1. Antiproliferative effects of creatine – non-stabilized or optimally stabilized in a panel of human tumor cell lines.

The antiproliferative effects of a optimally stabilized formulation of creatine was investigated in a comparative fashion vs. non-processed creatine against a panel of human tumor cell lines, representative for some important types of human malignant diseases – leukemias, lymphomas and solid tumors. The panel included the acute promyelocyte leukemia HL-60, the chronic myeloid leukemia LAMA-84, the Hodgkin-lymphoma HD-MY-Z, the multiple myeloma-derived cell lines OPM-2, U-266 and RPMI-23366, the bladder carcinoma MGH-U1 (formerly designated as EJ), and the breast carcinoma MCF-7. In order to evaluate the selectivity of cytotoxic/antiproliferative effects we investigated the effects of tested compounds against human umbilical vein endothelial cells (HUVEC) as an example of a normal, non-tumor cellular population MGH-U1 originated from the American Type Cell Culture (Rockvill, MD, USA), whereas all other tumor cells where obtained from the German Collection of Microorganisms and Cell Cultures (Brounschweig, Germany) and were routinely maintained under standard conditions – RPMI-1640 medium, supplemented with 10% fetal calf serum and L-glutamine, in a 5% CO_2 humidified atmosphere (at 37°C).

HUVEC were purchased from Cambrex (GB) and were maintained according to the instructions of the manufacturer in a ECM-growth medium supplemented with a Bullet kit (containing fetal calf serum, different growth factors and amphotericin B).

For the cytotoxicity assessment exponentially growing cells were plated in 96-well flat-bottomed microplates and after 24 h were treated with the tested creatine formulations. The tested compounds were dissolved in phosphate buffered saline and serially diluted in RPMI-1640 to the desired level. For each concentration at least 8 wells were used.

After a 72 h continuous exposure period the cellular viability was monitored by the standard MTT-dye reduction assay, as described elsewhere [1], with minor modifications [2, 3].

As evident from the results summarized in Tables 1.1-1.9 and figures 1.1-1.9, the treatment of malignant cells with either non-processed or stabilized creatine was consistent with a concentration-dependent decrease in the cellular viability. In all of the tested cell lines the administration of tested compounds failed to prodice 50% inhibition of cellular proliferation and hence IC_{50} values were not calculated. Nevertheless the point-to point juxtaposition of the effects unambiguously indicates that the optimally stabilized formulation is superior in terms of antiproliferative effects – at all concentration levels, throughout the panel of tumor cell lines this creatine product exerted greater inhibitory activity at a statistically significant level.

Moreover both processed and non-stabilized creatine products failed to induce any antiproliferative effects against the normal HUVEC cells, which is indicative for a tumor-specific antineoplastic effects.

The antiproliferative effects of high concentrations of macroergic compounds such as ATP and creatine is firmly established [4, 5]. On this ground a significant number of creatine and cyclocreatine analogues have been synthesized and evaluated for cytotoxic effects, whereby the established sensitivity of cancer cells is found to be dependent on the levels of creatine-kinase expression [6, 7]. The creatine kinase (CK) isozymes and their substrates, creatine and creatine phosphate, are believed to play a pivotal role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, and brain [7, 8]. This enzyme system, however may also be involved in the process of cellular transformation. Inhibition of tumor cell growth by creatine analogues has been observed and may be due to the ability of these analogues to impair cellular energy generation and utilization [6, 9]. Most probably creatine acts in a similar way when applied at high, milimolar concentrations.

Our results unambiguously show that the processing of creatine significantly inceases its antiproliferative effects, presumably due to better stability, conditioning superior exposure as compared to the conventional creatine formulation.

Appendix 1: Antiproliferative effects of creatine – non-stabilized or optimally stabilized in a panel of human tumor cell lines. MTT-data and dose –response plots.

Table 1.1. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human acute promyelocyte leukemia HL-60 as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	3.4		
0.2	100.0	0.7	89.8*#	3.6
0.3	96.2	2.7	83.7*#	4.5
0.4	90.4*	4.0	84.2*#	2.5
0.8	91.1*	5.2	78.8*#	2.0
1.0	86.9*	3.3	76.5*#	1.4

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

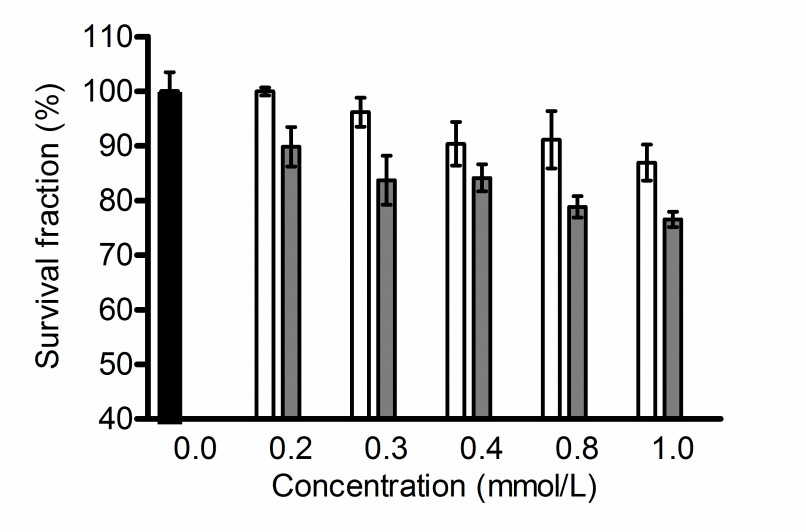

Figure 1. 1. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human acute promyelocyte leukemia HL-60. as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.2. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human chronic myeloid leukemia LAMA-84 as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	1.4		
0.2	98.3	4.7	94.1*	1.4
0.3	97.2	4.4	87.3*#	3.6
0.4	94.5*	2.4	85.9*#	3.8
0.8	94.2*	2.7	85.4*#	3.1
1.0	89.9*	5.8	80.9*#	3.3

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

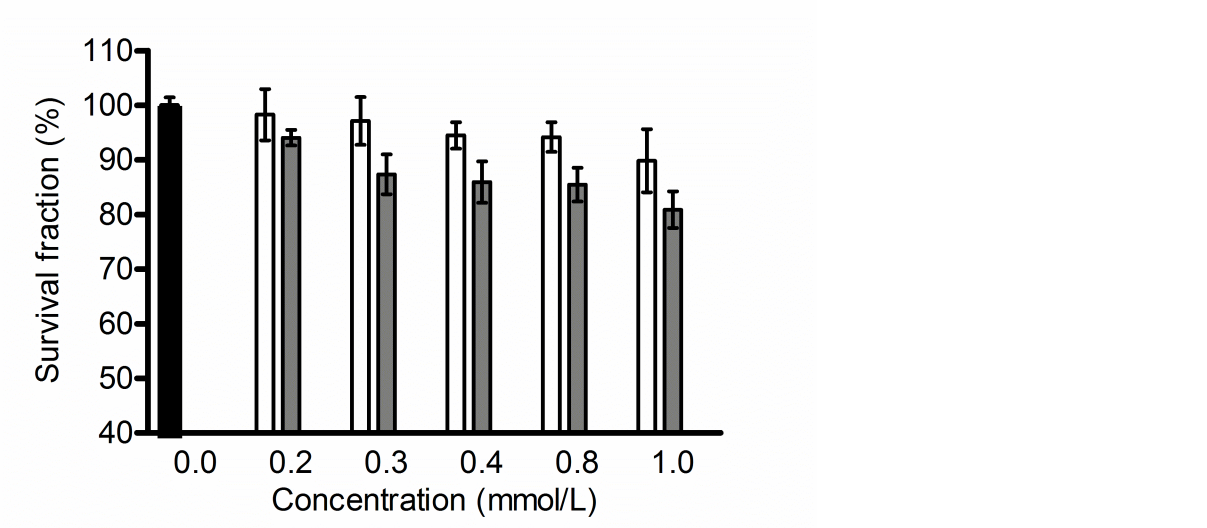

Figure 1.2. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human chronic myeloid leukemia LAMA-84, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.3. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human Hodgkin lymphoma HD-MY-Z as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	2.3		
0.2	100.0	0.7	85.9*#	3.9
0.3	97.7	2.8	85.4*#	3.4
0.4	95.1	7.4	80.3*#	2.1
0.8	86.5*	3.0	78.0*#	3.1
1.0	80.2*	2.3	73.6*#	3,1

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0*#.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

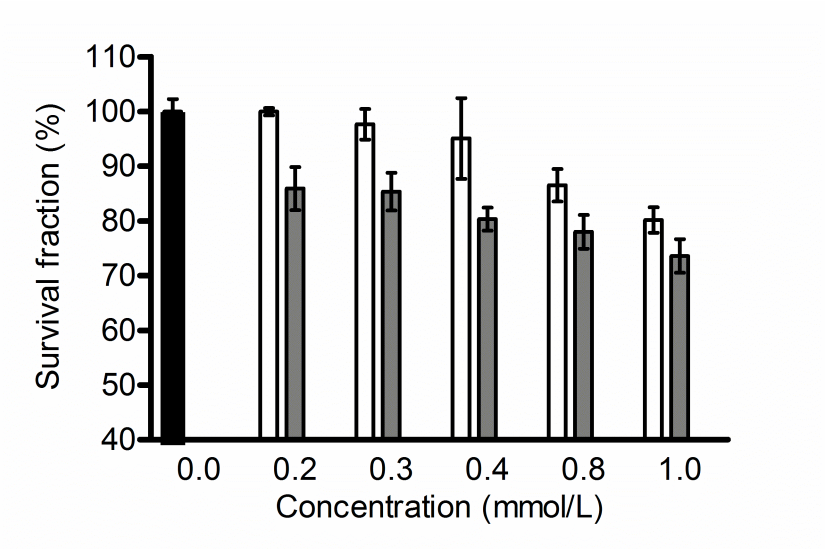

Figure 1.3. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human Hodgkin lymphoma HD-MY-Z, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.4. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human multiple myeloma OPM-2 as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	3.8		
0.2	88.5*	4.8	83.9*	1.5
0.3	88.6*	1.4	82.0*#	2.5
0.4	81.8*	1.2	75.4*#	2.8
0.8	80.0*	2.8	76.7*	1.9
1.0	79.5*	4.3	70.7*#	2.1

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

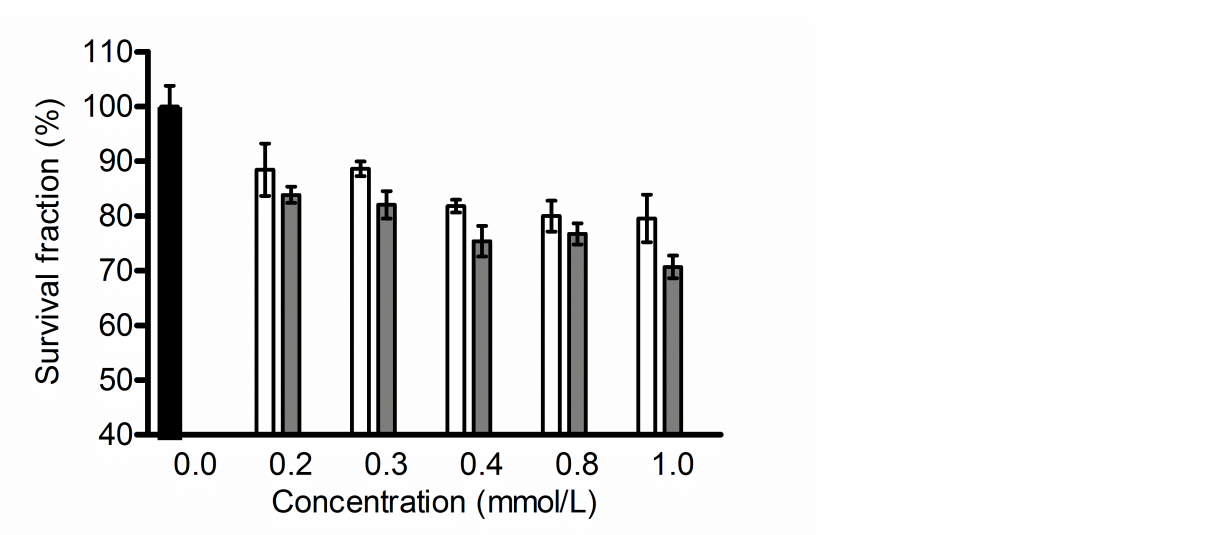

Figure 1.4. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human multiple myeloma OPM-2, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.5. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human multiple myeloma RPMI-8226 as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100,0	1,7		
0.2	99,2	3,7	97,9	2,2
0.3	98,2	3,0	93,6*	2,6
0.4	96,0*	2,3	92,0*	2,2
0.8	97,8	4,0	91,8*	3,2
1.0	95,2*	2,4	88,0*#	1,3

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

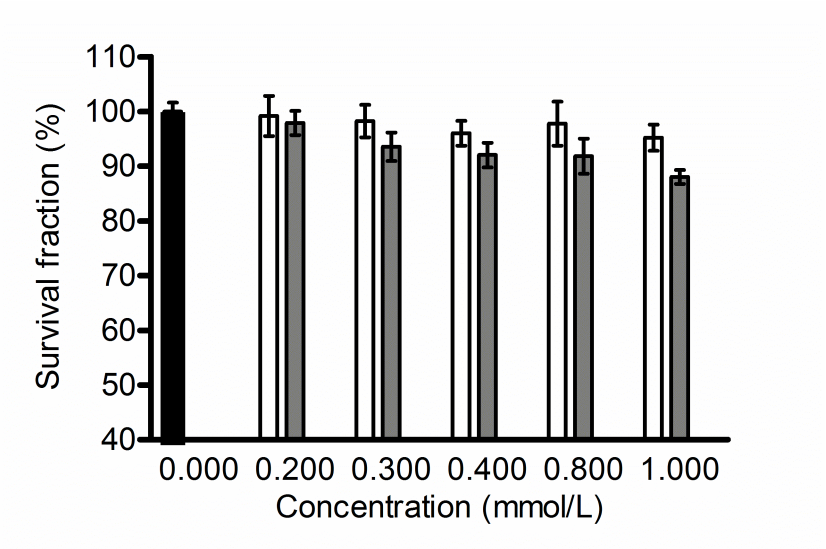

Figure 1.5. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human multiple myeloma RPMI-8226, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.6. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human multiple myeloma U-266 as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	5.2		
0.2	88.3*	3.7	88.1*	5.4
0.3	91.3*	3.3	87.3*	1.6
0.4	84.0*	2.4	79.9*#	1.1
0.8	86.1*	4.7	77.8*#	1.4
1.0	82.5*	6.3	71.4*#	2.3

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

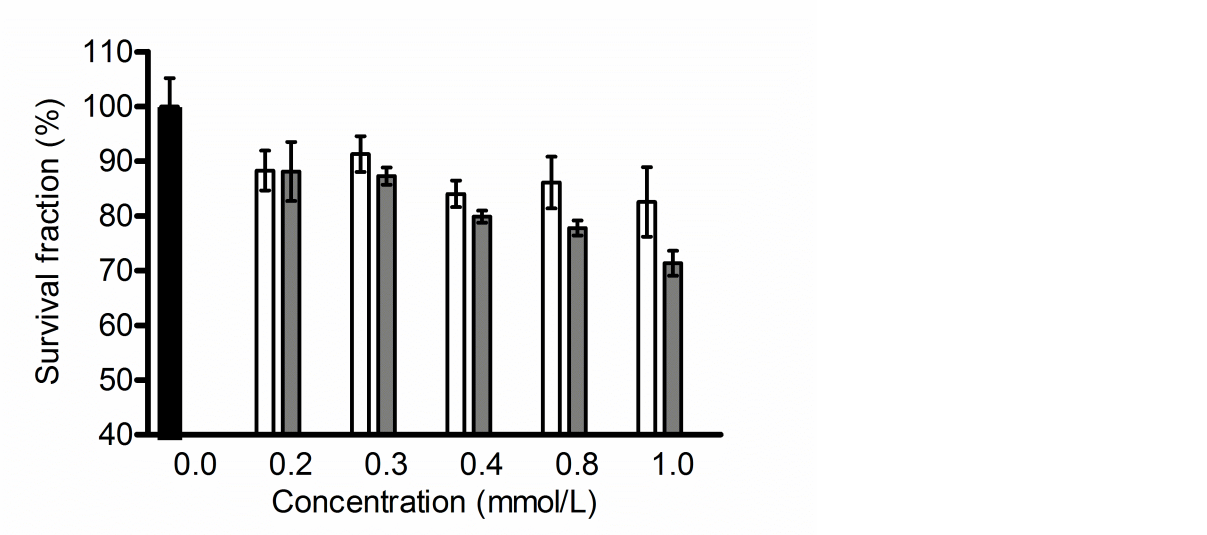

Figure 1.6. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human multiple myeloma U-266, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.7. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human bladder cancer MGH-U1, as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100,0	5,3		
0.2	89,9*	3,9	81,2*#	1,4
0.3	87,2*	5,0	73,7*#	2,8
0.4	78,1*	4,1	68,8*#	2,3
0.8	77,9*	6,1	64,9*#	3,6
1.0	77,2*	2,5	63,3*#	3,5

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

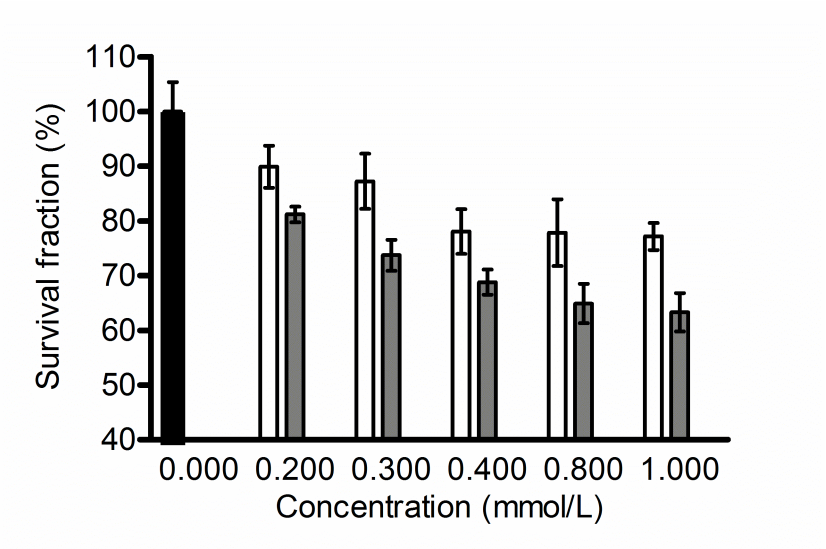

Figure 1.7. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human bladder cancer MGH-U1, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.8. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against the human breast cancer MCF-7, as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	2.6		
0.2	99.4	2.6	94.1*	2.3
0.3	96.6	2.8	82.2*#	2.8
0.4	85.6*	2.7	82.2*#	0.5
0.8	81.5*	1.9	74.5*#	3.4
1.0	81.1*	1.5	69.8*#	1.7

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

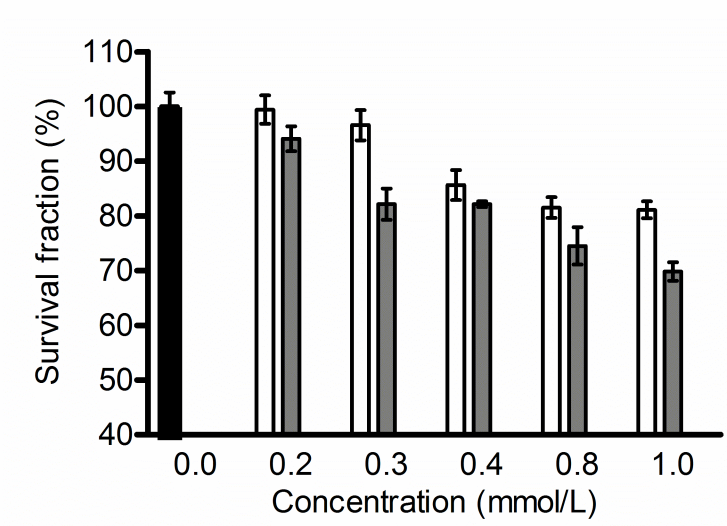

Figure1. 8. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against the human breast cancer MCF-7, as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.

Table 1.9. Antiproliferative effects of the optimally stabilized creatine formulation vs. conventional creatine against human umbilical vein endothelial cells (HUVEC), as assessed by the MTT-dye reduction assay after 72 h continuous exposure.

Concentration (mmol/L)	Conventional creatine		Optimally stabilized creatine	
	% of viable cells		% of viable cells	
	Mean	sd	Mean	sd
0.0	100.0	3.1		
0.2	103.6	3.2	99.2	6.9
0.3	98.2	1.5	98.6	7.4
0.4	96.5	4.1	99.0	1.4
0.8	98.2	7.6	98.6	8.6
1.0	100.6	4.3	100.3	7.6

* Statistically significant ($p<0.05$) vs. the untreated control; # Statistically significant ($p<0.05$) vs. the equivalent concentration of conventional creatine (Student's t-test).

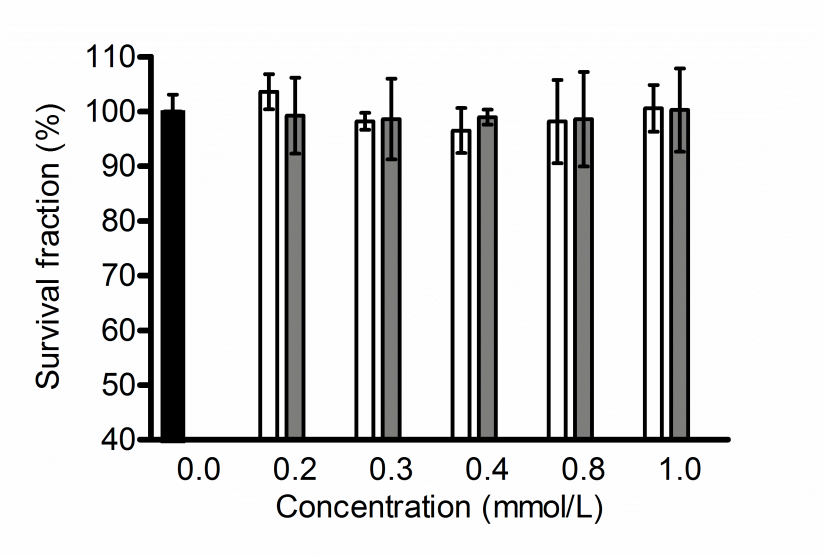

Figure 1.9. Antiproliferative effects of the optimally stabilized creatine formulation (grey bars). vs. conventional creatine (white bars) against human umbilical vein endothelial cells (HUVEC), as assessed by the MTT-dye reduction assay after 72 h continuous exposure. Each bar represents the arithmetic mean ± sd of eight separate experiments.