The anti-tumoral effectivness of **Biocrush®** by IGV institute, Germany

IGV GmbH

ANALYTIK / QUALITÄTSMANAGEMENT
BACKWAREN
BIOTECHNOLOGIE / UMWELT
NACHWACHSENDE ROHSTOFFE / LEBENSMITTELTECHNOLOGIE
TECHNOLOGIETRANSFER

The test of the anti-tumoural effectivness of the preparation *Biocrush®* in vitro

by
IGV GmbH
Arthur-Scheunert-Allee 40-41
14558 Nuthetal
Germany

Approved by: Prof. Dr. Dr. h.c. Otto Pulz

IGV INSTITUT FÜR GETREIDEVERARBEITUNG GMBH
Arthur-Scheunert-Allee 40/41
14558 Nuthetal OT Bergholz-Rehbrücke

Nuthetal, March 1, 2007

The cytotoxicity of **Biocrush®** on cancer cells was done by the MTT test. This a colorimetric test, which bases on the reduction of the yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) to purple formazan crystals through cellular dehydrogenase. Because dehydrogenase is only active in living cells, the transformation of MTT is a measure for the degree of viability and the metabolic activity of the cells (Mosmannet al.1983). The absorption of the formed formazan is determined photometrically at the wave length - 550 nm and reference filter - 690 nm.

The four cell lines (Table 1) were procured at the German collection for microorganisms and cell cultures (DSMZ, Braunschweig, Germany). All tumour cells grew adherent as mono-layer and were cultivated in T 25 cell culture flasks at 37°C and 5% CO_2 in an incubator.

Table 1. Overview of the tested cell lines

cell line	origin	growth	source	media
HELA-S3	human cervix carcinoma	adherent	ATCC-161	Hams F12+10%FCS
EN	human endometrial carcinoma	adherent	ATCC-564	DMEM+20%FCS
EFO-27	human ovary adenocarcinoma	adherent	ATCC-191	RPMI 1640 + 20%FCS+MEM+ purivate
EFM-192A	human breast carcinoma	adherent	ATCC-258	RPMI 1640 + 20%FCS+L-glutamine

EFO-27 and EFM-192A were cultivated in RPMI 1640 Medium, HELA-S3 in Hams F12 and EN in DMEM-media. To all media were added respectively 10-20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml L-glutamine.

Half of the respective media (table1) were exchanged every 2-3 days by fresh media. The cell passaging took place shortly before the confluence (80%-90%) and then the cells were splitted weekly according their growth rate 2-3 x in the ratio 1:5 to 1:10. Therefore the cells were washed twice with PBS and then enzymatically flaked of with 2.5 ml trypsin/EDTA from the growth surface. The flaking was controlled by microscopy. The trypsination was inactivated by addition of 10% FCS-containing media. The cell suspension was poured into sterile 15ml-centrifuge tubes and centrifuged for 4 minutes at 220 x g. The excess liquid was carefully sucked out and the cell pellet was resuspensed in 5 ml of fresh media. After this, the number of cells per ml was counted

with a Neubauer counting chamber. The cells were disseminated to the requested cell number in new cell culture flasks.

Procedure

To test the effect of **Biocrush®**, 5000 cells per well were disseminated in a 96 well plate in a volume of 200 µl. After 24 h the media was take away and replaced by 200 µl fresh media with 1 % FCS, which additionally contained the testing substance.

After 72h incubation were added to the cells with the test substance per well 20 µl MTT-solution (5 mg/ml) and the cells were incubated for 4 h at 37°C and at 5%-CO2 in an incubator. The cells then were disintegrated with 100 µl MTT-lysis buffer per well and the formed formazan crystals were given time to dissolve over night. Then the extinctions of each well at 550-690 nm were determined with a titer-plate-photometer.

As 0-control, the untreated cells was used and defined as non-toxic level. SDS (sodium dodecyl sulphate) as known cytotoxic reference substance was used for comparison in the concentrations 6.3, 12.5, 25, 50 and 100 µg/ml. **Biocrush®** was used respectively in 5 different concentrations.

The extinction of the untreated cells was set to 100 %, the other values are related percentually. Showed are the mean value from n = 8 parallels with the standard deviation. The level of significance relates to the comparison of the control.

Results

The test showed that the preparation **Biocrush®** has a cytotoxic effect on tumour cells. The effect depends on the concentration and increases with an enlarging concentration. In the concentration level of 1 to 1000 mg/ml the cytotoxic effect on various tumour cells varies.

For the Cervix cancer cell HELA-S3 the cytotoxic effect of **Biocrush®** in a concentration level of 1 to 10 mg/ml was negligible small. At a concentration level of 100 to 10000mg/ml a maximum inhibition of the cancer cells of app. 15% was determined.

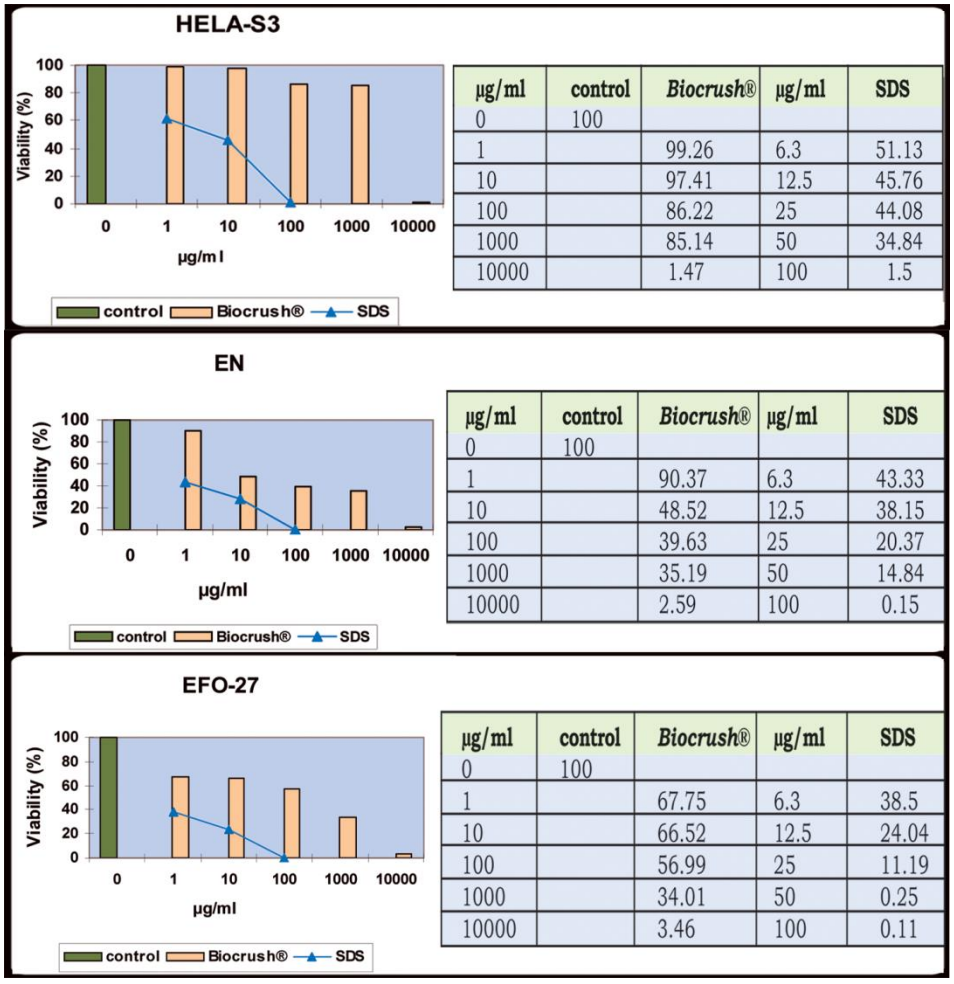
For the breast cancer cells EFM-192A **Biocrush®** in the concentration level of 1, 10 and 100 mg/ml also only a small effect was observed, i.e. the inhibition was at app. 3, 5 and 8%. At a concentration of 1000 mg/ml a clear inhibition of the cancer cells at app. 34% was observed.

A good inhibition effect was observed for the endometrial cancer cells EN and the ovary adenocarcinoma cells EFO-27. In the tested concentrations of 1, 10, 100 and 1000 mg/ml were

observed for EN cells inhibition rates of app. 10, 51, 60 and 65 % and for the EFO-27 cells of app. 33, 34, 44 and 66%.

At the maximum tested concentration of 10000 mg **Biocrush®**/ml the inhibition effect for all cancer cells was > 95%. In comparison to the influence of **Biocrush®** on cancer cells furthermore human dermal fibroblasts NHDF-c adult were tested. The influence of **Biocrush®** is negligible in a concentration level of 1 to 1000 mg/ml, i.e. no toxic effects on human fibroblasts were observed.

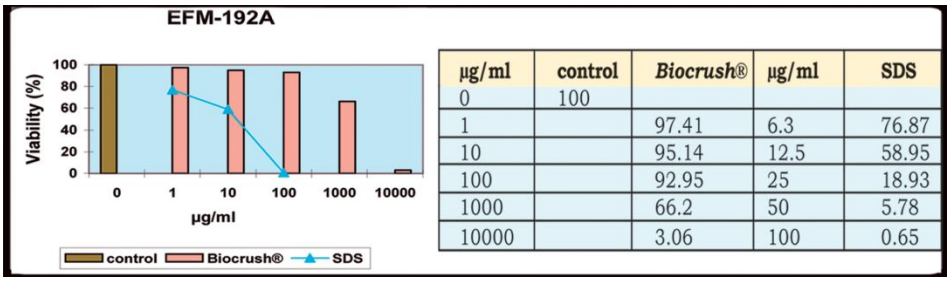
Cytotoxic effect of Biocrush® on the four tumor cell lines



µg/ml	control	*Biocrush®*	µg/ml	SDS
0	100			
1		99.26	6.3	51.13
10		97.41	12.5	45.76
100		86.22	25	44.08
1000		85.14	50	34.84
10000		1.47	100	1.5

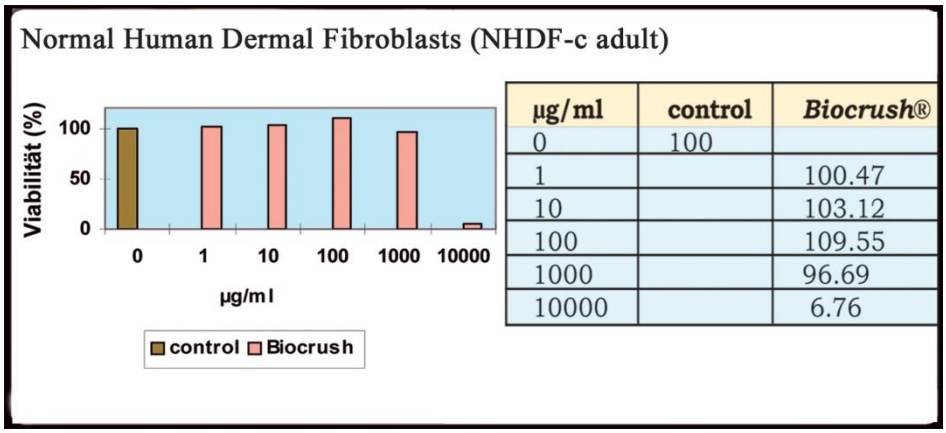
µg/ml	control	*Biocrush®*	µg/ml	SDS
0	100			
1		90.37	6.3	43.33
10		48.52	12.5	38.15
100		39.63	25	20.37
1000		35.19	50	14.84
10000		2.59	100	0.15

µg/ml	control	*Biocrush®*	µg/ml	SDS
0	100			
1		67.75	6.3	38.5
10		66.52	12.5	24.04
100		56.99	25	11.19
1000		34.01	50	0.25
10000		3.46	100	0.11



µg/ml	control	*Biocrush®*	µg/ml	SDS
0	100			
1		97.41	6.3	76.87
10		95.14	12.5	58.95
100		92.95	25	18.93
1000		66.2	50	5.78
10000		3.06	100	0.65

Testing of Biocrush® metabolic activity on human fibroblast



µg/ml	control	*Biocrush®*
0	100	
1		100.47
10		103.12
100		109.55
1000		96.69
10000		6.76

DMEM : Dulbecco´s Modified Eagle Medium

EDTA : Ethyldiamintetraessigsäure

FCS : Fetal calf serum

MEM : Amino acids solution

MTT : - 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide

PBS : Phosphate-buffered saline

RPMI 1640 : Medium was developed at Roswell Park Memorial Institute

Ham‘s F12 : Ham‘s Modified Medium with Hageman factor (coagulation factor XII)