 km/h	12.40 km/h	12.40 km/h	10.71%
3 mmol/l	13.10 km/h	13.60 km/h	13.90 km/h	6.11%
4 mmol/l	14.50 km/h	14.40 km/h	14.90 km/h	2.76%

Daberer Rosamunde	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9,60 km/h	9,60 km/h	10,20 km/h	6,25%
3 mmol/l	10,90 km/h	11,10 km/h	11,60 km/h	6,42%
4 mmol/l	11,80 km/h	12,20 km/h	12,60 km/h	6,78%

Daberer Christine	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	8,10 km/h	9,10 km/h	10,20 km/h	25,93%
3 mmol/l	9,80 km/h	10,50 km/h	11,50 km/h	17,35%
4 mmol/l	11,10 km/h	11,40 km/h	12,50 km/h	12,61%

Stocker Roland	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	14,20 km/h	16,00 km/h	15,90 km/h	11,97%
3 mmol/l	16,80 km/h	18,00 km/h	17,60 km/h	4,76%
4 mmol/l	17,90 km/h	19,00 km/h	18,70 km/h	4,47%

Vorwalder Alfred	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9.80 km/h	12.50 km/h	12.40 km/h	26.53%
3 mmol/l	11.80 km/h	13.90 km/h	13.80 km/h	16.95%
4 mmol/l	13.10 km/h	14.90 km/h	14.80 km/h	12.98%

Kautschitz Josef	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9.90 km/h	11.60 km/h	10.90 km/h	10.10%
3 mmol/l	12.20 km/h	14.40 km/h	13.70 km/h	12.30%
4 mmol/l	13.50 km/h	15.90 km/h	15.60 km/h	15.56%

Schmidt Charlotte	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	7.90 km/h	8.60 km/h	8.30 km/h	5.06%
3 mmol/l	9.30 km/h	9.80 km/h	10.10 km/h	8.60%
4 mmol/l	9.90 km/h	10.60 km/h	11.60 km/h	17.17%

Name	Test 1	Test 2	Test 3	Average change
Katsch Sabine	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	8.10 km/h	9.40 km/h	9.10 km/h	12.35%
3 mmol/l	9.70 km/h	11.20 km/h	11.10 km/h	14.43%
4 mmol/l	10.90 km/h	12.40 km/h	12.40 km/h	13.76%

Käfer Karoline	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	12.20 km/h	11.00 km/h	13.80 km/h	13.11%
3 mmol/l	13.20 km/h	12.50 km/h	14.90 km/h	12.88%
4 mmol/l	14.00 km/h	13.50 km/h	15.50 km/h	10.71%

Zirnig Dieter, jun.	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	10.30 km/h	11.00 km/h	11.00 km/h	6.80%
3 mmol/l	12.90 km/h	13.30 km/h	13.00 km/h	0.78%
4 mmol/l	14.10 km/h	15.00 km/h	15.00 km/h	6.38%

PLACEBOS				
Name	Test 1	Test 2	Test 3	Average change
Average values	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	12.13 km/h	11.85 km/h	11.82 km/h	-2.53%
3 mmol/l	13.56 km/h	13.63 km/h	13.32 km/h	-1.77%
4 mmol/l	14.68 km/h	14.77 km/h	14.35 km/h	-2.30%

Breitenhuber Martina	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9.30 km/h	8.50 km/h	9.60 km/h	3.23%
3 mmol/l	10.80 km/h	10.20 km/h	10.90 km/h	0.93%
4 mmol/l	11.80 km/h	11.30 km/h	11.70 km/h	-0.85%

Andretsch Nicola	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	10.70 km/h	11.60 km/h	11.30 km/h	5.61%
3 mmol/l	12.30 km/h	13.00 km/h	12.60 km/h	2.44%
4 mmol/l	13.50 km/h	14.00 km/h	13.40 km/h	-0.74%

Rasinger Lorenz	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9.50 km/h	9.50 km/h	10.30 km/h	8.42%
3 mmol/l	11.50 km/h	13.60 km/h	12.40 km/h	7.83%
4 mmol/l	13.20 km/h	15.10 km/h	13.80 km/h	4.55%

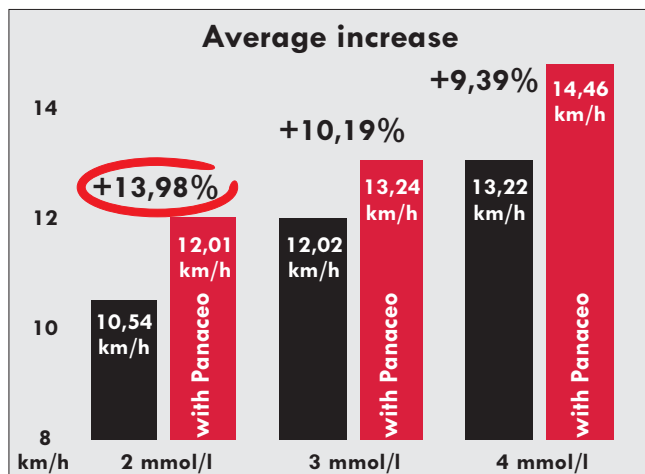
Doujak Valentin	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	15.00 km/h	15.00 km/h	15.10 km/h	0.67%
3 mmol/l	16.50 km/h	16.70 km/h	16.70 km/h	1.21%
4 mmol/l	17.40 km/h	17.80 km/h	17.80 km/h	2.30%

Kreimer Peter	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	12.00 km/h	12.40 km/h	11.60 km/h	-3.33%
3 mmol/l	13.50 km/h	13.70 km/h	13.70 km/h	1.48%
4 mmol/l	14.60 km/h	14.60 km/h	15.00 km/h	2.74%

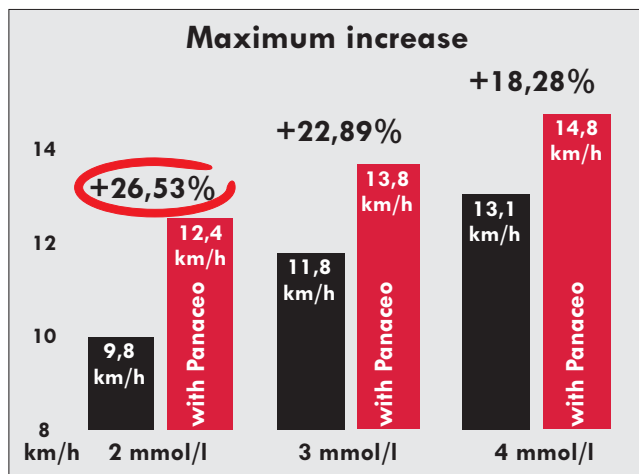
Hafner Richard	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	16.00 km/h	16.10 km/h	-	-
3 mmol/l	17.90 km/h	18.50 km/h	-	-
4 mmol/l	19.10 km/h	19.80 km/h	-	-

Name	Test 1	Test 2	Test 3	Average change
Roth Andreas	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	11.10 km/h	9.40 km/h	9.90 km/h	-10.81%
3 mmol/l	12.90 km/h	12.70 km/h	12.80 km/h	-0.78%
4 mmol/l	14.10 km/h	14.30 km/h	14.20 km/h	0.71%
Breitenhuber Herbert	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	15.70 km/h	14.00 km/h	15.60 km/h	-0.64%
3 mmol/l	17.20 km/h	16.00 km/h	17.20 km/h	0.00%
4 mmol/l	18.00 km/h	17.20 km/h	18.20 km/h	1.11%
Visconti Ursula	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	12.20 km/h	11.80 km/h	11.70 km/h	-4.10%
3 mmol/l	13.40 km/h	12.90 km/h	12.80 km/h	-4.48%
4 mmol/l	14.00 km/h	13.50 km/h	13.50 km/h	-3.57%
Hammer Eva	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	9.20 km/h	9.90 km/h	10.20 km/h	10.87%
3 mmol/l	10.70 km/h	10.90 km/h	11.10 km/h	3.74%
4 mmol/l	11.80 km/h	11.50 km/h	11.70 km/h	-0.85%
Hermann Leo	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	11.70 km/h	11.50 km/h	11.90 km/h	1.71%
3 mmol/l	13.10 km/h	13.30 km/h	13.30 km/h	1.53%
4 mmol/l	14.00 km/h	14.00 km/h	13.90 km/h	-0.71%
Schmidt Hubert	Test 1	Test 2	Test 3	Test 1-3
2 mmol/l	12.70 km/h	12.20 km/h	12.90 km/h	1.57%
3 mmol/l	14.30 km/h	13.90 km/h	14.40 km/h	0.70%
4 mmol/l	15.60 km/h	14.80 km/h	15.20 km/h	-2.56%

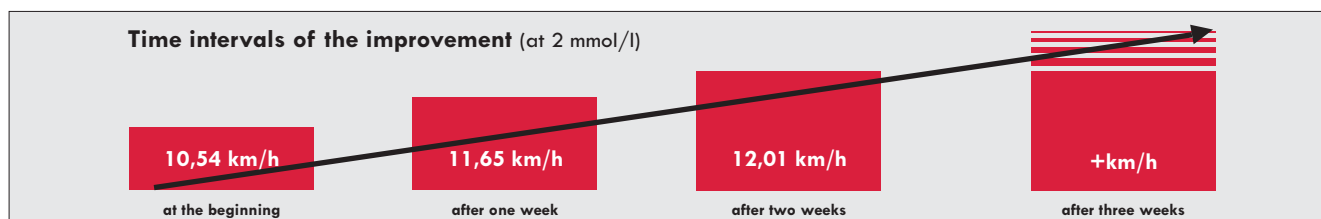
Diagram showing the results:



The group which was given Panaceo Sport showed an average increase in performance of 13.98 % at 2 mmol lactate.



Without exceeding the lactate threshold of 2 mmol, maximum increase was 2.6 km/h.



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## Natural zeolite clinoptilolite: new adjuvant in anticancer therapy

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**Abstract** Natural silicate materials, including zeolite clinoptilolite, have been shown to exhibit diverse biological activities and have been used successfully as a vaccine adjuvant and for the treatment of diarrhea. We report a novel use of finely ground clinoptilolite as a potential adjuvant in anticancer therapy. Clinoptilolite treatment of mice and dogs suffering from a variety of tumor types led to improvement in the overall health status, prolongation of life-span, and decrease in tumors size. Local application of clinoptilolite to skin cancers of dogs effectively reduced tumor formation and growth. In addition, toxicology studies on mice and rats demonstrated that the treatment does not have negative effects. In vitro tissue culture studies showed that finely ground clinoptilolite inhibits protein kinase B (c-Akt), induces expression of p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> tumor suppressor proteins, and blocks cell growth in several cancer cell lines. These data indicate that clinoptilolite treatment might affect cancer growth by attenuating survival signals and inducing tumor suppressor genes in treated cells.

**Keywords** Clinoptilolite · Adjuvant · Anticancer · Treatment

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**Abbreviations** *EGF*: Epidermal growth factor · *FBS*: Fetal bovine serum · *MAPK*: Mitogen-activated protein kinases · *PDGF*: Platelet-derived growth factor · *SDS*: Sodium dodecyl sulfate

## Introduction

Zeolites are hydrated natural and synthetic microporous crystals with well-defined structures containing  $\text{AlO}_4$  and  $\text{SiO}_4$  tetrahedra linked through the common oxygen atoms [1]. Zeolites have been extensively used in various industrial applications based on their properties to act as catalysts, ion exchangers, adsorbents, and detergent builders [2, 3, 4, 5, 6]. It is also known that silicates and aluminosilicates possess biological activity, either positive or negative. Talc and silica have been used in skin care for many decades, while well defined structures and catalytic activity make aluminosilicates an attractive model system for protein and enzyme mimetics [7]. Recent results have also demonstrated that natural, biologically nontoxic clinoptilolite from Cuba deposits is very effective as glucose adsorbent, and this has been suggested as a potential medication for individuals suffering from diabetes mellitus [8].

The best known positive biological activity of natural clinoptilolite is its action as antidiarrheal drug (see [9] and references therein). Clinoptilolite lowers the incidence of death and sickness (diarrheal syndrome) produced by intestinal diseases in swine, rats, and calves (see [9] and references therein). Based on these results a comprehensive study was carried out on antidiarrheal drugs based on natural clinoptilolite as an active material, in the therapy of acute diarrheal diseases in humans [9]. The research lead to approval of the antidiarrheal drug Enterex for use in humans. In addition, accumulating evidence has indicated that zeolites play an important role in regulating the immune system. Ueki et al. [10] and Aikoh et al. [11] have reported that silica, silicates, and aluminosilicates act as nonspecific immunostimulators similarly to superantigens. Superantigens are a class of immunostimulatory and disease-causing 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 superantigens with V $\beta$  domain of T cell receptor and with major histocompatibility complex class II molecules on the surface of antigen presenting cells [10]. Pro-inflammatory macrophages, which belong to class II MHC antigen-presenting cells, are activated by fibrogenic silicate particulates [12, 13, 14, 15]. Indeed, experiments carried out by Ueki and coworkers [10] have shown that removal of MHC class II DP/DR positive cells results in a lack of macrophage stimulation by asbestos.

Direct interaction of silicate particles with cells other than lymphocytes has also been identified and described. It seems that mineral particles can trigger alterations in gene expression by initiating signaling events upstream

of gene transactivation [16]. Exposure of cells to silicate particles has been shown to lead to activation of mitogen-activated protein kinases (MAPK), protein kinase C, and stress-activated protein kinases [17]. Important transcription factors such as activator protein 1 and nuclear factor  $\kappa$ B are also activated, and expression of proinflammatory cytokines such as interleukin 1 $\alpha$ , interleukin 6, and tumor necrosis factor  $\alpha$  is enhanced [18]. Modifications in receptor activation kinetics or activity of integrins may be responsible for the observed behavior. Alternatively, particles engulfed by phagocytosis have been reported to stimulate production of reactive oxygen species [19]. It was recently shown that redox regulation of gene expression is a general phenomenon in most cells.

The above knowledge of zeolites and other silicates led us to test the biological activity of natural clinoptilolite. Mechanical treatment of natural clinoptilolite was used to produce small-sized particles (MZ) that were tested for possible toxicity and anticancer activity in vivo. Here we provide evidence that orally administered natural clinoptilolite is nontoxic and useful in cancer treatment in animal models. Additional in vitro tissue culture experiments with various cancer cell lines indicated that MZ treatment modifies intracellular signaling pathways leading to inhibition of survival signals and induction of tumor suppressor genes.

## Materials and methods

### Natural clinoptilolite

The fine powder of natural clinoptilolite was obtained by tribomechanical micronization. Chemical composition of the MZ was determined by the atomic absorption spectroscopy. Qualitative and quantitative phase analyses of the MZ were performed by powder X-ray diffractometry using a Siemens 5000D diffractometer with  $\text{CuK}\alpha$  radiation in the region  $2\theta = 4\text{--}80^\circ$ . Thermogravimetric and differential thermogravimetric analysis of the MZ was performed using a TA 4000 System (Mettler-Toledo) apparatus. The heating rate was 10 K/min in nitrogen atmosphere. Particle size distribution curves of the MZ were taken by a Mastersize XLB (Malvern) laser light-scattering particle-size analyzer.

### Cell lines and proliferation assay

Effect of MZ on in vitro cell proliferation was studied on several human cell lines: diploid fibroblasts (Hef522), cervical carcinoma (HeLa), colon carcinomas (CaCo-2, HT-29, and SW 620), mammary carcinomas (MCF-7 and SkBr-3), and one mouse fibrosarcoma cell line. The cells were maintained by culturing in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . For the purpose of proliferation assay experiments the cells were plated at a concentration of  $1 \cdot 10^4$  cells/ml onto 96-microwell plates (200  $\mu\text{l}/\text{well}$ ). After overnight incubation the standard medium was replaced with the medium which was pretreated with either 0.5, 5, or 50 mg/ml MZ. For this purpose the medium and MZ were mixed, and after 18 h of shaking MZ was pelleted by centrifugation (5000 g for 10 min).

The cells were then incubated for additional 72 h, when cell viability (cell growth) was measured using MTT assay which detects dehydrogenase activity in viable cells. For this purpose the medium was discarded, and MTT was added to each well at con-



centration of 20 µg/40 µl. After 4 h of incubation at 37°C the precipitates were dissolved in 160 µl DMSO. The absorbance was measured on an enzyme-linked immunosorbent assay reader at 570 nm. The cell proliferation is expressed as a percentage of absorbance, recorded in cell line treated with particular concentration of MZ, in relation to the absorbance of control, nontreated, cells which was expressed as 100%.

#### Analysis of p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup>

Experiments with p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> were carried out on human adenocarcinoma (CaCo-2) and human cervical carcinoma (HeLa) cell lines. The cells, originally grown in tissue culture flasks, were collected and seeded onto glass slides. After 24 h the medium was replaced either with the fresh standard medium (control cells) or with the medium pretreated with 50 mg/ml MZ. After 72 h of incubation the cells were washed with PBS and fixed in methanol with 3% hydrogen peroxide (Kemika, Zagreb, Croatia).

Proteins, p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup>, expression was analyzed immunocytochemically. Nonspecific binding was blocked by applying normal rabbit serum (1:10) for 30 min. Primary antibodies p21 (5 µg/ml, PharMingen) and p27 (2 µg/ml, Transduction Laboratories) were allowed to bind overnight at 4°C. Slides were washed three times in PBS. Secondary antibody (rabbit anti-mouse; Dako, Denmark) was applied for 1 h at room temperature. Finally, peroxidase-antiperoxidase (Dako) conjugate diluted 1:100 in PBS was applied for 1 h at room temperature. After washing with PBS the slides were stained with 0.025% diaminobenzidine tetrahydrochloride (Sigma) containing 4% H<sub>2</sub>O<sub>2</sub> for 7 min and counterstained with hematoxylin for 30 s. The slides were analyzed with a light microscope (Olympus). The level of nonspecific background staining was established for each measurement using control cells processed in the same way but without exposure to the primary antibody.

The concentration of antigen was assessed by estimating the relative visual intensity of a chromogenic label, and the results are expressed on a three-point scale as follows: –, negative staining; +, weak staining and ++, moderate staining.

#### Biochemical studies of signaling pathways

The following were used: epidermal growth factor (EGF; Inter-gen), platelet-derived growth factor (PDGF) BB (Amgen), protein ladder markers (10–200 kDa; Life Technologies), leupeptin and a miniprotease inhibitor kit (Boehringer-Mannheim), Pefabloc (Fluka), aprotinin (Trasylol, Bayer), and nitrocellulose membranes (Millipore). Affinity-purified rabbit polyclonal anti-Akt, anti-pAkt, anti-JNK, anti-pJNK and anti-pERK2 (MAPK) antibodies were purchased from New England Biolabs. The rabbit polyclonal anti-ERK2 (C-14) antibodies were from Santa Cruz Biotechnology. Secondary antibodies, peroxide-conjugated swine anti-rabbit were from New England Biolabs, peroxide-conjugated sheep anti-mouse immunoglobulin from Amersham/Pharmacia, and peroxide-conjugated protein A from Kirkegaard and Perry Laboratories.

Murine fibrosarcoma cells were grown in Petri dishes (6 cm in diameter) in RPMI medium with 10% FBS up to the 80% confluence. Before starting the experiments the cells were starved for 24 h. Subsequently the cells were treated with MZ pretreated medium with or without 10% FBS for 0, 5, 30, and 60 min or with EGF (100 µg/ml) and PDGF (40 µg/ml). After the indicated time of treatment the cells were washed with PBS and scraped into ice-cold lysis buffer containing 50 mM hydroxyethylpiperazine ethane sulfonic acid, pH 7.2, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, and protease inhibitors (1 mM Pefabloc, 10 µg/ml leupeptin, and 1% Trasylol). Following 45 min at 4°C with gentle rocking a soluble fraction was prepared by centrifugation at 4°C for 15 min at 13,000 g. Equal amounts of cell lysates (measured by the Bradford assay) were mixed with 3× sodium dodecyl sulfate (SDS) sample buffer and heated for 2 min at 98°C. Proteins

were separated by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Immunoblots were blocked with 5% bovine serum albumin in TBS (10 mM Tris-HCl, pH 7.4; 150 mM NaCl) for 1 h, incubated for 1 h with primary antibodies (anti-pAkt, anti-pJNK, anti-pERK2) in TBS, washed six times for 10 min each in TBS 0.05% Triton X-100, and then incubated for 1 h with appropriate secondary antibody. Following further washes, immunoblots were visualized by using enhanced chemiluminescence reagents. To reprobe blots they were incubated in stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM 2-mercaptoethanol) at 58°C for 25 min, washed extensively with TBS, reblocked as described above, and reblotted with the appropriate antibodies.

#### Isolation of apoptotic DNA fragments

HeLa cells (1·10<sup>5</sup>) were grown in a 10-ml flask for 24 h, after which the medium was discarded and replaced with the MZ pretreated medium (see above). After 24 h the cells were trypsinized, pelleted by centrifugation (1200 g), and washed twice in PBS. Afterwards the cells were resuspended 10 s in 100 µl lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) and centrifuged 5 min at 3000 g. The supernatant was transferred to a new Eppendorf tube while the pellet was incubated once more with 100 µl lysis buffer and centrifuged as before. The supernatants were pooled together and incubated 2 h in 1% SDS and RNase (5 µg/µl) at 56°C, after which the proteinase K was added in final concentration 2.5 µg/µl overnight. DNA fragments were pelleted by addition of 1/2 volume of 10 M ammonium acetate and 2.5 volume of prechilled absolute ethanol. After centrifugation (30 min, 12,000 g), the pellet was washed with 70% ethanol, centrifuged 10 min at 12,000 g, dried, and dissolved in 20 µl TE buffer (10 mM Tris-HCl pH 7.4; 1 mM EDTA pH 8). The DNA was visualized on 1.5% agarose gel.

#### Animals

##### Mice

CBA/HZgr and C57BL/6 mice of both sexes were used. Toxicity study experiments were performed on the CBA/HZgr strain, while experiments with tumors were performed on both strains. For non-clinical tolerance testing male mice of the BALB/c strain were used. At the beginning of the experiments the animals were about 4 months old, weighing 25–28 g. Until beginning the experiments the mice were maintained in standard conditions with unrestricted access to food and water.

##### Rats

Wistar rats of both sexes from the animal breeding colony at the Institute for Medical Research, Zagreb, Croatia were used for toxicity and nonclinical tolerance testing studies. At the beginning of the experiments they were 2–3 months old, weighing in average 300 g (males) and 200 g (females).

##### Dogs

Twenty-two dogs were used in the experiments. They were of various breeds, weighing from 3 to 42 kg. The animals were of both sexes, 5–14 years old. The data on the 14 dogs in which disease improvement was observed, are presented in Table 2.

#### Application of mechanically treated natural clinoptilolite (MZ)

Because of the insolubility of the tested substance, it was administered to the animals either orally by gavage or in their diet (mice,



rats), supplemented as powder to the conventional food, or in capsules (dogs) which were again admixed to food. When testing the growth of mammary aplastic carcinoma or mammary aplastic carcinoma metastases formation MZ and standard food for laboratory mice (Pliva, Zagreb, Croatia) were mixed in the ratio 20%:80%. Each mouse on average ate about 4 g food daily, thus consuming about 800 mg MZ. When testing the growth of melanoma, MZ was given to mice orally (gavage) at doses of 20, 30, and 40 mg/mice five times per day (tested doses were 100, 150, and 200 mg/mice, respectively). In toxicity studies MZ was applied in diet mixed with standard food.

## Tumors

Mammary carcinoma occurred spontaneously in CBA/HZgr mice, maintained in the animal breeding section of the Division of Molecular Medicine, Ruder Bošković Institute, Zagreb, Croatia. The tumor is a highly anaplastic carcinoma with very high incidence of mitoses; it does not form any glandular structures and leads to spontaneous metastases in the lungs. After transplantation of  $1 \cdot 10^6$  viable tumor cells into the animals a growing tumor is obtained which causes the mouse's death after about 4 weeks. For the purpose of the experiments tumor cell suspension was always prepared from *in vivo* growing tumor.

Melanoma B16, originally obtained from Holt Radium Institute, Manchester, United Kingdom, has been maintained at the Ruder Bošković Institute since 1975 by subcutaneous inoculations of suspension containing  $2 \cdot 10^6$  tumor cells into flanks of C57BL/6 mice.

Spontaneous tumors in dogs were of various origins, sizes, and locations. The data on 14 tumors are presented in Table 2. In another 8 tumors, not presented in Table 2, there were two lymphomas, two autoimmune hemolytic anemias, and one each of prostate tumor, osteosarcoma, mammary fibrochondroadenocarcinoma, and epulis.

To obtain tumor cells in suspension large pieces of tumor removed from the mice were cut up in very small pieces (Hank's solution). The particles were allowed to settle, and the supernatant (cell suspension) was removed and spun down at 150 g for 10 min. The pellet was resuspended and cell viability was tested by Trypan blue exclusion test: more than 90% of tumor cells were scored as viable. To obtain locally growing tumor, an inoculum of 0.1 ml, containing  $1 \cdot 10^6$  viable tumor cells, was injected subcutaneously into the right thigh of recipient mice. Tumor growth was checked each day after tumor cell inoculation into the mice. When the tumor was established, its size was measured by a caliper. Three diameters were measured, and tumor volume was calculated. To obtain experimental lung metastases 0.25 ml, containing  $1 \cdot 10^5$  mammary aplastic carcinoma cells, was injected into mouse tail vein. The mice were killed 18 days later. The lungs were removed, washed in water, separated into lobules, and immersed in a fixative. Macroscopically visible nodules on lung's surface were counted.

## Toxicology studies

Preclinical toxicology was performed according to standards and regulations of the Organization for Economic Cooperation and Development principles of food laboratory practice (Paris 1998). The testing was approached by setting the "limit" test – applying the high doses of MZ,  $2 \cdot 200$  and  $2 \cdot 500$  mg/mouse per day orally (gavage) for 6, 14, and 30 days. Since the MZ did not cause the death of mice in a "limit" test, an "up and down" test was performed on mice, with daily doses ranging from 60 to 400 mg/mouse (MZ given orally, gavage, for 30 days). Again, no toxicity was observed. Therefore a classical acute, subchronic and chronic toxicity study of mice and rats of both sexes (separately) was performed.

## Mice

The mice were of the CBA/HZgr strain. MZ was given in a diet (powdered MZ mixed with standard food at the ratio of 25:75%). The duration of study was as follows: acute toxicity, 1 month; subchronic toxicity, up to 3 months; chronic toxicity, up to 6 months. Animals were monitored for: phenotypic changes, changes in behavior, and survival (every day), changes in body weight (weekly), amount of food and water consumed (checked on days 14 and 28 when mice were kept for 24 h in metabolic cages, five mice per cage), changes in hematological and serum clinical chemistry parameters (erythrocytes, leukocytes, platelets, hematocrit, hemoglobin, glucose, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, bilirubin, inorganic phosphorous, and calcium; after 1, 3, and 6 months); and urine clinical chemistry parameters (glucose, proteins, urobilinogen, bilirubin, nitrites, erythrocytes, leukocytes, pH, and specific gravity; urine was collected while the animals were kept, once a month for 24 h, in metabolic cages). Pathohistological analysis of liver, spleen, kidney, brain, lung, testes, ovary, duodenum, eye, stomach, large and small intestine, muscles, myocard, pancreas, thymus and axillary lymph node was carried out on killed experimental and control mice.

## Rats

Wistar rats were used. MZ was given in a diet (mixed with standard food at ratios of 25:75 and 50:50). The duration of study was as follows: acute toxicity, 1 month; subchronic toxicity, 3 months; chronic toxicity, 12 months. Animals were monitored for: phenotypic changes, changes in behavior and survival (every day), changes in body weight (every 4 days), amount of food (every day) and water consumed (every 4 days), and changes in hematological and serum clinical chemistry parameters (the same as for mice; once a month). Pathohistological analysis of liver, spleen, lung, kidney, testes, ovary and brain, was performed on killed experimental and control rats after 1, 6, and 12 months.

The reproductive/developmental toxicity was tested on mice (CBA/HZgr) due to their short gestation period and larger litter size. MZ was given in a diet (powdered MZ mixed with standard food at the ratio of 25:75%). For reproductive toxicity study ten male and ten female mice were fed with the food supplemented with the MZ for 50 and at least 14 days, respectively, before mating. The treatment continued during the pre-pregnancy and pregnancy period (one cycle) and to the point of weaning offspring. The same pair of animals was fed with the MZ and monitored during four consecutive cycles (approximately 4–5 months). The same schedule was applied for control, nontreated, animals. The parental generation was monitored for duration of cycle period (pre-pregnancy and pregnancy period), fertility (presence or absence of litter in particular cycle), delivery incidence, mortality, and pathohistological appearance of ovaries, after 4th cycle. Number of total and viable pups born as well gain in pups body weight and pups mortality until weaning was also scored.

For teratology study healthy, untreated pregnant mice were fed with MZ mixed to the conventional food from day 6 through day 16 of gestation and the mice were killed 1 day before parturition. The fetuses were analyzed for microscopic pathology.

Local tolerance was evaluated to ascertain whether the test substance is tolerated at the sites in the body which may come into contact with the product as a result of its administration.

Repeated-dose dermal tolerance testing was performed on male Wistar rats and male BALB/c mice. MZ was applied on the shaved skin of the whole dorsal region of animals in three ways: (a) as original powder, (b) mixed with neutral creme at the ratio of 1:1, (c) mixed with paraffin oil at the ratio of 1:1. The animals were treated twice a day during 28 days. Macroscopic changes in the treated skin were examined daily. The left dorsal region of the animal was used as control. For microscopic analysis of the possible changes the skin samples were collected 1 day after the last treatment.

## Results

### Properties of mechanically treated natural clinoptilolite

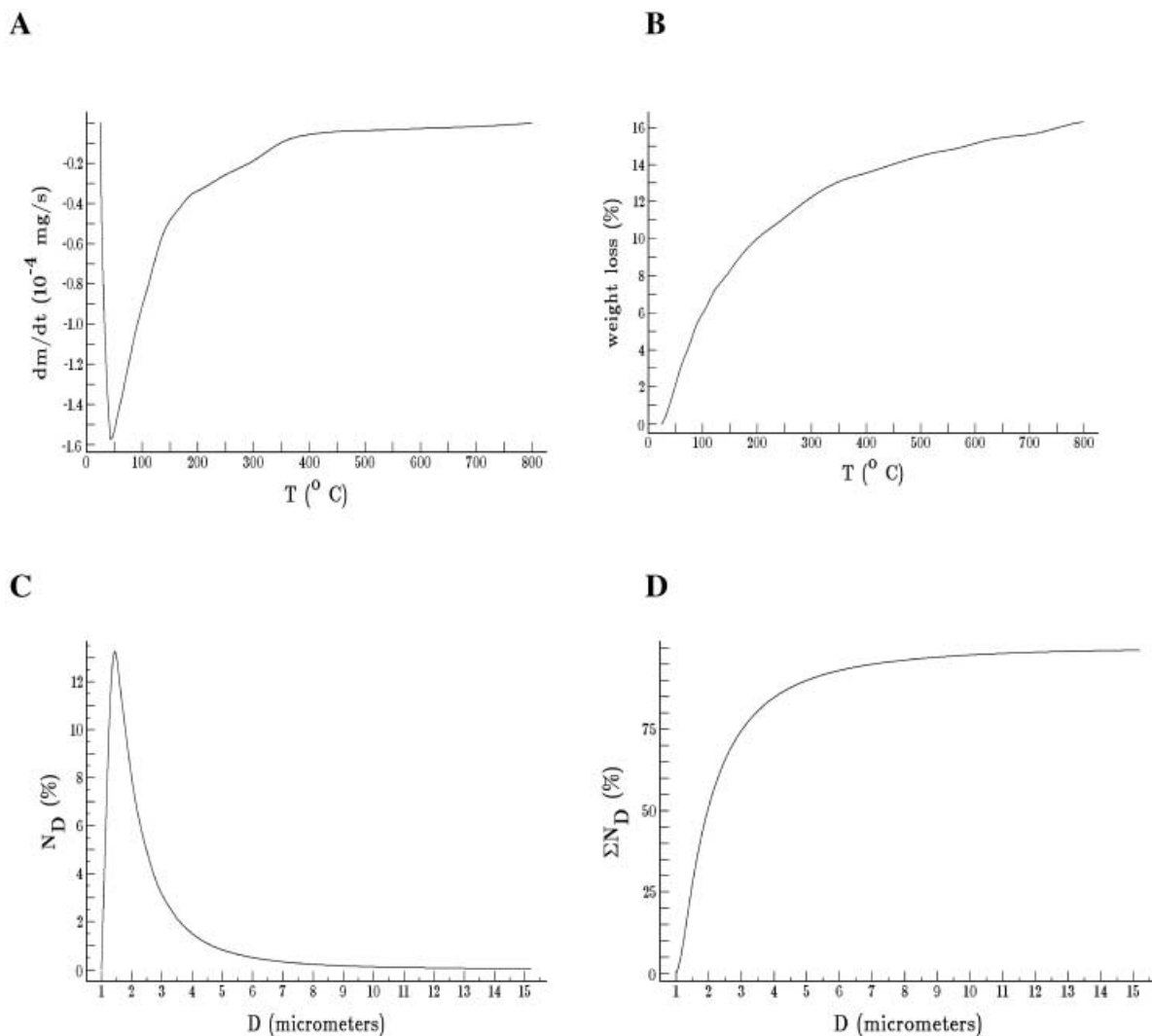
Mechanically treated natural clinoptilolite (MZ) contained approximately 85 wt.% clinoptilolite. The remaining 15% consisted of silica, montmorillonite and mainly mordenite zeolite. The chemical composition of the natural clinoptilolite is presented in Table 1. Differential thermal analysis (differential thermogravimetric) of the MZ shows that the maximum rate of water desorption occurred at 50°C, indicating that the change in sample weight during heating to 50°C corresponds to the removal of loosely held moisture within the solid microstruc-

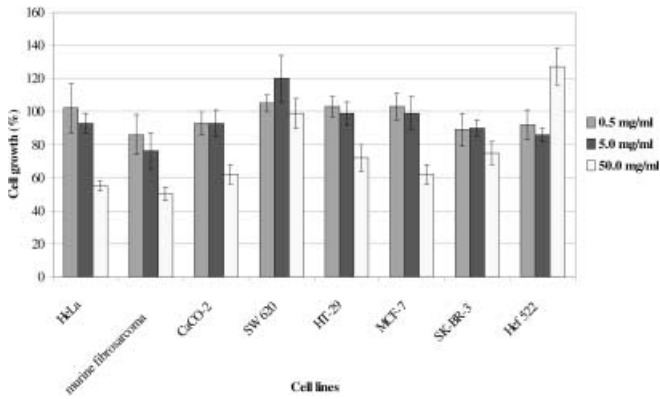
ture. Analysis of the water desorption curve shows that the MZ contains approx. 16 wt.% of water (loosely held moisture + zeolitic water) of which approx. 2 wt.% is loosely held moisture (Fig. 1A, B). No phase transformation was observed during the heating of MZ to 800°C. Particle size analysis of the MZ showed that maximum frequency of particles (approx. 13%) appeared at 1.5  $\mu\text{m}$  with average size of 2.9  $\mu\text{m}$ . In 25% of particles the size was up to 1.5  $\mu\text{m}$ , in 50% up to 2  $\mu\text{m}$ , and in 75% up to 3  $\mu\text{m}$  (Fig. 1C, D).

**Table 1** Chemical composition of the mechanically treated natural clinoptilolite (MZ)

Oxide	wt.%
SiO <sub>2</sub>	50–55
Al <sub>2</sub> O <sub>3</sub>	9.3–11.4
Fe <sub>2</sub> O <sub>3</sub>	2.2–2.8
Na <sub>2</sub> O	0.8–1.1
K <sub>2</sub> O	2.9–4.3
MgO	0.8–1–2
CaO	13.7–17.2
MnO	0.07–0.90
TiO <sub>2</sub>	0.14–0.22
Water (800°C)	14–16

**Fig. 1** **A** Differential thermogravimetric curve of MZ;  $dm/dt$  differential change ( $dm$ ) in the mass of sample in differential time interval ( $dt$ );  $T$  temperature of heating. **B** Weight loss during controlled heating of the MZ from ambient temperature ( $T=25^\circ\text{C}$ ) up to  $T=800^\circ\text{C}$ . **C** Distribution of differential particle sizes by number of MZ.  $N_D$  Number percentage of particles of the corresponding diameter  $D$ . **D** Cumulative particle size distribution by number of the MZ.  $\Sigma N_D$  Percentage of the particles having diameters between  $D=0$  and  $D$





**Fig. 2** Effect of the medium pretreated with 0.5, 5.0, and 50.0 mg/ml MZ on growth of various cell lines. Vertical bars Standard deviations; all white bars (50 mg/ml) are statistically different in comparison to control ( $P < 0.001$ , Student's  $t$  test)

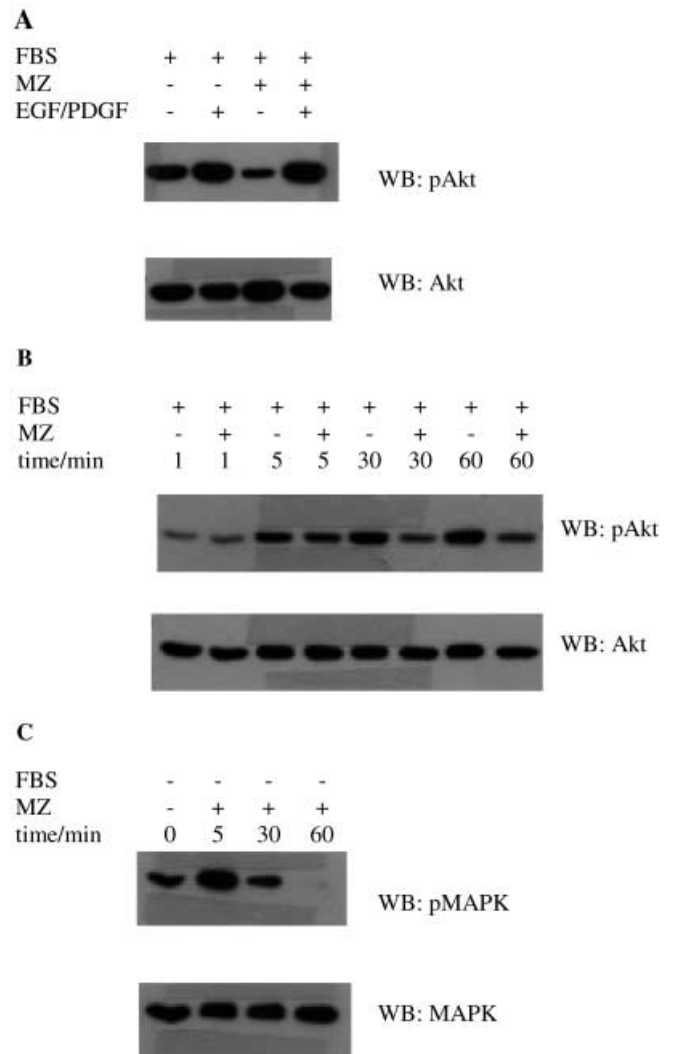
The effect of MZ on proliferation of cell lines grown in vitro

Figure 2 presents the cell proliferation state of Hef522, HeLa, CaCo-2, SW620, HT-29, MCF-7, SKBR-3, and mouse fibrosarcoma cells after 3 days of treatment. The growth of all cell lines except Hef522 and SW620 was significantly inhibited with the dose of 50 mg/ml. The strongest inhibition (for 50%) was seen on mouse fibrosarcoma cells, the growth of SW620 cells was unchanged, and that of Hef522 cells was slightly stimulated. Similar results were observed measuring [ $^3\text{H}$ ]thymidine incorporation assay in the presence of 10% FBS in mouse fibrosarcoma cells (data not shown).

Analysis of intracellular signaling pathways in MZ-treated cells

Since previous studies have indicated that exposure of cells to silicate particles leads to activation of MAPK, protein kinase C, and stress-activated protein kinases/JNK [17], we further analyzed whether MZ treatment also affects mitogenic and survival signaling pathways in these cell models.

The most significant results were detected measuring the activity of Akt protein. Akt, or protein kinase B, has been recently shown to mediate survival signals downstream of phosphoinositide-3 kinase by phosphorylating Bad proteins. We have observed an increase in Akt phosphorylation in response to serum, EGF, or insulin treatment. The addition of the MZ pretreated medium containing 10% FBS to the cells decreased Akt phosphorylation in comparison to the cells treated with only serum containing medium, while the addition of growth factors EGF and PDGF restored its activity (Fig. 3A) and overcame the effects of MZ on cell growth. Determination of the activity of Akt at various times after the addition of MZ pretreated medium with 10% FBS showed slight decrease in pAkt level after 5 min. This decrease was more

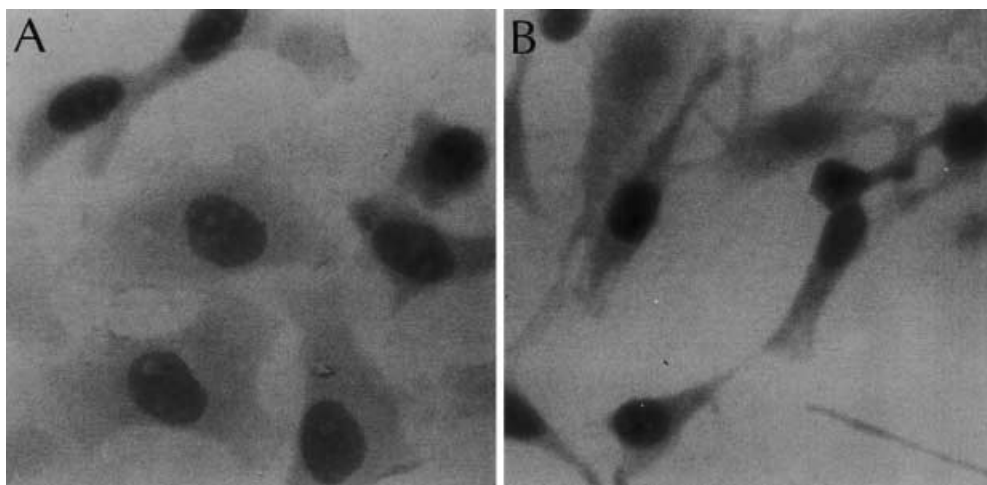


**Fig. 3** **A** Activity of Akt protein 5 min after addition of the MZ pretreated medium to murine fibrosarcoma cells. **B** Decreased Akt protein activity at various times after treatment of murine fibrosarcoma cells with MZ pretreated medium. **C** Effect of serum free MZ-pretreated medium on the activity of MAPK in murine fibrosarcoma cells. WB Western blot; FBS fetal bovine serum; MZ mechanically activated clinoptilolite zeolite; pAkt phosphorylated Akt; EGF epidermal growth factor; PDGF platelet-derived growth factor; MAPK mitogen-activated protein kinase; pMAPK phosphorylated mitogen-activated protein kinase

pronounced after 30 and 60 min of treatment (Fig. 3B). However, the addition of MZ pretreated medium without serum to the cells increased activity of Akt compared only to the serum-starved cells. Overnight treatment of the cells with EGF also increased Akt activity. However, combined overnight treatment of the cells with EGF and MZ pretreated medium decreased Akt activity, indicating that inhibition of Akt might be linked to MZ inhibition of the EGF-triggered pathways.

MAP kinase activity was increased in serum-starved cells in response to EGF, PDGF, or serum. Addition of only MZ pretreated medium to the serum-starved cells increased MAPK activity only temporarily (after 5 min);

**Fig. 4** Immunohistochemical analysis of p27<sup>KIP1</sup> on control HeLa cells (A) and HeLa cells after incubation with the MZ pretreated medium (B). *Brown staining* Cells expressing p27<sup>KIP1</sup>



in the next 30 min MAPK activity returned to the normal level (Fig. 3C). In contrast, addition of MZ pretreated medium plus 10% serum slightly decreased MAPK activity compared only to serum-treated cells or cells incubated only with MZ pretreated medium. These results are in agreement with those of the previously performed thymidine test.

Medium pretreated with MZ added to the cells either alone or in combination with serum caused no change in JNK activity (data not shown).

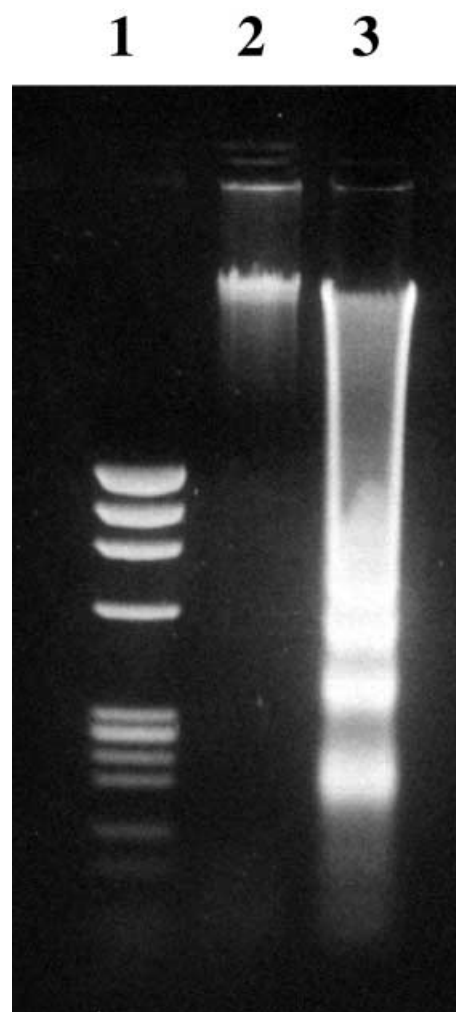
The effect of MZ on expression of inhibitors of cycline-dependent kinases, p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup>, was tested using immunocytochemical method, in HeLa and CaCo-2 cells. Treatment with MZ induced the expression of p21<sup>WAF1/CIP1</sup> in CaCo-2 cells and p27<sup>KIP1</sup> in HeLa cells, while nontreated cells were negative for expression of p21<sup>WAF1/CIP1</sup>/p27<sup>KIP1</sup> (Fig. 4).

#### Induction of programmed cell death-apoptosis

To evaluate whether the inhibition of cell growth by MZ is due to programmed cell death, i.e., apoptosis, an attempt was made to isolate small DNA fragments. Large amount of small (degraded) DNA fragments in DNA isolate would indicate that MZ induces programmed cell death in treated cells. The result of small DNA fragment isolation from HeLa cell is shown in Fig. 5. DNA isolated from MZ treated cells exhibited significant degradation (lane 3a bulk of low-molecular, degraded DNA indicated with an arrow) in comparison to DNA from untreated cells (lane 2). The DNA degradation in MZ treated cells is most probably due to induced programmed cell death (apoptosis).

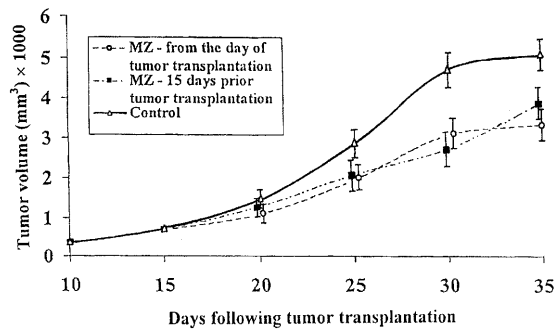
#### Toxicology

Oral (in diet) administration of MZ to mice and rats for 6 and 12 months, respectively, caused no changes that could be considered a toxic effect of treatment. The MZ



**Fig. 5** Apoptotic DNA fragments in 1.5% agarose gel. *Lane 1* DNA molecular weight marker IX (Øx 174/*HindIII*); *lane 2* DNA isolated from untreated HeLa cells; *lane 3* DNA isolated from the MZ-treated HeLa cells; degraded, low-molecular DNA fragments





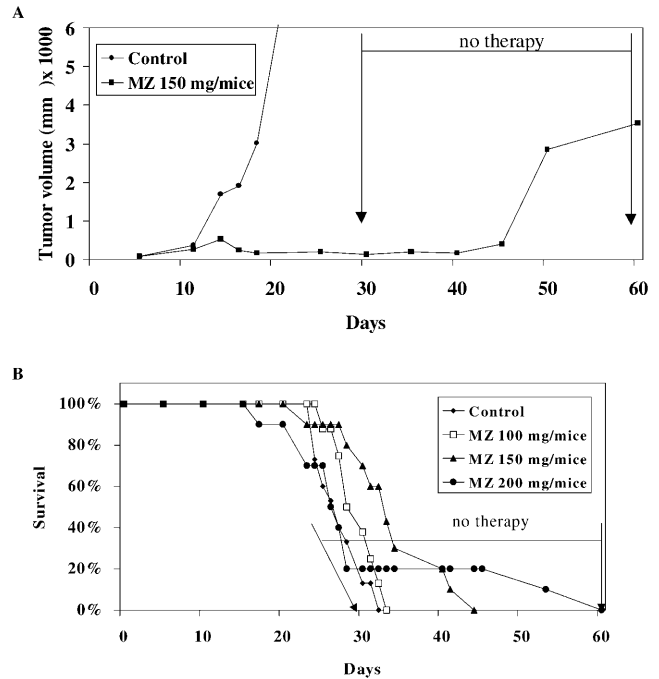
**Fig. 6** Tumor growth following injection of  $1 \cdot 10^6$  mammary aplastic carcinoma cells into the right thigh of CBA/HZgr mice. The animals were exposed to 20% of MZ in the food either from the day of tumor transplantation ( $n=14$ ) or 15 days prior to tumor transplantation ( $n=14$ ). Control mice received standard food. Vertical bars Standard deviation. The differences between control and both experimental groups were statistically significant ( $P<0.001$ , Student's  $t$  test) for the days 25, 30, and 35

equalized (regulated) and shortened the prepregnancy period. The number of pups per litter was increased in MZ-treated mice. Probably for this reason the gain in pups' body weight until weaning was decreased. As a final consequence higher mortality of pups between days 8 and 21 of the neonatal period was observed. However, there are no differences between control and treated animals that would suggest reproductive toxicity attributable to the MZ administration. The MZ did not elicit toxicity during the period of organogenesis. The test substance, MZ, was not toxic or allergenic for the skin.

#### Effect of MZ on tumor growth in animal models

Previous studies in cultured cells have suggested that MZ inhibits growth of cancer cells in vitro. To study the effect of MZ in vivo studies on mice, rats, and dogs were undertaken. Subsequent studies were performed on murine transplantable tumors, melanoma B16, and mammary carcinoma. Mammary aplastic carcinoma cells were injected into the right thigh of two groups of mice. One group ( $n=14$ ) was fed with food supplemented with MZ starting from 15 days prior tumor transplantation until the animal's death; the other group ( $n=14$ ) was fed with MZ from the day of tumor transplantation until the animal's death. A group of five tumor-bearing mice receiving standard food was used as control. Tumor growth was significantly inhibited in both groups of animals fed with MZ supplemented food (Fig. 6). The tumor growth curves for individual animals were uniform, particularly when MZ was given prior to the tumor transplantation. However, there was no difference in mice survival among the groups.

Melanoma B16 cells were inoculated subcutaneously in C57BL mice on day 0. For the next 30 days the mice were given MZ orally five times per day. Tumor volume was recorded; it was markedly lower in 5 of 80 mice (daily dose 150 mg/mouse) than in the control group



**Fig. 7** Growth rate (A) of melanoma B16 treated with 150 mg MZ/mouse per day and survival (B) of melanoma-bearing mice treated with three different doses of MZ

(Fig. 7A). Despite the fact that the tumors started to grow more rapidly after the therapy with MZ was abrogated (between days 30 and 60 after tumor transplantation), the mice lived a statistically significantly longer period when treated with 200 and 150 mg MZ than control animals (Fig. 7B). The mice used for experimental mammary aplastic carcinoma lung metastases formation were fed with MZ diet from 15 days prior to tumor cell injection until to the end of the experiment, i.e., 18 days after tumor transplantation. The controls consumed standard food. Each of these two groups comprised 20 animals. About 20–40 nodules per animal were scored, but there was no difference between the groups (data not shown).

There was no effect of MZ treatment on in vivo growth of two mammary carcinomas which differed from that showed in Fig. 6 (data not shown).

Of 22 dogs suffering from various kinds of spontaneous tumors that were treated with MZ, 14 responded to therapy, i.e., the tumor disappeared completely, or the tumor size was significantly reduced (presented in Table 2). Among three dogs which had prostate tumor there was one that was stated sonography showed to have (in addition to prostate tumor) a prostate cyst (case 3). The dog was conspicuously quiet, without appetite, and hardly moved. When the usual therapy did not work, MZ therapy was started. After only 2 days of treatment the dog became active; on the third day it began eating normally, and on the fourth day the dog urinated normally, blood-free urine. On day 10 the cyst and the tumor were reduced in size, and after 1 month they had disappeared completely. Although the prostate be-

**Table 2** The effect of MZ treatment on growth of spontaneous tumors of dogs (. values before and after treatment with MZ, *a.t.* after beginning of treatment, *HMT* hematocrit, *ALT* alanine amino-

transferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *GGT* .-glutamyl transferase, *L* number of leukocytes)

No.	Breed	Age (years) <sup>a</sup>	Weight (kg)	Sex	Diagnosis	Previous treatment	MZ treatment	Biochemical and hematological changes	Therapeutic effects
1	Schnauzer	8	15	M	Prostate adenocarcinoma <sup>b</sup>	Castration	3· 200 mg/day, 28 days	HMT 61. 45; ALT 103. 62	7 days a.t. general improvement; withdraw of catheter; 14 days a.t. no signs of disease
2	Poodle	12	16	M	Prostate adenocarcinoma (4· 3 cm) and testis tumor (20 cm)	—	3· 200 and 2· 200 mg/day, 6 months	AST 55. 10; GGT 4. 1	90 days a.t. reduction in tumor mass (testis) to 1/3
3	German shepherd	8	42	M	Prostate adenocarcinoma (5· 5 cm) and cyst	Antibiotics	3· 1200 mg/day	Bilirubin 25.8. 6.2; AST 497. 16; ALT 433. 43; ALP 79. 33	29 days a.t. tumor disappeared
4	Mixed German shepherd	14	20	F	Mammary adenocarcinoma, multiple – 5 nodes (0.5–3 cm)	—	3· 400 mg/day, 1 month	No changes	10 days a.t. all nodes disappeared; 12 months later no signs of disease
5	English cocker spaniel	8	15	F	Mammary adenocarcinoma, multiple – 4 nodes (0.5–3 cm)	—	3· 400 mg/day, 58 days	No changes	58 days a.t. all tumor nodes reduced in size 50%
6	Poodle	11		F	Mammary adenocarcinoma, multiple – 4 nodes (0.5–3 cm)	—	3· 400 mg/day, 2.5 months	No changes	2–3 months (smaller nodules); 4–6 months (larger nodules)
7	Dobermann pinscher	8		F	Mammary adenocarcinoma, multiple – 4 nodes (0.5–3 cm)	—	5· 400 mg/day, 3 months	No changes	2–3 months (smaller nodules); 4–6 months (larger nodules)
8	English cocker spaniel	9		F	Mammary adenocarcinoma, multiple – 4 nodes (0.5–3 cm)	—	3· 400 mg/day, 4 months	No changes	2–3 months (smaller nodules); 4–6 months (larger nodules)
9	Airedale terrier	9		F	Mammary adenocarcinoma, multiple – 4 nodes (0.5–3 cm)	Antibiotics	5· 400 mg/day, 10 months	No changes	2–3 months (smaller nodules); 4–6 months (larger nodules)
10	German shepherd	8	38	M	Skin adenocarcinoma (tail)	Surgically removed, resection wound did not heal	6· 400 mg/day, 62 days, and local appl. of powdered substance	Glucose 6.9. 3.8; AST 50. 38	3 days a.t. normal healing started and completed 7 days later

Table 2 (continued)

No.	Breed	Age (years) <sup>a</sup>	Weight (kg)	Sex	Diagnosis	Previous treatment	MZ treatment	Biochemical and hematological changes	Therapeutic effects
11	Mixed German shepherd	10	35	M	Carcinoma planocellulare of the skin (tail), 3 tumor	Two nodes surgically removed	4· 100 mg/day, 93 days	Urea 17.5. 6.3	remaining node disappeared 67 days a.t.
12	Malamute	12	40	M	Carcinoma planocellulare of the tongue	Surgically removed, resection wound did not heal	3· 100 mg/day, 32 days	—	3 days after treatment wound started to heal, and 2 days later no further signs of wound visible; dog started to eat
13	German pinch	5	3	M	Hypertrophy and hyperplasia of salivary gland	Antibiotics	3· 100 mg/day, 147 days	Urea 9.5. 7.5; AST 40. 27; ALT 54. 36; L 3.1. 12	7 days a.t. node became softer and smaller (75%); 14 days later no signs of hypertrophy
14	Berner sennenhund	8	40	M	Lung cancer	—	4· 400 mg/day, 35 days	AST 35. 16; bilirubin 8.5. 2.8	7 days a.t. general improvement; 7 days later no signs of tumor (obtained by X-ray)

<sup>a</sup> At the beginning of therapy<sup>b</sup> Hormone dependent

came only insignificantly smaller, the dog showed no signs of illness. At this point it is interesting to note that the very high pretherapy serum values for aspartate aminotransferase (497  $\mu\text{mol/l}$ ) and alanine aminotransferase (433  $\mu\text{mol/l}$ ) decreased after 1 month of MZ therapy to normal levels (16 and 43  $\mu\text{mol/l}$ ) and remained in the normal range for entire observation period (5 months).

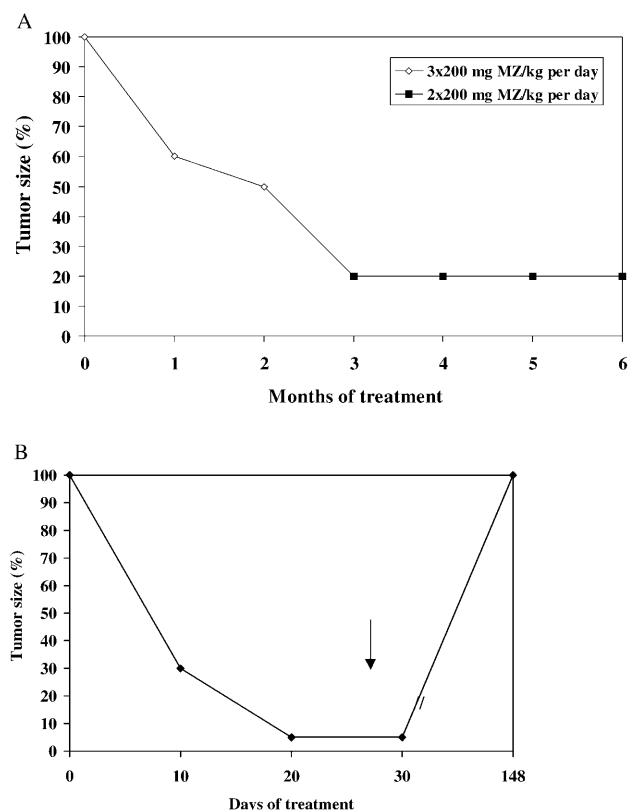
Another dog (case 2) had, in addition to prostate tumor, a testis tumor. The testis was approximately 20 cm in diameter when the therapy with MZ was started. After 1 month therapy the testis size was reduced by one-third. After 2 months of therapy the testis was reduced in size to one-half and after 3 months to one-third of its pre-treatment size (Fig. 8A). However, the prostate remained equally large.

The third dog (case 1) diagnosed to have prostate adenocarcinoma came to the clinic in a very bad general condition. It urinated only with great difficulty. After 1 month of classical therapy no improvement was observed. A catheter was placed in the dog's urethra. The therapy was continued for a further 2 weeks but did not work. The dog was *ante finem* and the owners asked for euthanasia. Classical therapy was then replaced by MZ

therapy (3· 200 mg/day). After 1 week a general improvement was observed, and the catheter was removed. After 14 days of therapy no signs of disease were still visible. The therapy continued for an additional 14 days, with daily health improvement. Then the owners decided on castration (in most cases castration eliminates problems related to the prostate), and the therapy with MZ was stopped. Eight months later the dog is still alive without any major health problems.

Three dogs suffered from skin tumors. One of these (case 11) had three lesions nodules on the skin above the tail. Two were removed, and the third, the smallest, was left. Histologically the tumor was diagnosed as carcinoma planocellulare. After 1 month of therapy with MZ the cherry-sized tumor was reduced in size by one-third. Over following 5 weeks the lesion disappeared completely. The dog is still (7 months latter) under therapy. The presently 11-year-old dog is very vivacious and in unusually good condition.

Another dog (case 10) suffered from adenocarcinoma on the skin of the tail, which was surgically removed. However, even 2 weeks after surgery the wound did not heal, and amputation was considered. The dog was then



**Fig. 8** Growth rate of testicular tumor (A) and salivary gland hyperplasia (B) in dogs. **A** Poodle, 12 years old, case 2. **B** Pinch, 5 years old, case 13. *Arrow* Day of therapy cessation. All other details are indicated in Table 1

given MZ in capsules, and powdered MZ was also scattered on the wound. The wound healed within 1 week.

The third dog (case 12) had a growth on its tongue of approx. 2 cm diameter. Histologically it was carcinoma planocellulare. After surgical removal of the tumor the wound did not heal. The dog was given MZ orally in capsules, and powdered MZ was also applied locally. Five days later the biopsy wound was no longer visible.

A 5-year-old dog (case 13), diagnosed to have enlarged (walnut-size node) left salivary gland, was treated with conventional therapy for 4 months, without success. During that time the gland became larger and larger, and the dog developed serious problems with swallowing and salivation. After only 1 week of MZ therapy the node became softer and smaller by one-third. After a further 1 week the node disappeared completely, and only the capsule was palpable (Fig. 8B).

Mammary adenocarcinomas, in the form of multiple nodules (in sizes between that of green beans and large walnuts), were diagnosed in six female dogs. After the therapy with MZ was started, the nodules disappeared completely: in one dog after 10 days, with no signs of disease even after 12 months; in four dogs after 2–3 months (smaller nodules) and 4–6 months (larger nodules), with no signs of disease thereafter, at the present, 2 months; and in one dog the nodules were reduced in size to 50% after 58 days of treatment.

In one case of a dog (case 14) with lung cancer, again, after only 14 days of treatment with MZ (4·400 mg/day) signs of tumor disappeared completely.

In addition to the effects of MZ expressed on the primary disease, all dogs, even those in which primary disease was not cured, responded to MZ therapy in only about 7 days with general constitutional and behavioral improvement lasting even after the therapy was interrupted. The same was observed for some hematological and serum clinical parameters measured before and after the therapy. Hematocrit decreased to the normal range in case 1. Very high total serum bilirubin values fell to the normal range in cases 3 and 14, while serum urea concentration change was noted in cases 11 and 13. The most pronounced improvement was noted for aspartate aminotransferase, alanine aminotransferase, and alkaline leukocyte phosphatase, with pretherapy values normalized after the therapy was started in almost all cases (nos. 1, 2, 3, 10, 13, and 14; Table 2).

## Discussion

Numerous natural compounds are commonly used for the treatment of various diseases, including green tea and soybean extracts (for review see [20]). Recent findings indicate that dietetic products and antioxidant compounds also have a beneficial effect particularly in cancer patients. In many cases the exact mechanism of their action is not fully understood. In this report we studied the effect of natural clinoptilolite zeolite particles on development of several cancer models in vivo and in vitro. We found that mechanically activated clinoptilolite zeolites act as anticancer therapeutic agents in in vivo animal studies and in tissue culture cell models. Clinoptilolite applied orally in mice and dogs suffering from a variety of tumor types led to a significant shrinkage of some tumors and improvement in overall health status in some animals.

The range of effects was diverse, ranging from negative antitumor response, to normalization of biochemical parameters, prolongation of life span, and decrease in tumor size. The best results in animal models were observed in the treatment of skin cancer in dogs, suggesting that adsorption of some active components is responsible for MZ activity (direct contact action). Complementary studies performed in tissue culture indicated that MZ treatment affects proliferation and survival of several cancer cell lines. Addition of MZ inhibited cell proliferation in a concentration-dependent manner, in part due to induction of inhibitors of cyclin dependent kinases, inhibition of B/Akt expression and induction of programmed cell death.

The work described here was performed with the non-toxic natural, high silica content zeolite, clinoptilolite. The zeolite particles were negatively charged in the entire pH range studied (pH 1–11). Electron microscopy showed the absence of fibers, and most particles were round with very rough surface (data not shown). The ab-



sence of fibrous, positively charged particles was encouraging since such particles are present in asbestos and erionite zeolites, which are highly carcinogenic and mutagenic. In addition, activated zeolite particles did not catalyze the production of hydroxyl radicals, unlike asbestos or erionite (data not shown). It seems that absence of fibrous particles capable of producing hydroxyl radicals makes this zeolite sample nontoxic and noncarcinogenic, at least when applied orally.

Silicate and aluminosilicate particulates can interact directly with specific cells and modify their intracellular pathways, leading to the regulation of gene expression. MZ was particularly successful in inhibiting protein kinase B/Akt in *in vitro* experiments with cancer cells. Such inactivation resulted in growth inhibition and increase in apoptosis of cancer cells. Inhibition of Akt by MZ treatment was shown only in the presence of serum. This indicated that adsorption of serum components can be one of the mechanisms of MZ action in these experiments. Indeed, the addition of EGF to serum-free medium led to activation of Akt, which was also blocked by MZ pretreatment. Adsorption of molecules involved in signal transduction cascades, such as inositol phosphatides and calcium, might also contribute to its therapeutic efficiency. Preliminary lipid adsorption studies show that MZ are strong lipid sorbents. Similar results are observed with adsorption of proteins. Modifications of membrane ordering and interactions of other proteins with membrane proteins might also be involved [21], since membrane translocation is needed for activation of protein kinase B/Akt. It has also recently been shown that the activation of phosphoinositide-3 kinase and Akt is responsible for the ability of transformed epithelial cells to survive without cell attachment. Recent results indicate that constitutive activation of phosphoinositide-3 kinase in five small-cell lung cancers cell lines studied was responsible for fast growth and anchorage independence of small-cell lung cancer cells [22]. In accordance with this, MZ treatment leads to inhibition of protein kinase B/Akt pathways and subsequent apoptosis in our cell model. Akt has recently been demonstrated to inactivate an important cyclin inhibitor and tumor suppressor molecule, p27<sup>KIP1</sup> [22].

Here we provide evidence that MZ treatment increases levels of p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> in tumor cell models. It is not yet clear whether inhibition of Akt is involved in regulation of expression of p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> cell cycle inhibitors. Preliminary results also show that MZ adsorbs and deactivates nitric oxide and other oxidants. In addition, it has recently been reported that antioxidants stimulate the activation of cyclin inhibitor p21<sup>WAF1/CIP1</sup> [23]. This molecule is responsible for the arrest of cell growth, and its expression in adenocarcinomas of lung is positively correlated with optimistic survival prognosis. The present study observed that activated clinoptilolite induces tumor suppressor molecules (both p21 and p27).

The mechanisms of action of MZ *in vivo* remain largely unknown at this time. The results presented here

indicate that inhibition of proliferation and survival of cancer cells may be part of mechanisms involved in anticancer effect of MZ compounds. More studies on several other aspects of their action including possible immunomodulatory action of MZ will be performed in the future. Taken together, this report characterizes cellular effects of the MZ compounds in tissue culture cell models and provides data supporting a role of natural zeolite as an anticancer therapeutic agent in *in vivo* tumor models.

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## Immunostimulatory effect of natural clinoptilolite as a possible mechanism of its antimetastatic ability

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**Abstract Purpose:** 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. **Methods:** The experiments were performed on mice. Different biochemical and molecular methods were used. **Results:** Micronized zeolite (MZ) administered by gastric intubation to mice injected with melanoma cells significantly reduced the number of melanoma metastases. In mice fed MZ for 28 days, concentration of lipid-bound sialic acid (LSA) in serum increased, but lipid peroxidation in liver decreased. The lymphocytes from lymph nodes of these mice provoked a 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 superoxide anion, increased. However, NO generation was totally abolished. At the same time, translocation of p65 (NF $\kappa$ B subunit) to the nucleus of splenic cells was observed. **Conclusion:** Here we report antimetastatic and immunostimulatory effect of MZ and we propose a possible mechanism of its action.

**Keywords** Micronized zeolite · Clinoptilolite · Oxidative stress · Immunostimulation · T-lymphocyte NF $\kappa$ B

### Introduction

Zeolites are hydrated natural and synthetic microporous crystals with well-defined structures containing  $\text{AlO}_4$  and  $\text{SiO}_4$  tetrahedra linked through the common oxygen atoms (Breck 1964). Zeolites have properties to act as catalysts, ion-exchangers, adsorbents, and detergent builders (Colella 1999; Garces 1999; Flanigen 1980; Naber et al. 1994; Sersale 1985). Apart from being extensively used in different industrial applications, it is known that silicates and aluminosilicates also possess either positive or negative biological activity. Well-defined structures and catalytic activity make aluminosilicates an attractive model system for protein and enzyme mimetics (Bedioui 1995). Recent results have demonstrated that zeolite was very effective as a glucose adsorbent (Concepcion-Rosabal et al. 1997) as well as a potential adjuvant in anticancer therapy (Pavelic et al. 2001). 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 (Ozesmi et al. 1986).

Accumulating evidence has indicated that zeolites play an important role in regulation of the immune system. It was reported that silica, silicates, and aluminosilicates act as non-specific immunostimulators similarly to superantigens (Ueki et al. 1994). Superantigens (SAG) are a class of immunostimulatory and disease-causing proteins of bacterial and viral origin with the ability to activate a relatively large fraction (5–20%) of the T cell population. Activation requires simultaneous interaction of the SAG with the V $\beta$  domain of T cell complex (MHC) class II molecules on the surface of antigen-presenting cells (Ueki et al. 1994). Pro-inflammatory macrophages, that belong to class II MHC antigen-presenting cells, are activated by fibrinogen silicate particulate (Allison et al. 1996; Drumm et al. 1998).

It was shown that exposure of alveolar macrophages to silicate particles leads to activation of mitogen-activated protein kinases (MAPK), protein kinase C, and

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stress-activated protein kinases (SAPK) (Lim et al. 1997). Important transcription factors such as AP-1 and NF $\kappa$ B are also activated in lung epithelial cell, and expression of pro-inflammatory cytokines such as IL-1 $\alpha$ , IL-6 or TNF- $\alpha$  was enhanced (Simeonova et al. 1997). Modifications of receptor activation kinetics or activity of integrins can be responsible for the observed behavior. Alternatively, particles engulfed by phagocytosis were shown to stimulate production of reactive oxygen species (ROS) that have been found to be important second messengers for signal transduction in general (Martin et al. 1997). 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 (Gin-Pease and Whisler 1998; Kaul et al. 1998). 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 have shown that clinoptilolite treatment of mice and dogs suffering from various tumour types led to improvement of the overall health status, prolonged life-span, and decrease of tumour size (Pavelic et al. 2001). In addition, toxicology studies on mice and rats demonstrated that the same treatment did not have any negative effect (Pavelic et al. 2001). 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 decreased cell proliferation in several cancer cell lines. Here we present evidence for antimetastatic activity and immunostimulatory effect of clinoptilolite in vivo. In addition, we propose a possible mechanism of its action.

## Materials and methods

### Natural clinoptilolite

The 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 the silica, montmorillonite, and mordenite zeolite. Chemical composition of clinoptilolite: 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%, and 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, NH<sub>4</sub><sup>+</sup> substitution capacity 8,500 mg NH<sub>4</sub><sup>+</sup>/kg. Particle size analysis of the clinoptilolite showed that the maximum frequency of particles appeared at 1  $\mu$ m.

### Animals

C57Bl/6 mice were used for the experiment with B16 metastasis. CBA/HZgr and RFM mice were used for oxidative stress param-

eters and for cellular immune-response measurement. The mice were about 3 months old, weighing 21–26 g. Mice were bred in the Animal facility of the Ruđer Bošković Institute. Food (Domzale, Slovenia) and tap water were given ad libitum. Animals were kept in conventional circumstances: light/dark rhythms 12/12 h, temperature 22 °C, and humidity 55%.

### Application of MZ

Since MZ is insoluble, it was administered to the mice either orally by gavage (100 mg/mice per day) or in their diet given as standard food consisting of 12.5% or 25% MZ. Each mouse ate about 4 g food daily, thus consuming 0.5 g or 1 g MZ, respectively. In part of the experiments suspension of MZ was administered intraperitoneally (3 mg/mouse).

### 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 H<sub>2</sub>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 h after i.p. or 7, 14, 21, and 28 days after per os administration of MZ. Macrophages were collected with Hank's solution (without phenol red; Sigma) and red blood cells were removed by NH<sub>4</sub>Cl lysis. The remaining cells were washed three times, resuspended in RPMI 1640 (without phenol red; Sigma) supplemented with antibiotics and 10% fetal calf serum (FCS; Sigma), and adjusted to  $2 \times 10^6$  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. (Johnston et al. 1978). Samples contained 1 ml of cytochrome C (1 mg/ml) in phenol-free Hank's balanced salt solution and  $2 \times 10^6$  cells in 100  $\mu$ l of medium. The specificity of the reaction was tested by the addition of 60 IU SOD per millilitre 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 800 $\times$ g, 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. (Naslund et al. 1995). Briefly, cultures of isolated peritoneal macrophages were incubated in plastic 24-well flat-bottom microplates (Falcon, USA) for 48 h at 37 °C and 5% CO<sub>2</sub>. Aliquots (800  $\mu$ l) of each supernatant were placed in tubes and mixed with 800  $\mu$ l of GRIESS reagent (1% sulfanil amide 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  $\mu$ M) 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 exsanguination, sera from fed mice were collected and prepared for LSA measurement according to Katopodis et al. (Katopodis et al. 1982). The spleen and liver were removed from i.p. and per os treated mice. Concentration of TSA in the spleen was determined according to Hadzija et al. (Hadzija et al. 1992) 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. (Ohkawa et al. 1979). 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 (Shohat and Trainin 1980) was used. In our experiment, LAG-VHR was done on allogeneic mice instead of rats. For each experiment ten control mice (treated with conventional food) and ten mice in each experimental group were used. CBA mice were fed either 12.5% (0.25 g) or 25% (0.5 g) MZ per day, during a period of 21 or 28 days. Mice were killed by bleeding. The pool of lymphocytes from lymph nodes of 3–5 treated or control mice was prepared, washed two times with Hank's by centrifugation. 2×10<sup>7</sup> living cells were injected intradermally into the shaved abdominal skin of RFM mice (irradiated with 7 Gy, 24 h before), where lymphocytes 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 the blue-stained area was measured with caliper along two opposite diameters. A mean diameter of each spot was shown as a result.

#### NFκB activation in spleen

Twenty-four hours after i.p. injection of MZ to experimental mice, and Hank's solution to control mice, animals were killed by cervical dislocation. For preparation of cytoplasm and nuclear fractions of spleen, cells were isolated and a crude spleen extract was made. Erythrocytes were removed by ammonium chloride lysis. The nuclear and cytosolic fractionation procedure was a modification of the protocol of Lernbecher et al. (Lernbacher et al. 1993). 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 60-min lysis on ice, nuclei were spun down, and the supernatant, after additional centrifugation at 17,500×g, was stored as the cytoplasmic fraction. The nuclear pellet was resuspended 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 min. Centrifugation at 17,500×g was performed to remove insoluble debris. The supernatant was used as nuclear extract.

#### Western blot (immunoblot) analysis

Protein concentration in nuclear and cytoplasm fractions was determined by Bradford assay. Equal amounts of nuclear and cytoplasm proteins (20 µg and 80 µg, respectively) were separated by 9% – SDS PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). Levels of loaded proteins were 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 min with primary antibodies (anti-p50, anti-RelB, and anti-p65), washed in TBS/0.05% Triton X-100, and then incubated for 1 h 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, 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.

#### Statistical analysis

Statistical analysis for all experiments was performed by the Student's *t*-test. A level of *P* < 0.05 was accepted as statistically significant.

## Results

### Antimetastatic effect of MZ

In the experiment where 15×10<sup>4</sup> melanoma cells were injected into controls and mice treated with MZ, the number of lung metastases was reduced from 36.05 ± 13.09 (in the control group consisting of six mice) to 21.0 ± 4.96 for treated mice (consisting of five animals). This was not statistically significant (*P* = 0.056). On the other hand, if 7.5×10<sup>4</sup> melanoma cells were injected, MZ-treated mice (ten animals) had a reduced number of lung metastasis compared to controls (five animals). While the number of metastasis in the control group was 5.2 ± 1.64, in treated mice the number of metastasis was strongly reduced to 0.7 ± 1.06. In addition, statistical significance was *P* < 0.001.

Influence of MZ on macrophages O<sub>2</sub><sup>−</sup> production, lipid peroxidation (LPO) in liver, and lipid-bound sialic acid (LSA) in serum of healthy mice fed with MZ

O<sub>2</sub><sup>−</sup> production, TBARS, and LSA concentration were measured in healthy mice fed with MZ (0.5 g or 1 g/day) for 7, 14, 21, and 28 days. The results are shown in Table 1. Concentration of O<sub>2</sub><sup>−</sup> (in peritoneal macrophages) started to change slightly 14 days after administration of 1 g of MZ. However, TBARS concentration (in liver) started to change significantly 21 days after administration regardless of MZ concentration. Significant results regarding LSA concentration in serum was obtained with 1 g MZ on day 28.

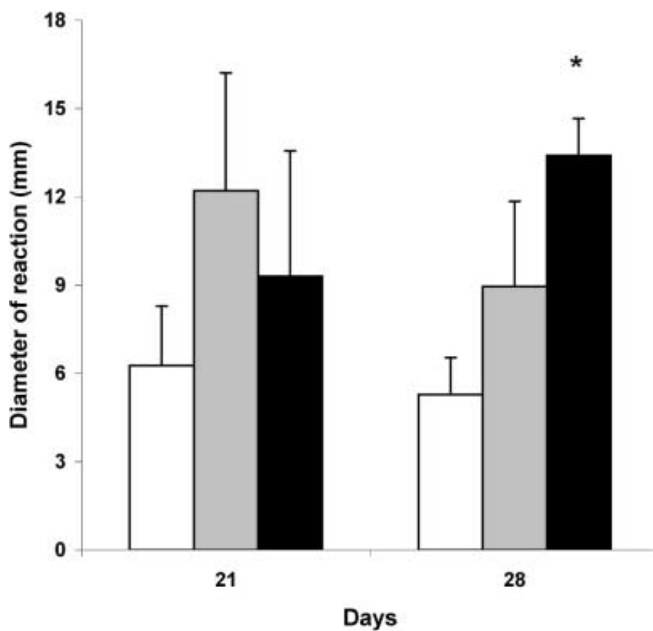
### Effect of MZ on local allogeneic graft versus host reaction

Results of two separately prepared experiments on healthy mice fed MZ 21 and 28 days are shown in Fig. 1. The cells from lymph nodes of mice fed 28 days with MZ (1 g/day) provoked a significantly higher GVH reaction than cells of control group mice. A treatment with a lower dose (0.5 g) of MZ for 21 or 28 days also

**Table 1** Influence of MZ on  $O_2^-$  production, lipid peroxidation (TBARS), and lipid bound sialic acid (LSA) in healthy mice fed with MZ

	Days	7	14	21	28
$O_2^-$ nmol/ $2 \times 10^6$ macrophages	Control	$3.10 \pm 0.14$	$1.50 \pm 0.23$	$0.45 \pm 0.07$	$1.20 \pm 0.9$
	0.5 g MZ	$3.95 \pm 0.8$	$0.68 \pm 0.2^a$	$2.55 \pm 0.21^a$	$0.55 \pm 0.07^a$
	1 g MZ	$3.20 \pm 0.56$	$0.95 \pm 0.35$	$0.57 \pm 0.39$	$1.10 \pm 0.14$
	Control	$0.97 \pm 0.11$	$0.94 \pm 0.22$	$1.89 \pm 0.18$	$1.78 \pm 0.09$
TBARS nmol/mg liver proteins	0.5 g MZ	$0.95 \pm 0.11$	$0.72 \pm 0.04$	$1.20 \pm 0.29^a$	$1.16 \pm 0.08^a$
	1 g MZ	$0.94 \pm 0.13$	$0.61 \pm 0.09$	$0.85 \pm 0.11^a$	$0.85 \pm 0.12^a$
	Control	$0.18 \pm 0.07$	$0.26 \pm 0.11$	$0.29 \pm 0.03$	$0.27 \pm 0.03$
LSA nmol/l	0.5 g MZ	$0.29 \pm 0.13$	$0.25 \pm 0.04$	$0.42 \pm 0.16$	$0.51 \pm 0.17^a$
	1 g MZ	$0.28 \pm 0.06$	$0.28 \pm 0.08$	$0.34 \pm 0.06$	$0.49 \pm 0.15^a$
	Control				

<sup>a</sup>Indicates significantly different (Student's *t*-test,  $P < 0.05$ ) result compared to relevant control. The values are  $\Sigma \pm SD$



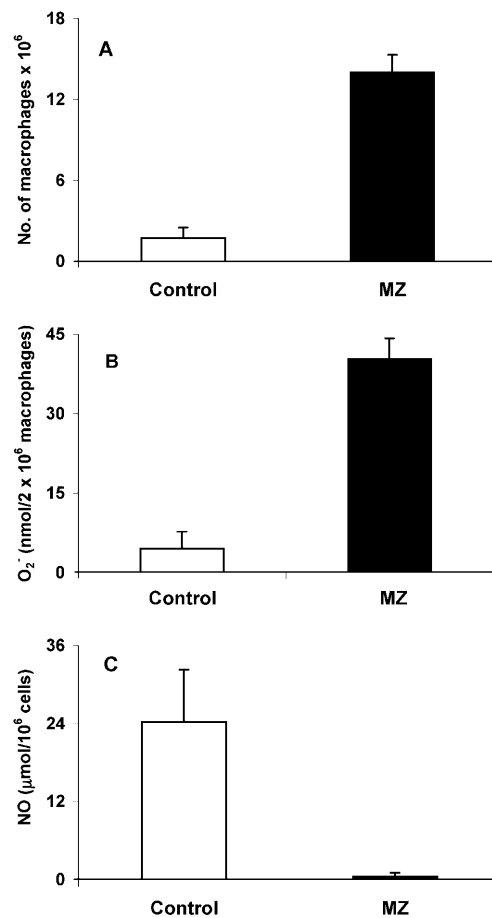
**Fig. 1** Effect of MZ on allogeneic graft versus host reaction. The GVH reaction of lymph node cells of control mice (empty column), mice fed with 0.5 g (gray column), and 1 g (black column) MZ were tested

showed the higher reaction than the control group, but it was not significant.

Effect of i.p. administration of MZ on number of peritoneal macrophages, ROIs generation, and oxidative stress (OS) parameters

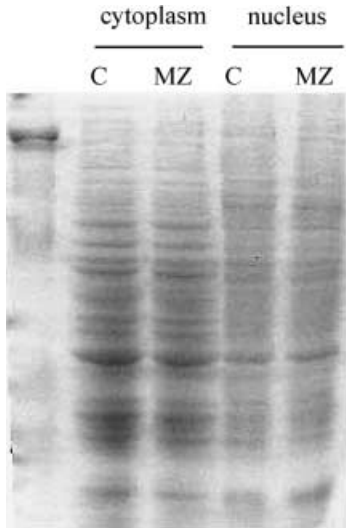
Intraperitoneal administration of MZ at a dose higher than 3 mg was lethal for mice and a dose of 3 mg was sublethal but proinflammatory. In experiments, a dose of 3 mg was used and the number of macrophages, production of  $O_2^-$  and NO, as well as TSA measurement in spleen and TBARS concentration in liver was performed 24 h after intraperitoneal injection of MZ.

MZ provoked accumulation of macrophages in peritoneum. The number of peritoneal macrophages (PM) after treatment was seven times higher than in control mice (Fig. 2a). The concentration of  $O_2^-$  was ten



**Fig. 2** a Number of peritoneal macrophages per mouse, b superoxide generation in macrophages, and c nitric oxide concentration in peritoneal macrophages ex vivo 24 h after treatment with 3 mg MZ/mouse. There were 18 control and 21 mice treated with MZ in two experiments

times higher in macrophages of treated mice than in controls (Fig. 2b). 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 h ex vivo, strongly decreased (Fig. 2c). There was no change in liver TBARS concentrations (expressed in nmol/mg protein) between the control

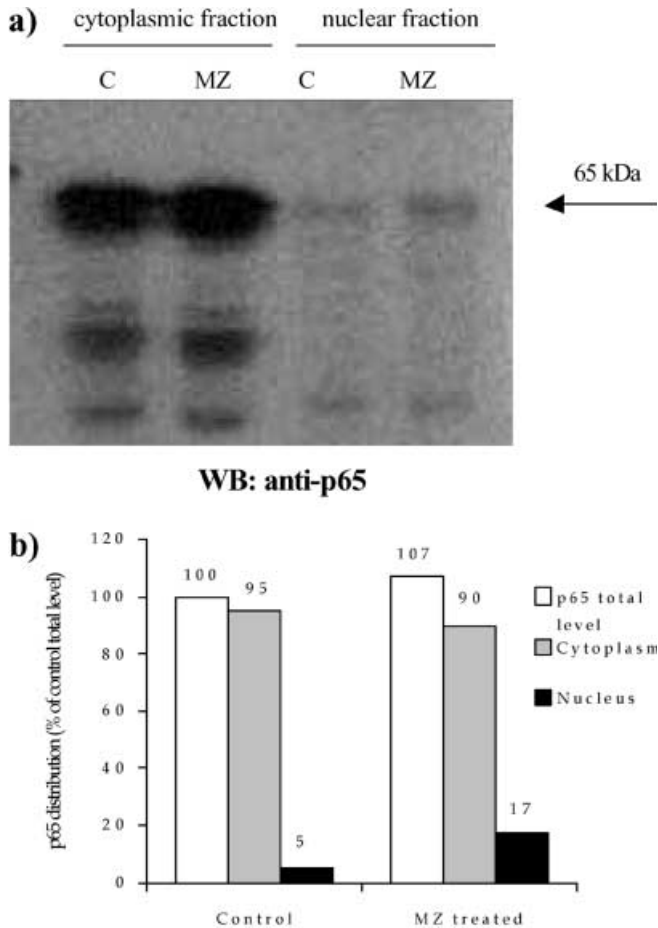


**Fig. 3** Amounts of nuclear and cytoplasmic proteins were checked by Coomassie blue staining. *C* indicates splenic proteins of control group, whereas *MZ* indicates splenic proteins of treated animals

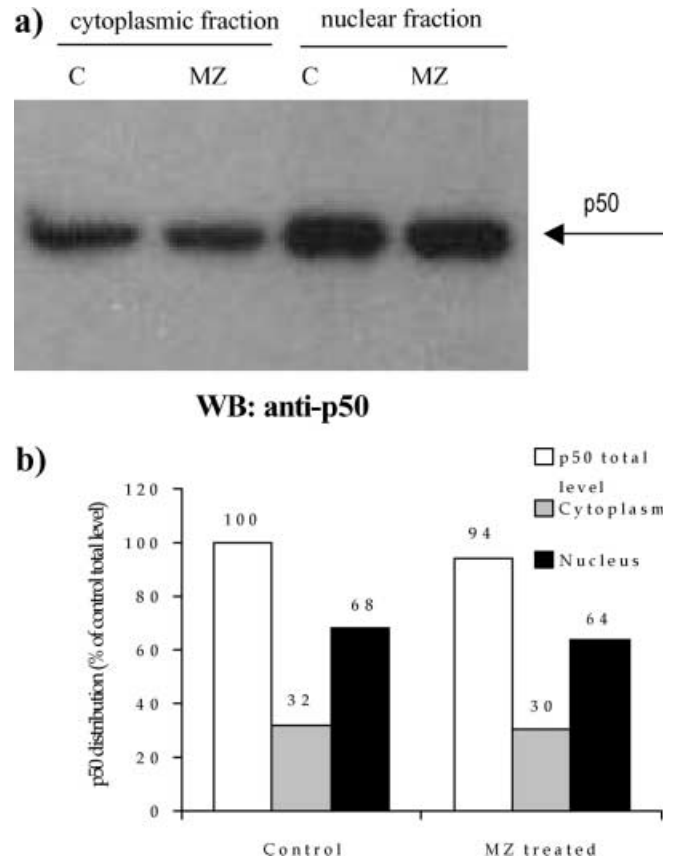
group ( $0.907 \pm 0.17$ ) and treated ( $0.886 \pm 0.16$ ) group. In addition, TSA concentration (expressed as  $\mu\text{g}/10^6$  splenocytes) was not changed after treatment with MZ. The value of the control group was  $5.25 \pm 0.73$ , and  $5.58 \pm 0.78$  for the treated group.

#### Effect of MZ on NF $\kappa$ B/Rel proteins

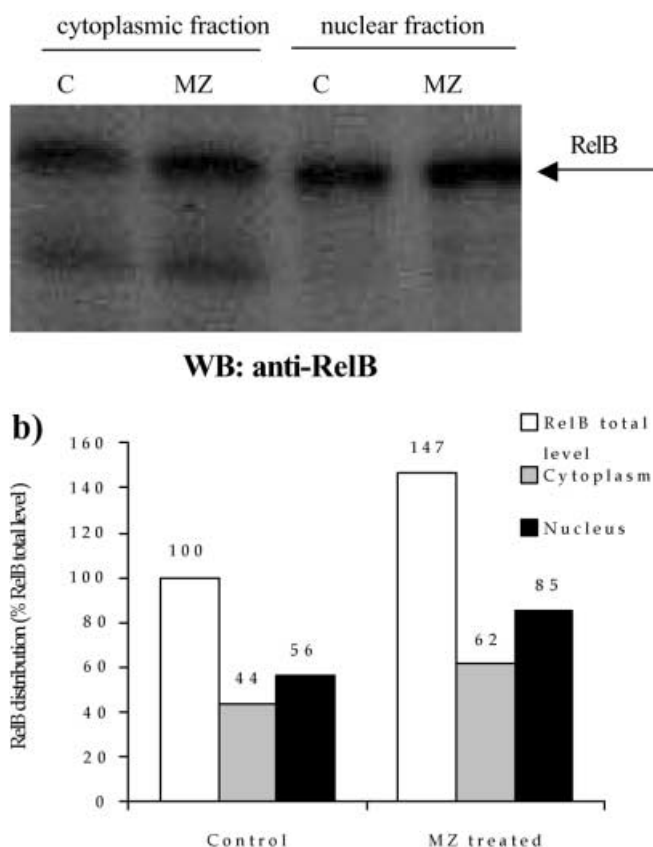
A mass of spleens isolated from animals treated i.p. with 3 mg MZ was significantly higher than that of control animals ( $120 \pm 18$  mg for control compared to  $175 \pm 23.6$  mg for MZ-treated mice). The effect of MZ on NF $\kappa$ B/Rel proteins could be seen after preparation of cytoplasm and nuclear fractions, electrophoresis, and Western blot. Equal levels of nuclear and cytoplasm fractions ( $20 \mu\text{g}$  and  $80 \mu\text{g}$ , respectively) isolated from spleen of control and MZ-treated mice were loaded on gel, which was confirmed by Commase blue staining (Fig. 3). MZ treatment increased translocation of p65 subunit into nucleus (Fig. 4) suggesting that the NF $\kappa$ B-containing p65 subunit had been activated. However, MZ treatment did not have any effect on the p50 or RelB subunit concerning translocation into the nucleus (Fig. 5



**Fig. 4a,b** Effect of MZ on level and distribution of p65 subunit of NF- $\kappa$ B. **a** Western blot analysis of p65 in nuclear and cytoplasmic fractions. *C* indicates control group of animals, whereas *MZ* indicates treated animals; **b** densitometric quantification of signal intensities. p65 total level indicates sum of cytoplasmic and nuclear fraction signal intensities in each group (*C* and *MZ*). Results are expressed as percentage of control p65 total level



**Fig. 5a,b** Effect of MZ on level and distribution of p50 subunit of NF- $\kappa$ B. **a** Western blot analysis of p50 in nuclear and cytoplasmic fractions; **b** densitometric quantification of signal intensities. p50 total level indicates sum of signal intensities of cytoplasmic and nuclear fraction in each group (*C* and *MZ*). Results are expressed as percentage of control p50 total level



**Fig. 6a,b** Effect of MZ on level and distribution of RelB subunit of NF- $\kappa$ B. **a** Western blot analysis of RelB in nuclear and cytoplasmic fractions; **b** densitometric quantification of signal intensities. RelB total level indicates sum of signal intensities of cytoplasmic and nuclear fraction in each group (C and MZ). Results are expressed as percentage of control RelB total level

and 6). However, the total amount of the RelB subunit increased by about 40% and the total amount of p50 slightly decreased compared to controls.

## Discussion

### Antitumor effect of MZ in vivo

Previous in vitro and in vivo animal studies showed that MZ was a non-toxic compound that acted as an adjuvant in anticancer treatment (Pavelic et al. 2001). The range of effects was diverse, ranging from negative antitumor response, to normalization of biochemical parameters and prolonged life span.

Our previous in vitro experiments (Pavelic et al. 2001) 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 (50 mg/ml). We analyzed mitogenic and survival signaling pathways in tumour cells (Pavelic et al. 2001) and the most significant results were observed in the activity of Akt protein that was highly inhibited after incubation of cancer cells with MZ-pre-treated medium. That resulted in growth inhi-

bition and increased apoptosis of cancer cells, but only in the presence of serum in medium.

Since previous in vitro results indicated an indirect effect on tumour cell lines growth and that MZ is not resorbed from gastrointestinal system, the MZ effect in vivo cannot be due to some direct biochemical interaction. The result of the antimetastatic effect of MZ presented here is concomitant with previous results and shows reduction of metastasis in mice. However, since the antimetastatic effect is dependent on the number of tumour cells injected into mice, we assumed that an immunological response is involved in MZ in vivo action. To confirm the possible mechanism of MZ in vivo effect, we used different ways of applying MZ to healthy mice.

### Immunostimulatory effect of MZ in vivo

Inoculation of immunostimulators such as BCG or Freund's adjuvant as well as antigens or Silasorb results in a two- to threefold increase of the serum sialic acid level in mice (Sydow et al. 1989). Being a marker for inflammation, sialic acid may have a regulatory role in immunological processes. Inoculation of BCG also provokes an increased formation of macrophages in peritoneum (Sydow et al. 1989). The data presented here demonstrated that application of MZ in food for 28 days increased serum LSA concentration in healthy mice, which is probably associated with inflammatory process, i.e., activation of macrophages. That is confirmed by our data of elevated  $\text{O}_2^-$  in peritoneal macrophages of MZ-fed mice. We suspect, according to previous results (Sydow et al. 1989), 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 acute phase proteins.

Although a parenteral route of application is not suitable, to confirm the immunomodulatory effect of MZ we examined the processes that happened after MZ intraperitoneal application. For this purpose, different amounts of MZ were injected in normal, healthy mice. It was shown that effect of MZ was dose dependent. Doses higher than 3 mg/mouse were toxic. However, since lower doses (3 mg/mouse) had a non-toxic, but proinflammatory effect, the immunological parameters were measured. In the acute phase of an inflammatory process, a large number of polymorphonuclear leukocytes (PMNs) migrate from the blood and accumulate in the exudate (Hambleton and Miller 1989). In our experiments, 24 h after MZ administration, a high accumulation of macrophages was found in peritoneum of treated animals. Our results confirmed that macrophages of treated mice were activated since they generated a nine-times higher amount of  $\text{O}_2^-$  than those of control mice.



Normally, NO reacts with  $O_2^-$  in a quite fast reaction, which is completed within less than 1  $\mu$ s (Huie and Padmaja 1993). 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 peroxynitrous acid, a relatively long-lived, strong oxidant, which could initiate oxidation of lipids. These reactions could explain the toxic (at higher doses) and inflammatory (at lower doses) effect of MZ administrated in peritoneum. In addition, the shown depletion of NO from peritoneal macrophages might have significantly increased superoxide generation and in this way could have intensified the effect of MZ. Since the 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 (Chaudhri and Clark 1989). One ubiquitous transcription factor of particular importance in immune and inflammatory responses is nuclear factor kappa B (NF $\kappa$ B) (Kopp and Ghosh 1995). 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 an immunoactivator, activating NF $\kappa$ B, and therefore inducing transcription of genes regulated with NF $\kappa$ B.

A decreased amount of p50 and increased amount of RelB proteins in treated mice compared to control mice could be due to a changed number and/or ratio of B- and T-lymphocytes. The fact that spleens of treated mice were 11% heavier contributes to this assumption. B-lymphocytes have a basal level of p50 homodimer that is not inducible upon stimulation (Liou et al. 1994) and/or serve as regulators of NF $\kappa$ B activity (Kang et al. 1992). A decreased total amount of p50 protein, as well as unchanged translocation into nucleus, could imply that B-lymphocytes were not stimulated by MZ. In addition, 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 (Lernbecher et al. 1993; Lernbecher et al. 1994). 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 NF $\kappa$ B activation, only inducible NF $\kappa$ B activity has been described in T-cells or T cell lines (Schreck et al. 1991). However, as complete T-cell activation requires at least two signals provided by the T-cell receptor (TCR) complex and another stimulatory molecule, optimal NF $\kappa$ B activation in the T-cell is also dependent on dual signaling mechanisms (Crabtree and Clipstone 1994). Many agents have been shown to

promote activation of NF $\kappa$ B in T-cells including TNF- $\alpha$  (Menon et al. 1995), calcium ionophores (Ginn-Pease and Whisler 1998), and  $H_2O_2$  (Schreck et al. 1991). However, maximal NF $\kappa$ B activation has been observed in response to combinations of stimulants which fulfil the dual signaling requirements of T-cells (Crabtree and Clipstone 1994; Kanno and Siebenlist 1996). ROS have been found to act as second messengers in activation of NF $\kappa$ B/Rel proteins (Schreck et al. 1991) and oxidative stress can modulate the activity of NF $\kappa$ B in T-cells (Ginn-Pease and Whisler 1998). In addition, previous results have shown that NF $\kappa$ B is activated in inflammatory diseases. Therefore, according to all these results, as well as to ours, we concluded that MZ, in our experiments, activated a T-cell immunological (cellular) response that could be involved in an anticancer effect of MZ in vivo.

### Possible mechanism of MZ in vivo action

Due to our results, we propose the mechanism of MZ in vivo action. MZ caused local inflammation at the place of application that attracted peritoneal macrophages. Macrophages were activated, which has been shown with increased  $O_2^-$  production. We suggest that activated macrophages produced TNF- $\alpha$  that, together with the other stimulants (e.g., other cytokines, ROS or changed calcium concentration), stimulated spleen T-cells. Since products of the genes that are regulated by NF $\kappa$ B also cause its activation, this type of positive regulatory loop may amplify and perpetuate the local inflammatory response. Our hypothesis is that MZ acted the same way after per os administration, affecting intestinal macrophages. The results of experiments with metastases reduction, when a lower number of tumour cells were injected, as well as local allogeneic graft versus host reaction, can partially confirm this hypothesis. Our results are in agreement with the accumulating evidence that zeolites could play an important role in regulation of 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- $\alpha$  in serum and B- and T-lymphocytes should be measured separately, as well as activation of NF $\kappa$ B in macrophages.

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