The influence of yeast killer toxins on the cytotoxicity of shiga-like toxins. Part I – The effect of killer toxins on mammalian cells

Anna Teresa Salek

MILAN-SCIENCE, Institute für Microbiologie GmbH & Co., KG, Passau, Germany

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Summary

It is evident that the results of preliminary experiments with 5 different yeast killer proteins did not show emphatic cytotoxicity or any adverse effect on any mammalian and embryo-cells. Moreover, they are likely to be harmless to animals' and humans' tissue cells. Therefore, they could be used to explain the pre-therapeutic effect on mammalian cells (mostly animals) in the case of infections by strains *Escherichia coli*, called EHEC.

It was found that the yeast killer toxin from *Williopsis mrakii* can protect mammalian cells such as HeLa and Vero cells against the challenge by Shiga-Like-Toxins (derived from cultures of pathogenic strains of *Escherichia coli).* The final activities of tested mammalian cells are better when they are pre-treated by the killer protein, i.e. before the challenge with Shiga-Like-Toxins. It appears that this prophylactic effect could be very interesting for veterinary, which has been proved on a big population (about 2000) of healthy and ill (with diarrhoea, i.e. haemorrhagic colitis) pigs (manuscript – confidential data).

We can conclude that the yeast killer strains are **probiotic**, i.e. could reduce or eliminate fecal shedding of EHEC strains in pigs prior treated with the developed yeast toxins.

Key words:

cell receptors, yeast killer toxins, Shiga-Like-Toxins Verocytotoxins, EHEC, mammalian cells, diarrhoea, haemorrhagic colitis, uremic syndrome, prophylactic, therapeutic effect.

Adres do korespondencji

Anna Teresa Salek,
Research Associate
Passauer Str. 92
94121 Salzweg bei Passau
Germany
e-mail:
anna.salek@T-Online.de

1. Introduction

It has been observed that an increasing number of pathogens are becoming resistant to antibiotics in current use. Therefore, biotechnology is turning to the natural product to find new biotherapeutic agents, active against pathogenic bacteria or yeasts. Recently, a prophylactic and therapeutic anti-microbial strategy, based on a specific physiological target, has become effective due to the use of killer yeasts directed against their natural competition.

The killer phenomenon has been reported for strains of the genera *Saccharomyces, Kluyveromyces, Hansenula* (or *Pichia*), *Hanseniaspora, Candida, Torulopsis, Debaromyces, Cryptococcus* and *Ustilago* (1-8). The above mentioned yeasts produce toxins which are mostly glycoproteins and similar in structure to lectins. They act against sensitive strains of the same or closely related species as well as against unrelated microorganisms, including pathogenic yeasts or bacteria such as enterohemorrhagic *Escherichia coli* (EHEC), producing Shiga-Like-Toxins (SLT). The activity of yeast killer toxins are lethal due to the presence of specific cell wall receptors. Therefore, they have been proposed as a potentially useful biotherapeutic agents for improvement of humans' or animals' health (9–15) as well as for environmental control (16–17).

The toxic effect of killer protein cannot be comprehensively utilised without a very specific study. Such studies aim at characterising:

– an influence of yeast killer proteins on standard mammalian cells (examination of cell viability/cytotoxicity),

– the biochemistry of killer toxins, as well as

– receptors (ligands) from pathogens or those which bind the killer glycoprotein.

Cytotoxicity is a complex event *in vivo*, where its expression may be manifest in a wide spectrum of effects, from simple cell death, as in a toxic effect of anticancer drugs on both the cells of the cancer and normal cells of the bone marrow, skin, or gut, to complex metabolic aberrations such as neuro- or nephrotoxicity, where no cell death may occur, only functional change. Of course, the definition of cytotoxicity will tend to vary depending on the nature of the study, whether cells are killed or simply have their metabolism altered.

Current legislation demands that new veterinary medicines go through extensive cytotoxicity testing before they are released. This usually involves a large number of experiments on animals, which are very costly and raise considerable public concern. Therefore, more human and economic is to perform at least part of cytotoxicity testing *in vitro* on specialised mammalian cell lines or embryo cells to explain a mechanism of proved drug.

Experiments in these studies were carried out *in vitro* to determine the potential cytotoxicity of different yeast killer toxins (proteins) to mammalian cells (or some change in their activity). Since killer toxins can be used as veterinary agents for oral

application during a therapy of microbial infections (18), they must be to be non-toxic, without antigenic and harmless effect on intestinal as well as kidney cells.

2. Principle and methods

The choice of cytotoxicity assay (the short-term-perturbation of a specific metabolic pathway) depends on the agent/drug under the study, the nature of the response, and the particular target mammalian cells.

The alterations in respiration, assayed by the MTT-test (**mi**cro**tit**ration test), were used to measure the metabolic response to potentially toxic yeast killer proteins. The MTT-test measures the activity of mitochondrial succinate dehydrogenase in living cells, independent of whether they are carrying out DNA synthesis or not. The method has the advantage of rapid completion of large experiments with counting of death cells.

After the weak, yellow-coloured, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) has diffuses into cells, dehydrogenases of active (live) mitochondria are able to open the tetrazolium ring (reduction of MTT), and the water-insoluble dark-blue formazan is produced. Cells are lysed with DMSO (**dim**ethyl-**sulfoox**id), setting the water-insoluble MTT-formazan crystals free (dissolved), and the intensity of the blue colour is assayed by spectrophotometric means. A large number of samples can be measured and the results evaluated according to various criteria with the help of an on-line computer.

Eventually, in the context of killer toxin cytotoxicity and activity tested cells, it should be kept in mind that effects of killer toxin on metabolism must be interpreted only as such. To establish an irreversible effect on cell survival, the culture should be continued in the absence of that agent.

3. Materials and methods

3.1. Cultivation of mammalian cells

3.1.1. Material

– **Mammalian cell lines**: **HeLa** (human uterus cancer cells), **PK-15** (pork kidney cells), **Vero** (monkey kidney cells), **Hep 2** (human liver cells), **V-79** (Chinese hamster kidney cells).

– **Embryo cells**: **EBL** (embryo bovine lung) cells.

– **Growth medium**: for normal mammalian cells D´MEM (**D**ulbecco´s **m**odified **E**agle **M**edium), supplemented with 1% penicillin/streptomycin (each 1000 μg/ml), 5 or 10% FCS (**F**oetal-**C**alf-**S**erum) and 1 or 2% L-glutamine. For PK-15, Vero, HeLa and EBL cells, D´MEM supplemented with 5% FCS, 1% antibiotic mixture (streptomycin + penicillin) and 1% L-glutamine was used. For Hep-2 cells D´MEM with 1% antibiotic mix, 10% FCS and 2% l-glutamine was used. Cells V-79 were grown on RPMI-1640 AUTO MOD™ commercial medium (Sigma, Cell Culture Catalog No. R-7755).

– **T 175 (Nunck) flasks**: containing 20 ml of the appropriate medium.

– **Thawing of mammalian cells**: For very rapid thawing of cells, a warm water bath (35°C) was used. The DMSO (**dim**ethyl **sulfox**ide) was washed out by washing and centrifugation with 8 ml D´MEM (+FCS). The cells were re-suspended in a small volume of medium.

3.1.2. Methods

– **Trypsinization**: after 24 hours of cultivation the old medium (20 ml) was removed and 2.5 ml of STV (**S**aline-**T**rypsin-**V**ersen Solution) was added, followed by incubation at 37°C for 3-5 min. D´MEM medium with FCS was used to stop the trypsinization. Then, cells were centrifugated (1200 rpm for 5 min) and thoroughly re-suspended in fresh D´MEM (+FCS) medium.

– **Cell suspension**: a high concentration of live cells, i.e. 10^6 per 1 ml of proper medium was prepared, with assay by hemocytometer (e.g. Thoma): trypsinized cells were re-suspended (100-times) in trypan blue, i.e. 0.99 ml PBS (**p**hosphate-**s**aline-**b**uffer)-Dulbecco´s medium + 0.010 ml 0.5% trypan blue); only unstained cells were counted (10× objective).

– **Preparation of microtitration plates with cultures**: 96-well plates (each well having 28-32 mm^2 growth area) were seeded with 100 μl of the suspension of cells (10^6 cells/ml) prepared above, and incubated for 24 hours at 37°C in 5% CO_2 incubator until the mid-log phase of growth.

3.2. An outline of MTT test for the viability of cells (ev. cytotoxicity of toxins)

3.2.1. Materials

– Cells to be tested, adherent or in suspension from the log-phase of a stock culture.

– MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS, sterile filtered.

– PBS without Ca^{2+} or Mg^{2+}.

- DMSO for cell lysis.
- 96-well Plates.
- Multi-pipette.
- Mikroplate shaker.
- ELISA (Enzyme-Linked Immuno Sorbent Assay)-reader, 492 nm.

3.2.2. Methods

The monolayer mammalian cultures in the microtitration plates were incubated (at 37°C) in different concentration ranges of the yeast killer toxin. Then, MTT was added to each well and after that, plates were incubated in the dark for 4 h. Next, the medium with killer toxins and MTT were removed. The water-insoluble MTT-formazan crystals were dissolved in DMSO. Absorbance was recorded using an ELISA reader. The procedure of determination was following:

- Cells (in T 175 Flask) were trypsinised as above and pipetted into the wells of a microtitration plate at 1×10^6 cells/ml in a volume of 100 µl: it is best to allow the outside wells to remain empty! Four rows of 10 wells were set up.
- The plate was incubated for ca. 24 h at 37°C, 5% CO_2 and 95% humidity.
- 100 µl of the killer toxin to be tested was added to each well. The controls contained culture medium (e.g. D´MEM, Dulbecco´s Modified Eagle Medium).
- The cells were cultivated for the following 24 h.
- 20 µl MTT-solution (5 mg/ml) was added to each well and the cells were incubated for the following 4 h in the incubator.
- The culture medium was pipetted away.
- Then, the cells were lysed by the addition of 100 µl DMSO, which also dissolved the dye. This process was favoured by 5 min shaking.
- After thorough mixing (all cell fragments should have disappeared), the absorption at 492 nm was measured by ELISA reader.

3.3. Yeast killer toxin preparations

3.3.1. The killer toxins from strains

- *Williopsis mrakii* NCYC 500 or AS/15ρ⁻, grown in YNB-glu 2% medium (Yeast-Nitrogen-Base medium+ Glucose),
- *Pichia anomala* UCSC 25F, grown as *Williopsis mrakii* NCYC 500,
- *Pichia subpelliculosa* NCYC 16, grown as *Williopsis mrakii* NCYC 500,
- *Saccharomyces globosus* BKM y 438, grown in YNB-glu 2% + 0.5% peptone medium,

– *Hanseniaspora valbyensis* 13cs/6ρ⁻, grown in YNB-glu 2% medium.

3.3.2. Preparation of killer toxins

– After purification by IEX (ion exchange) chromatography the following fractions of the killer toxins were collected:

• *W. mrakii* NCYC 500: fractions 12-33, with activity 160 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain);

• *P. anomala* UCSC 25F: fractions 12-23, with activity 190 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain);

• *P. subpelliculosa* NCYC 16: fractions 12-22, with activity 160 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain);

• *S. globosus* BKM y 438: fr. 21-26, with activity 255 Units/ml (against *S. cerevisiae* S-6/1, sensitive strain). The killer toxins in the above fractions were about 2000 times concentrated (with respect to the fresh culture).

– Preparations of the killer toxin from *Hanseniaspora valbyensis* 13cs/6ρ⁻ which were obtained after the gel filtration chromatography (a second purification method). For the purification, the 2000-times concentrated and dialysed culture-supernatant (from YNB-glu 2% medium) was used. For the measurement of potential cytotoxicity the solutions which derived from collected gel filtration fractions were used:

• 10-15 (sample S-III),

• 16-20 (sample S-IV),

• 21-25 (sample S-V).

Unfortunately, the 3 above samples did not show any killer activity against 8 pathogenic bacteria, such as:

– *Pseudomonas aeruginosa* DSM 1117,

– *Escherichia coli* DSM 1103,

– *Escherichia coli* DSM 2430,

– *Staphylococcus aureus* DSM 2569 (for test),

– *Salmonella goldcoast* PA^{1+},

– *Enterococcus faecalis* DSM 2570,

– *Streptococcus agalactiae* DSM 2134 (for CAMP test),

– *Paseurella multocida* 4c (a strain from the Collection of the Institute of Animal Hygiene, University of Munich, Germany).

– In each case, 100 μl of the toxin solution was given to the first well, and to further wells the same volume at successive dilutions: as 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 was given.

3.3.3. Microtitration plates with the killer toxins

The following controls were made:

- w/o cells, for each sample of the killer toxin (wells at the edge of the plate),
- for growing of cells (wells w/o toxins), containing 0.5 M NaCl, buffered by 0.01 M citric (Na^+) buffer, as used in IEX (ion exchange) chromatography for elution of the killer proteins.

In order to be able to compare the measurements from various microtitration plates (with 5 various killer proteins) with each other, the extinction value of the cell control from each microtitration plate was set to 100% and the measured values of the individual wells of the dilution series adjusted in relationship to it.

Cytotoxic effects of the killer toxins on cells could be recognised when the MTT-ring-opening activity was significantly less than 100%. Within a dilution series, the cytotoxic threshold concentration was taken to be the one that gave <80% of the formazan production of the cell control. A formazan production of >120% was taken to indicate a stimulatory activity.

For each plate, two additional controls were set up which contained the mammalian cell medium only. The results from this trial were included in the final evaluation.

4. Results and discussion

It was found that killer toxins from the yeasts *Williopsis mrakii* NCYC 500, *Pichia anomala* UCSC 25F, *Pichia subpelliculosa* NCYC 16 and *Hanseniaspora valbyensis* 13cs/ρ^- do not have inhibitory effects on mammalian cells (Fig. 1-10). However, some emphatic inhibition of mammalian cells activity (except for human liver cells, Hep-2 and monkey kidney cells, Vero-79) has been observed when 100-times concentrated killer toxin from *Saccharomyces globosus* BKM y 438 was used.

The most sensitive cells towards that toxin were HeLa – human uterus cancer cells and, even then, only at 100-times concentration. Further dilution of the killer toxin from *Saccharomyces globosus*, i.e. to relative concentrations of 50, 25, 12.5, 6.25 and 3.12-times, showed some stimulation of activity in the case of all tested mammalian, as well as embryo bovine lung cells (EBL). A similar tendency has been demonstrated in the case of other killer toxins. The strength of this stimulation is not clear.

The proof of yeast killer toxins cytotoxicity (or their absence) may require more wide-ranging investigation including full particulars of changes in metabolism, viability or in cell-cell signalling. Such evidence could be the fact that they might give rise to an inflammatory or allergic responses.

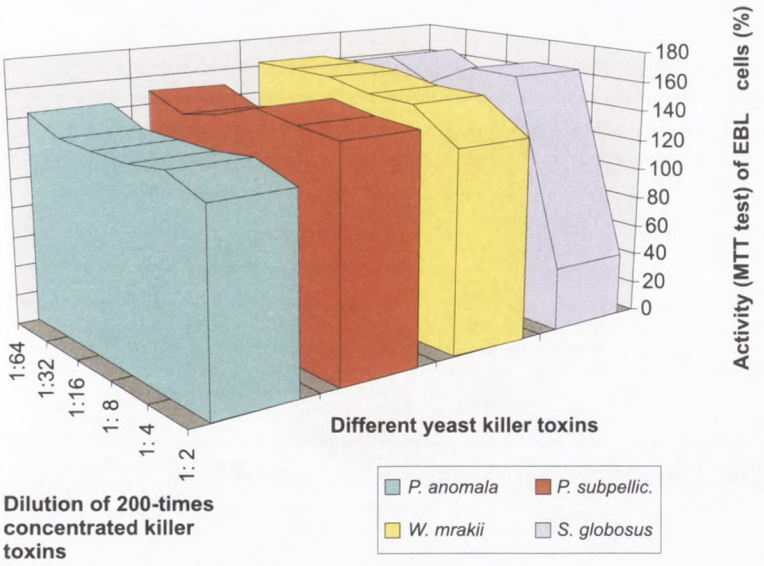

Fig. 1. Toxicity of different killer toxin for EBL cells.

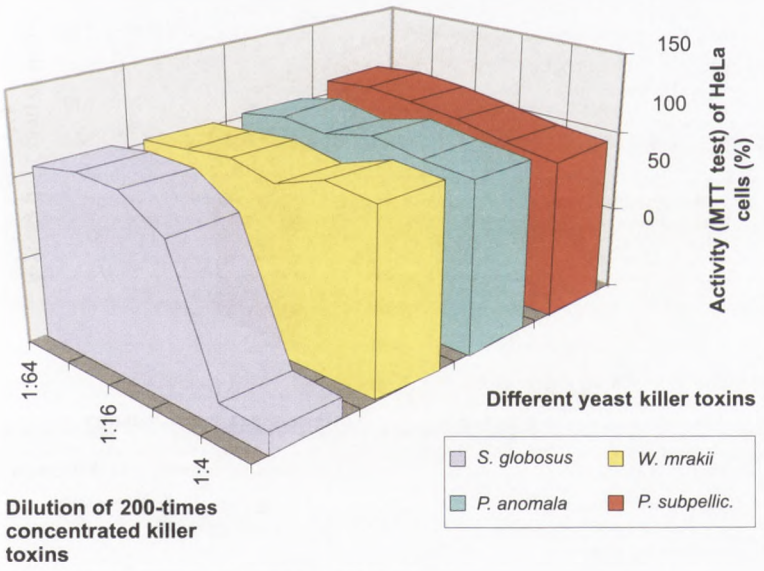

Fig. 2. Toxicity of different killer toxins for HeLa cells.

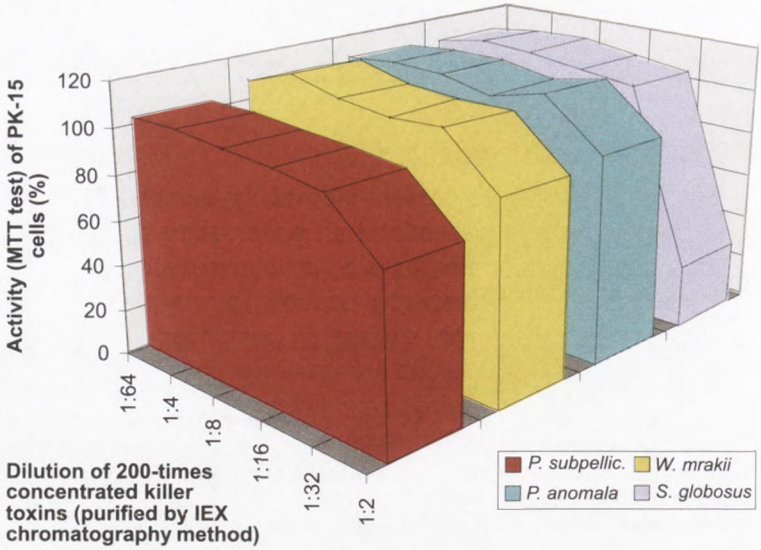

Fig. 3. Toxicity of different killer toxins for PK-15 cells.

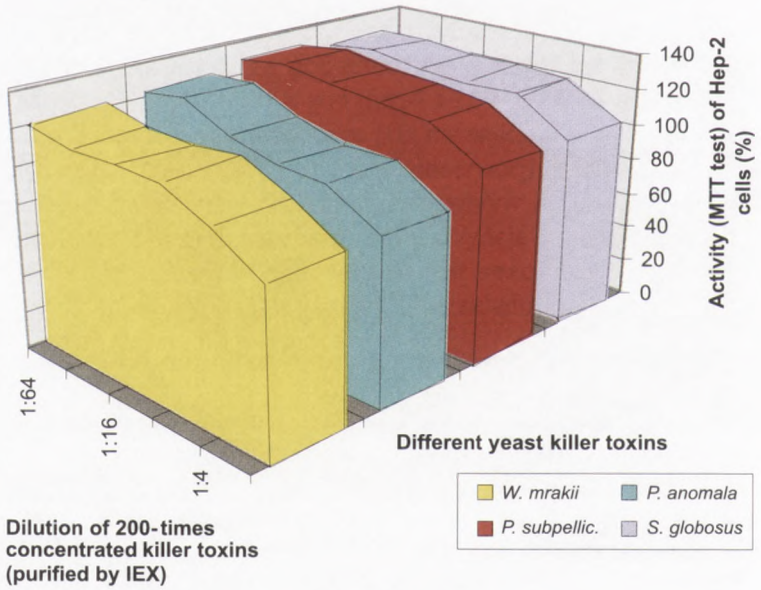

Fig. 4. Toxicity of different killer toxins for HEP-2 cells.

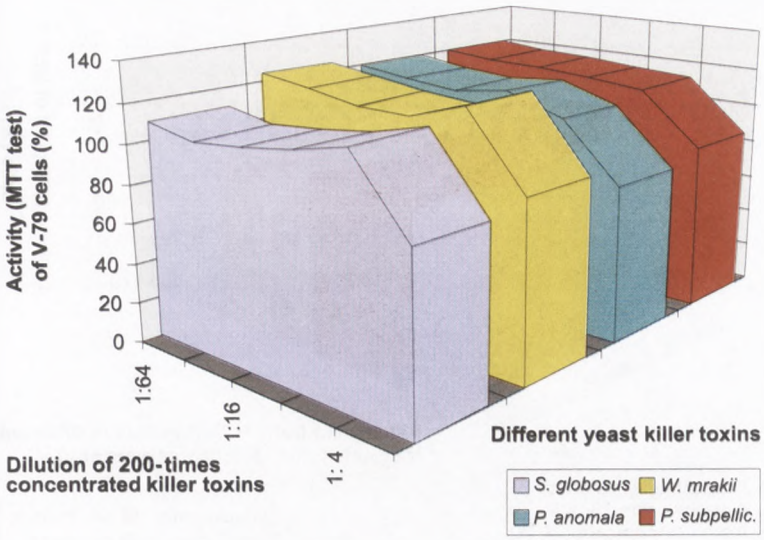

Fig. 5. Toxicity of different killer toxins for V-79 cells.

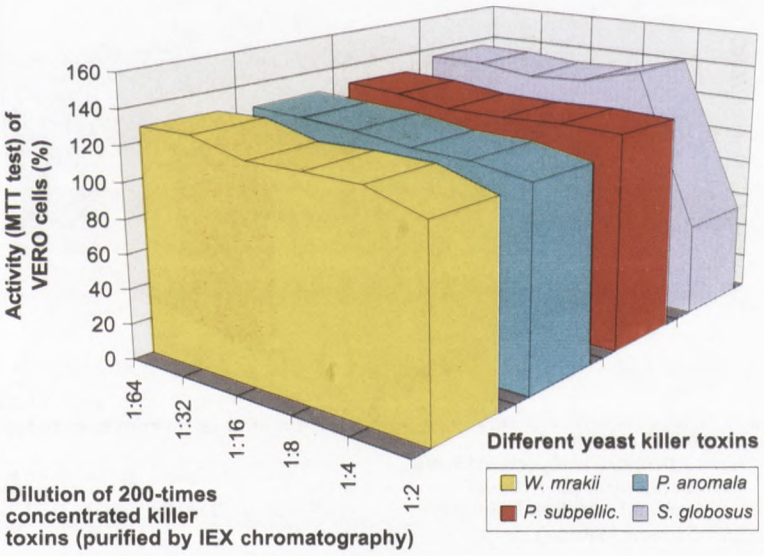

Fig. 6. Toxicity of different killer toxins for VERO cells.

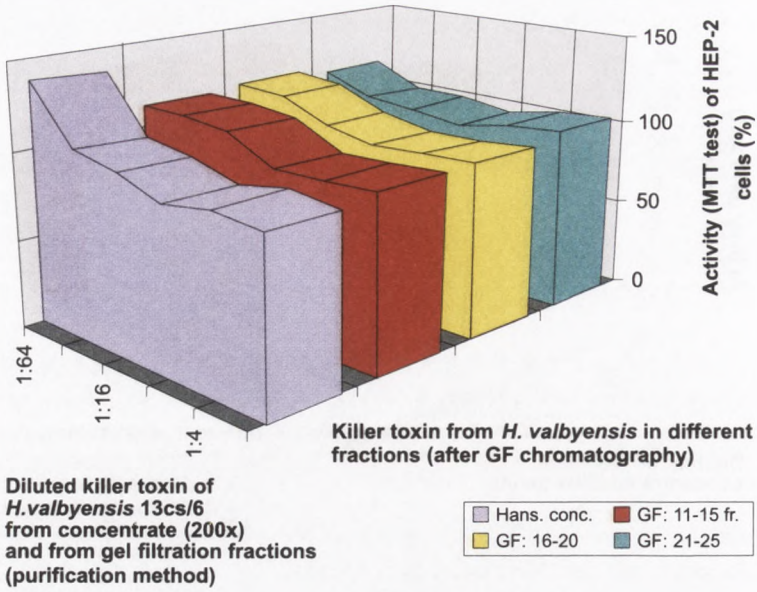

Fig. 7. Toxicity of killer toxins from *Hanseniaspora valbyensis* 13cs/6 for HEP-2 cells.

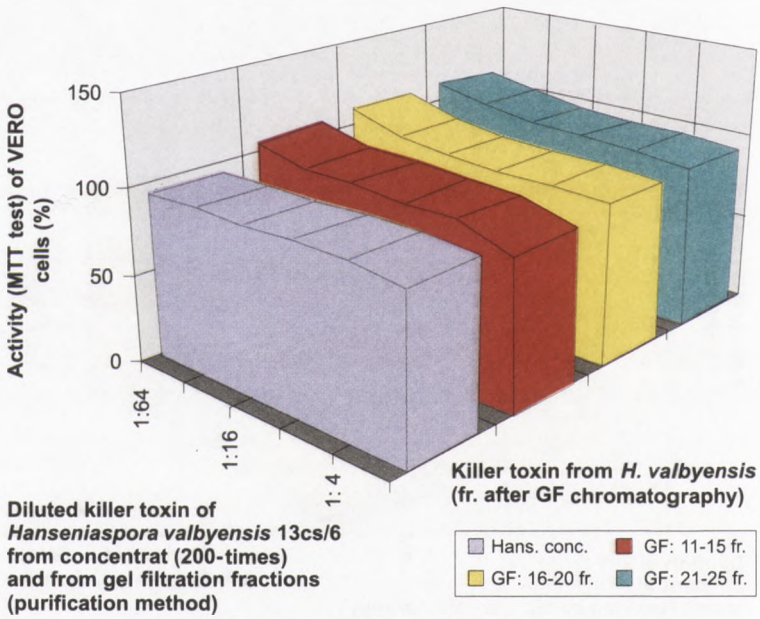

Fig. 8. Toxicity of killer toxins from *Hanseniaspora valbyensis* 13cs/6 for VERO cells.

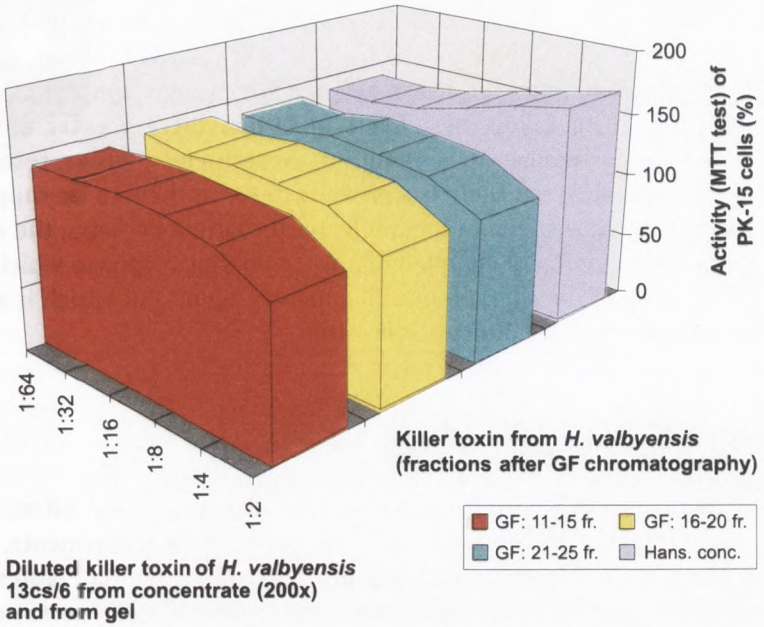

Fig. 9. Toxicity of killer toxins from *Hanseniaspora valbyensis* 13cs/6 for PK-15 cells.

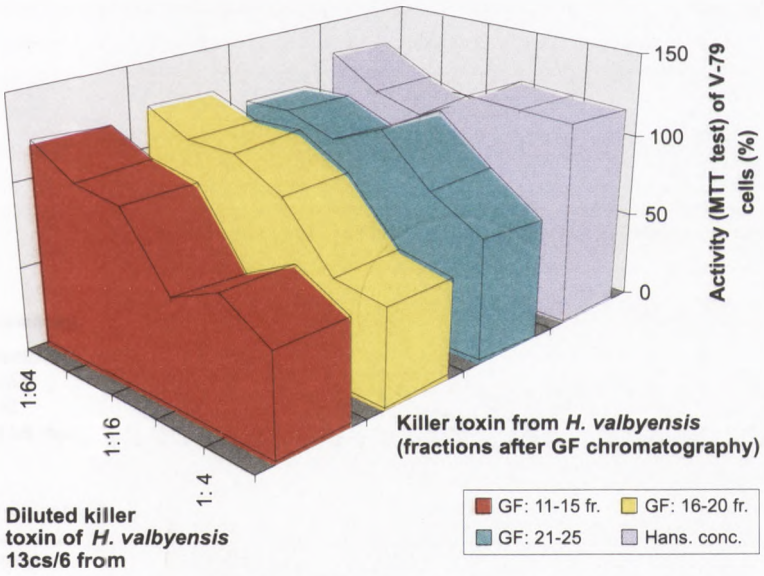

Fig. 10. Toxicity of killer toxins from *Hanseniaspora valbyensis* 13cs/6 for V-79 cells.

The cytotoxicity assay (the level of activity/viability of cells) employed here for the preliminary investigation was chosen because it is relatively cheap, easily quantified, and reproducible. However, it has become increasingly apparent that, generally, it is not enough to follow the effects of drugs where greater emphasis on changes in metabolic regulation is required. Nevertheless, gross tests of cytotoxicity are still necessary, but there is a growing need for them to be supplemented with more subtle measurements of metabolic perturbation. Perhaps, the most obvious of these is the induction of an inflammatory or allergic response which need not to imply cytotoxicity of the allergen or inflammatory agent, but which is still one of the hardest endpoints to demonstrate *in vitro*.

5. Conclusion

Killer toxins which are used for examinations display a lethal effect to a wide spectrum of pathogenic microorganisms. From preliminary experiments, it is clear that the results with 5 different yeast killer proteins, presented on Figures 1-10, did not show emphatic cytotoxicity (especially toxin from *Williopsis mrakii*) or any adverse effect, in any mammalian and embryo-cells. In other words, they are likely to be harmless to animals' and humans' tissue cells. Therefore, they could be used for the explanation of the pre-therapeutic effect on animal cells in the case of EHEC infections.