SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Biocompatibility investigations of pharmaceutical excipients on different cell culture model systems

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I. Introduction

Regulation (EC) No 1333/2008 of the European Parliament and of the Council defines preservatives as substances „which prolong the shelf-life of foods by protecting them against deterioration caused by micro-organisms and/or which protect against growth of pathogenic micro-organisms". It is a basic fact that the aqueous medium is advantageous to the growth of various microorganisms, therefore, the addition of preservatives is necessary in case of multi-dose pharmaceutical preparations – even more for those which contain sugars as flavour enhancers - foods and products marketed as food supplements. Today in Hungary, parabens, benzoates and sorbates are the most common microbiological preservatives in approved preparations.

Esters of para-hydroxybenzoic acid are the parabens, the antimicrobial activity and use of which as a preservative have been known since the 1950s. Since then, they have been widely used as preservatives in the food, pharmaceutical and cosmetic industries as they are tasteless, odorless and chemically essentially stable at microbiologically effective concentrations. They are typically used in combination. Regarding the antimicrobial effect of parabens, it can be said that the MIC value must be determined for each species, the spectrum of the compound family cannot be described in general, as we can find equally resistant and susceptible species among fungi, Gram positive and Gram-negative bacteria.

The harmful effects of parabens can be basically divided into two major groups, one is their allergenic nature which can cause contact dermatitis, and the other is their various effects on the human endocrine system. In connection with the former, the literature nowadays concluded that paraben allergy is much rarer than we thought, and some researchers have already raised the possibility of omitting these esters from dermatological allergen testing palette. This is due to more sensitive methods, more thorough and repeated testing of patients, and the limited concentration of parabens in cosmetic products which reduces the chance of developing allergy. The first scientific studies were published in 1997-98, which showed poor binding of the group to estrogen receptors based on *in vitro* receptor binding and *in vivo* animal experiments. Results from both human and animal experiments prove that the paraben level measured in urine (urine is the primary excretory pathway) correlates with menstrual disorders, thyroid hormone levels, but also with the anatomical parameters and behavior of offspring. However, it should be noted that the vast majority of data in the literature are retrospective rather than planned clinical studies, so a causal role of other factors in the listed and further observed biological effects cannot be ruled out.

Sorbates, are the esters and salt of 2,4-hexadienoic acid better known as sorbic acid. The mother molecule was named after the Latin name for rowan (*Sorbus aucuparia*) as the fruit of the tree is rich in the compound and the compound was isolated from it for the first time, however, it can be detected in several species. There have been publications on its use in food and pharmaceuticals as a preservative since 1945, describing its antifungal activity and toxicology. The results of various toxicity studies have been proving the safety of sorbic acid and potassium sorbate as excipients for decades without any serious concerns. Their allergenic effect is also negligible, toxic symptoms could only be produced by chronic overdosing of the maximum daily allowable amount. In terms of their antimicrobial activity, they act primarily on fungi and Gram-positive bacteria.

Nowadays, there is strong political and public pressure on researchers to reduce the extent of animal testing in research where possible. As early as the late 1950s, 3R appeared as a guiding principle to minimalize the number of animals used for scientific purposes and their suffering by a triple of replace, reduce, and refine. In line with these efforts, in my experiments I performed cytotoxicity studies on Caco-2 cell lines using MTT and NR tests, which methods and cell lines have different EU level approval in case of *in vitro* studies (European Commission Regulations 440/2008 and 2017/735)

The MTT test is an enzymatically catalysed redox reaction consisting of a reduced and oxidized form of a tetrazolium ring-containing dye (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT). based on a different colour. **Mosmann** developed it for measuring cell proliferation and cytotoxicity in 1983. Contrary to popular belief, the MTT test does not depend directly on the mitochondrial activity of the cells. Due to its positive charge, the molecule can pass through the cell membrane and NAD(P)H-dependent oxidoreductase and dehydrogenase enzymes localized anywhere within the cell catalyse the reaction, during which the tetrazolium ring opens and the compound loses its charge. In addition to NAD(P)H, other electron donors also play a role in the process, such as succinate or pyruvate. Due to the insolubility of the formazan salt formed in the reaction, the MTT test is an endpoint method. It is fast and easy to carry out since the concentration of the formed formazan salt can be measured spectrophotometrically. False results can be obtained in cases where the compound does not cause direct necrosis or apoptosis but has a lasting effect on the oxidoreductive potential of the cell, either by increasing or decreasing it. A further disadvantage is that the compound itself is toxic, irritating to the skin, eyes and lungs and is likely to cause genetic damage.

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, NR) is a phenazine derivative that is taken up by cells from the cell medium by phagocytosis, first used

in 1985 by **Borenfreund** and **Puerner** to measure cytotoxicity. The method is based on the fact that the dye is not or only weakly charged at neutral pH, so it easily crosses the cell membrane which has a negative membrane potential and becomes charged in the strongly acidic medium of lysosomes and since the inner membrane of lysosomes is neutral, it can no longer escape. Maintaining the acidic pH of lysosomes requires ATP-powered proton pumps. The amount of dye released or taken up by the cells can be measured with a spectrophotometer. The accuracy of the method is impaired when cells are treated with compounds that affect lysosomal membranes or ATP levels, as non-necrotic/apoptotic cells also show low viability. Well quantifiable, fast, simple, inexpensive, stable, non-toxic to the dye itself and can be used on a wide variety of cell lines under a variety of conditions.

Caco-2 is an epithelial, adherent, hypertetraploid cell line isolated from a primary colon tumor of a 72-year-old Caucasian male. During differentiation, a confluent monolayer is formed and the cells can be identical to polarised enterocytes in terms of morphology and functionality. Due to the the presence of specific transporter proteins and the cell-cell relationship similar to *in vivo* conditions, it is a widely used model system spread on cell culture inserts to study the absorption of various drugs and compounds. Measurement of cytotoxicity is also widespread application for a wide variety of compounds like microbial toxins. The disadvantage of the cell line is that the real small intestinal epithelium is composed of several interacting cell types, so it is not able to model accurately and does not produce mucus polymers characteristic of the small intestine.

Microbiology has begun in the last decade to use the larvae of *Galleria mellonella*, the large wax moth, to compare the virulence of pathogens of the same species but of different strains. The larva has both cellular and humoral immune responses that show fundamental similarities to certain elements of the mammalian immune system. A special feature of the immune system is melanisation, which means a local immune response, capsule formation, and tissue damage. It has also been used to measure the efficacy of *in vivo* therapies and antimicrobial agents, as well as to measure the tolerability of these compounds. However, the relative toxicity shown by the larvae was found to be proportionally similar to that observed in mice or rats. Since then, the model was used to assess the biocompatibility of ionic 1-alkyl-3-methylimidazole chlorides of various carbon chain lengths, lipid nanocapsules and metal nanoparticles. Its popularity as a toxicological modelling organization has been steadily growing in recent years. In practice, experiments are usually done by injection into the left last proleg with a thin needle but feeding and forced feeding are also possible. However, the biggest disadvantage of the species as a model organization is that there are currently no stable strains

available from traders characterized by known phenotypes (genotypes), which always produce the same larvae, they can only be obtained from some small producers.

II. Aims

The initial goal of my doctoral research was to get a more comprehensive view on the nature and modifiability of the cell-damaging effects of preservatives often used in liquid dosage forms with the help of routine cytotoxicity tests available at the Department of Pharmaceutical Technology. Our questions were the following:

1. **Do other excipients modify the cytotoxicity of preservatives?** Based on my previous undergraduate research, methyl paraben and benzalkonium chloride were examined on Caco-2 cells by MTT-test whether increased or decreased cell damage can be observed with the addition of surfactant and viscosity-increasing polymers.
2. **Do certain combinations of excipients modify the cell-damaging effect of preservatives on human and microbial cells?** Methyl, ethyl, *n*-propyl and *n*-butyl paraben were formulated in two complex cosolvent systems containing solvents and surfactants with different cytotoxic properties. Caco-2 cells were tested for cytotoxicity by MTT assay and antimicrobial activity in *P. aeruginosa*, *E. coli*, *S. aureus*, *C. albicans*, *C. parapsilosis*, *C. glabrata* was assessed by broth microdilution method.
3. **Do salts of carboxylic acids used as preservatives with different alkalis reduce cell viability to the same extent?** The sodium, potassium, and calcium salts of sorbic acid, benzoic acid, and propionic acid were compared on Caco-2 cells by NR and MTT assays to examine the effect of cations on cytotoxicity.
4. **How is the cell damaging effect of sorbate esters differ from sorbic acid?** Sorbic acid, potassium sorbate, ethyl sorbate and isopropyl sorbate were compared to examine the toxicity and inhibitory/killing effect of sorbate esters relative to the parent molecule. Biocompatibility was assessed on Caco-2 cells by MTT and NR assays and flow cytometry, injection on *G. mellonella* larvae, and antimicrobial activity on *E. coli*, *S. aureus*, *C. albicans* species by time-kill experiment.
5. **What biocompatibility interactions occur between essential oils and preservatives?** Lemon, rosemary and peppermint essential oils were formulated in emulsions as possible preservatives and their effect was investigated on Caco-2 cells by MTT and NR test, injection of *G. mellonella* larvae and *E. coli*, *S. aureus*, *C. albicans* species by both microdilution method. The emulsions were also supplemented with potassium sorbate to find synergisms.
6. **How different are the cell damaging effects of the constitutional isomer parabens?** The water solubility and cytotoxicity of *n*-propyl, isopropyl, *n*-butyl, isobutyl, *n*-pentyl,

isopentyl paraben were examined on Caco-2 cells by MTT and NR assays, and by *E. coli*, *S. aureus*, *C. albicans* species by broth microdilution to investigate structure-activity relationships between branched and unbranched carbon chain esters.

III. Materials and methods

All materials used in our of experiments were obtained in the finest quality commercially available in Hungary.

Labrasol® and Capryol® PGMC were kind gifts from Gattefossé.

The Caco-2 cells used for the experiments were obtained from ECACC and were cultured with modified DMEM cell culture media. After passage, cells were seeded into 96-well microplates. After 1 week, the old media was removed and the test solutions were pipetted into the plates. The cells were incubated uniformly with the test solutions for 30 minutes, then removed and diluted reagent solutions were pipetted onto the cells. The plates were then kept at 37 ° C for 3 hours for the MTT-assay and 2 hours for the NR-assay. After removal of the reagents, the cells were dissolved and homogeneous solutions were formed in the wells of the plate with detection reagent and were measured spectrophotometrically.

After arrival, *G. mellonella* larvae in the sixth developmental stage were were cleaned and stored at 10 ° C in a dark place for 1-2 days and then warmed to room temperature for the experiments. Dead or too small specimens were removed. The injection was given in the tail area after the last proleg. Their viability was checked at the indicated times by fine physical contact.

The antimicrobial studies were performed at the Department of Medical Microbiology, by the staff of the Department, Renátó Kovács Ph.D. lecturer, Fruzsina Nagy and Zoltán Tóth PhD students performed the experiments and the evaluation. I measured at the Department of Pharmaceutical Technology.

The instrumental part of the flow cytometric measurements was performed at the Department of Biophysics and Cell Biology of the Faculty of Medicine, the measurements were performed with the help of István Rebenku, and the evaluation was also performed by him.

The parabens with various unbranched and branched alkyl chains were tested in their diluted from their ethanolic stock solutions. For each experiment, solutions diluted with PBS were diluted, centrifuged, and the supernatant was filtered through a 0.2 μm pore size membrane filter. For HPLC measurements, solutions diluted to 0.1 $^{m}/_{V}$% and a Merck-Hitachi ELITE LaCrom apparatus with an Agilent HC-C18 (2) (150x4.6 mm) column was used. The mobile phase was methanol.. Water solubility was determined using a calibration curve previously recorded at 256 nm.

The measurements were performed at the Department of Pharmaceutical Technology by Dr. Ádám Haimhoffer, PhD student.

IV. Results

IV./1. Investigation of simple excipient-preservative interactions.

During the first series of experiments, I examined methyl paraben and benzalkonium chloride on Caco-2 cells together with surfactants using the MTT test. The study of mixtures with surfactants was based on our previous research in the Methyl paraben was tested with polysorbate 20, while benzalkonium chloride was tested with Labrasol®.

After the addition of polysorbate 20, extremely low cell viability of around 20% was measured at all concentrations, while *Mucilago hydroxyethylcellulosi*, which contains 2-hydroxyethylcellulose, was able to significantly reduce the cell-damaging effects of the other two compounds in a concentration-dependent manner.

Benzalkonium chloride and Labrasol® also potentiated the effects of each other, however, the polymer could not show any protective effect. Our experiments were also performed with methyl paraben - Labrasol® and benzalkonium chloride - polysorbate 20 pairings, however, we obtained a similar result - the mucus could not increase cell viability.

IV./2. Investigation of cytotoxicity and antimicrobial interactions of preservatives and excipients in complex cosolvent systems.

Continuing to research in the field of liquid dosage forms for oral application, we have decided to form complex cosolvent systems consisting a water-miscible solvent and a surfactant. Based on these, we constructed our systems from concentrations lower than the individual IC_{50} values of each component, so that we can observe the combined effect of the excipients and not the cytotoxicity of a given excipient would cover the possible interaction:

1. system: 30 $^{v}/_{v}$% glycerol, 0,002 $^{v}/_{v}$% polysorbate 20, 0,2 $^{m}/_{V}$% paraben, PBS as solvent

2. system: 1,4 $^{v}/_{v}$% ethanol, 0,5 $^{v}/_{v}$% Capryol® PGMC, 0,2 $^{m}/_{V}$% paraben, PBS as solvent

The systems were diluted to 0.2; 0.02; 0.002 and 0.0002 $^{m}/_{V}$% of paraben, so the solvents and surfactants were equally diluted.

However, in addition to the combined parabens tested in the systems, we were also interested in the cell-damaging effects of the alcoholic stock solutions of each compound on their own, without the other pharmaceutical excipients present in the systems. These results clearly show that carbon chain length increases cytotoxicity, with *n*-butyl paraben exerting the most cell-damaging effect, followed by *n*-propyl paraben, followed by ethyl and methyl paraben. The *n*-butyl-*n*-propyl and methyl-ethyl pairs were not significantly different at the concentrations studied, but the two tandems were significantly different at higher concentrations.

The parabens formulated in the first system showed very similar curves to the aqueous-ethanolic solutions, the ranking of cytotoxicity was the same.

In the second system, we obtained a significantly different result from the previous ones. At the most concentrated concentration, *n*-butyl paraben was less toxic than the other three derivatives, which caused nearly complete cell death. At the second highest concentration, methyl and *n*-butyl parabens were found to be slightly toxic, while ethyl and *n*-propyl parabens were not toxic. The effect of the formulated control was negligible here as well, except for the highest concentration.

Different concentrations were used in the antimicrobial studies, as based on the data in the literature, we certainly would not have observed an inhibitory effect at very low concentrations. Our chosen paraben concentrations are therefore 0.1; 0.15; 0.25 $^{m}/_{V}$%, so the ethanol contents of the alcoholic solutions and the second system were 0.7, 1.05 and 1.75 $^{m}/_{V}$%, respectively. The microdilution experiments were performed by the staff of the Department of Medical Microbiology, Renátó Kovács Ph.D. and Fruzsina Nagy PhD student.

In the study of the fungus species *C. albicans*, low viability was observed in all cases. In case of *C. parapsilosis* that while alcoholic solutions of parabens completely killed fungal cells, the formulations impaired the efficacy of parabens and increasing concentration increased cell viability in several cases, even by more than 50%. *C. glabrata* showed different sensitivity than before, as parabens formulated in the first system were less effective than alcoholic solutions or parabens formulated in the second system.

Gram-positive coccus *S. aureus* was resistant to alcoholic solutions, only *n*-butyl paraben was able to exert an inhibitory effect. Control solutions of both systems without parabens caused total cell death.

During the study of the Gram-negative rod *E. coli*, the longer-chain derivatives were less effective in ethanolic solutions, the first system caused complete cell death, while the second system also showed that the formulation alone was more effective than with *n*-propyl or *n*-butyl paraben.

In case of *P. aeruginosa* the first system again caused complete cell death, while in the other cases methyl paraben proved to be the most effective preservative. The second system effectively increased the effect of ethyl and *n*-butyl paraben compared to both itself and alcoholic solutions.

Overall, the results indicate that the cytotoxicity and antimicrobial activity of the four most commonly used alkyl esters of *p*-hydroxybenzoic acid are greatly modified by the

presence of other pharmaceutical excipients, and that a universal trend of efficacy of parabens for all conditions and species cannot be established.

IV./3. Cytotoxicity investigation of sorbate, benzoate and propionate salt.

Sorbic acid, benzoic acid and propionic acid and their salts are common preservatives, and we were interested in whether the solutions of each salt had a different cytotoxicity profile at the same anion concentration and whether the concomitant ion had some effect on cell viability. As a new cytotoxicity method, the Neutral Red (NR) test was introduced in addition to the MTT test. Our measurements were performed on Caco-2 cells.

The results revealed that a 50% decrease in viability was observed for the MTT test only for calcium salts, whereas no decrease in viability was observed for the NR test. In parallel, broth microdilution antimicrobial studies were performed, which did not show a significant difference between the results of each salt with respect to different alkali and alkaline earth metal ions.

IV./4. Biocompatibility and antimicrobial studies of various sorbic acid derivatives.

Our series of experiments was based on a 2007 paper in which sorbic acid esters with different side chains were investigated from an antimicrobial point of view to find a structure-activity correlation. Two compounds with simple alkyl chains, ethyl sorbate and isopropyl sorbate, were selected to be compared with sorbic acid and potassium sorbate. Ethyl sorbate was commercially available, while the synthesis and purification of isopropyl sorbate was assisted by the staff of the Department of Pharmaceutical Chemistry, Prof. Pál Herczegh DSc., Prof. Anikó Borbás DCs., Kelemen Viktor Pharm.D., Zsolt Szűcs Ph.D. Pharm.D., Erzsébet Rőth, Márta Bodzsa and member of the Department of Organic Chemistry Sára Balla. Cell viability was assessed on Caco-2 cells by MTT and NR assays. As a new method, we also studied in vivo toxicity in *Galleria mellonella* larvae, in which different concentrations of substances were injected, and we also examined by flow cytometry whether the four substances had a necrotic or apoptotic effect. The original publication had no toxicity data, so we aimed to describe the molecules with the above mentioned methods.

The results of the MTT and the NR assay show that most of the sorbic acid derivatives tested reduce cell viability in a concentration-dependent manner- The two esters were between the sorbic acid and the potassium sorbate regards toxicity, the latter being the less toxic and sorbic acid being the most toxic.

The different cytotoxic effects of each compound were measured at the Department of Biophysics and Cell Biology with the help of István Rebenku using flow cytometry with

annexin V and propidium iodide staining. 80% percent of the untreated cells were alive, and only 20% were either necrotic or apoptotic. Treatments for ethyl-, potassium-sorbate, and sorbic acid reduced the proportion of surviving cells to an average of 64–68% and increased the proportion of apoptotic cells to 28–29%. Isopropyl sorbate showed significantly different behavior, as 68% of the detected cells were apoptotic and only 27% survived.

Galleria mellonella larvae were used to supplement the *in vitro* experiments. The measurements were performed at the Department of Biotechnology and Microbiology under the training of Valter Pfliegler Pharm.D.. Although several animals from the ester-treated group died towards the end of the experiment, statistical analysis of the Kaplan-Meier curves over the entire duration of the experiment showed no significant difference between the treated groups.

Unlike before, these were not tested by broth microdilution, but by the more sensitive time-kill experiments. Antifungal experiments revealed that isopropyl sorbate had fungicide action at more concentrations, while the other compounds had a maximum of weak static effect until the half time of the experiment. *E. coli* had similar results.

The Gram-positive bacterium proved to be very resistant to various treatments, and even no bacteriostatic effect was observed in most cases.

Overall, sorbic acid alkyl esters, including isopropyl sorbate, showed significantly different biocompatibility and antimicrobial activity compared to the parent compound and its potassium salt.

IV./5. Formulation and biocompatibility investigation of emulsions containing different essential oils and their interaction with potassium sorbate.

It is known in the scientific literature that various plant essential oils have a significant antimicrobial effect even against resistant pathogens. However, this effect can be used not only for short-term, therapeutic or disinfection purposes, but also for long-term use, even for preservation purposes. For this reason, we thought we would formulate emulsion systems using modern pharmaceutical technology excipients from Gattefossé. Due to the standardization of the active ingredient, we used peppermint, lemon and rosemary essential oil of pharmaceutical quality. Although essential oils are known to be more effective as emulsions, we were also interested in how the antimicrobial and biocompatibility parameters of the formulation are modified by the addition of a conventional preservative, potassium sorbate. Cell viability studies were performed on Caco-2 cells by MTT and NR assays, in vivo toxicity in *G. mellonella* larvae, and pathogen efficacy by broth microdilution method.

Multiple excipients and formulations were tested, and the final, most stable recipes were the following:

1. 4 $^{V}/_{V}$% peppermint essential oil, 2 $^{V}/_{V}$% Labrasol ALF®, 30 $^{V}/_{V}$% glycerol, 64 $^{V}/_{V}$% water, 2 mg/ml xanthan gum

2. 4 $^{V}/_{V}$% rosemary essential oil, 2 $^{V}/_{V}$% Labrasol ALF®, 30 $^{V}/_{V}$% glycerol, 64 $^{V}/_{V}$% water, 2 mg/ml xanthan gum

3. 4 $^{V}/_{V}$% lemon essential oil, 1,5 $^{V}/_{V}$% Labrasol ALF®, 10 $^{V}/_{V}$% glycerol, 64 $^{V}/_{V}$% water, 2 mg/ml xanthan gum

All three emulsions were tested alone and in supplemented form with 2 mg/ml potassium sorbate. For *in vitro* and *in vivo* studies, samples were diluted with PBS to 50%, 10%, 5%, and 1% emulsion contents so that the original essential oil and potassium sorbate concentrations were diluted proportionally.

The results of the MTT, the NR tests and the *G. mellonella* injection studies were very similar. The most toxic emulsions were the ones with peppermin essential oils, followed by rosemary and lemon containing samples. The potassium sorbate elevated the cytotoxicity and the mortality in all cases.

After finding that some emulsions were highly cytotoxic at 5 $^{V}/_{V}$% and that significant *in vivo* toxicity could be expected in case of peppermint, microbial experiments were started at 2.5 $^{V}/_{V}$% with further halving dilutions using the broth microdilution method.

C. albicans cells were particularly sensitive to the essential oils, and even low concentrations resulted in significant inhibition. The first emulsion media had no effect and the second was not able to reduce the optical density of fungal cells surviving the treatment below 50%. Among the essential oils, the same order of effect set by the toxicity studies developed, so that the weakest antifungal properties were the emulsions containing lemon essential oil, followed by those containing rosemary and finally those containing peppermint essential oil. The addition of potassium sorbate significantly increased the inhibitory effect of all essential oils, and the effect of potassium sorbate could already be increased by very few essential oils.

The results of *S. aureus* and *E. coli* are varied, but overall no synergism could be detected, only additive action was seen between the essential oils and the potassium sorbate.

IV./6. Cytotoxicity and antimicrobial studies of different parabens.

Based on a comparative study of sorbates, we were curious to see what differences can be found if we also tested infrequently used compounds. In order to really observe the difference in chemical structure, we chose branching of the carbon chain as a structural factor and wanted

to study each derivative at the same concentration. Unbranched and branched esters with 3, 4 and 5 carbon atoms in the alkyl chain were paired and tested for cytotoxicity and antimicrobial activity in aqueous media. However, in the absence of literature data, it was first necessary to determine the maximum water solubility measured in aqueous media by HPLC, which was carried out by my colleague Ádám Haimhoffer Pharm.D..

The results show that the branched form has nearly twice the water solubility in case of the C3 and C5 esters than the unbranched chain form, while the C4 esters have nearly the same values. The parabens were then tested by number of carbon atoms in pairs at the maximum water-soluble concentration of the lower water-soluble ester. As such 0.025 $^{m}/_{V}$% for *n*-propyl and isopropyl paraben, 0.023 $^{m}/_{V}$% for *n*-butyl and isobutyl paraben, and 0.01 $^{m}/_{V}$% for *n*-pentyl and isopentyl paraben.

According to the MTT and the NR assays, there was a significant difference in cell viability for derivatives containing an odd carbon atom, with unbranched-chain ones always being more cytotoxic. There was no difference between the *n*-butyl and isobutyl derivatives at the same concentration, however, they proved to be more toxic than the other two pairs.

Using the broth microdilution method, we investigated the antimicrobial effect of ester pairs on the three previously used species at half concentrations. The inhibitory effect on *C. albicans* decreases with increasing carbon chain length and the unbranched form is less effective. An exception to this is the *n*-pentyl paraben-isopentyl paraben pair, where *n*-pentyl paraben was more effective, however, these compounds could no longer provide at least 50% inhibition at the lowest concentration, unlike the other compounds.

Our Gram-positive reference strain (*S. aureus*) proved to be relatively resistant to parabens. Isopropyl and isobutyl paraben were more effective than their unbranched counterparts, however, isopentyl had a lower inhibitory effect at higher concentrations than *n*-pentyl paraben, whereas at low concentrations the two compounds showed almost the same results.

E. coli was almost completely insensitive at the examined concentrations, however, the trends observed for the previous two species with respect to the effect of branched and unbranched derivatives were repeated in this species as well.

V. Megbeszélés

By the end of my doctoral studies, I was able to combine two *in vitro* and one *in vivo* toxicity measurement techniques in a routine application. In addition to the *G. mellonella* model organization, the application of MTT and NR tests combines three methodologies that can be performed quickly, cheaply, easily, in parallel in one protocol, and the different strengths and weaknesses complement each other well. In this way, better sensitivity and specificity can be achieved than with the use of the method alone, and beyond cytotoxicity, biocompatibility can be tested.

Do other excipients modify the cytotoxicity of preservatives? The results of the first series of experiments show that surfactants and preservatives significantly increase the cytotoxicity of each other, but these results are not surprising. However, the protective effect of 2-hydroxyethyl cellulose mucus needs to be explained. The Caco-2 cell culture model coated with the appropriate biopolymer is more resistant to cytotoxic effects. Benzalkonium chloride has also been described as being able to dissolve the mucus layer deposited on Caco-2 cells.

Do certain combinations of excipients modify the cell-damaging effect of preservatives on human and microbial cells? One of the most interesting phenomena of the second series of experiments is the different cytotoxicity of parabens measured in the two systems. Examples in the literature show that longer chain derivatives are more toxic than shorter ones. What explains the extraordinary result of butyl paraben at the highest concentration in the second system? Studies show that Caco-2 cells have special transesterase activity, and in the presence of ethanol, methyl, *n*-propyl, and *n*-butyl parabens are also converted to ethyl parabens. The enzymes have better activity towards shorter-chain than longer-chain derivatives, and higher concentrations of ethanol facilitate their activity, so the higher ethanol content of the second system presumably facilitated the conversion of methyl-paraben to ethyl-paraben. The latter is a more toxic derivative and may therefore explain the cell viability of methyl paraben, whereas less toxic ethyl paraben was formed in the case of *n*-butyl paraben. However, other effects should be assumed, as transesterification only explains the lower cell viability of methyl paraben, not *n*-butyl paraben, as it is converted to the still toxic ethyl paraben. All bacterial species were more sensitive to the first system containing glycerol and Labrasol® than to alcoholic solutions or the second system. It is known in the literature that Labrasol® has good antimicrobial activity against, for example, *Mycobacterium tubercolosis*, where complete cell wall disintegration has been described. It is hypothesized that this excipient explains the outstanding antimicrobial results of the formulation. However,

negative interactions between surfactants and parabens, such as cationic surfactants, have also been shown to reduce *n*-butyl paraben uptake and inhibitory activity in *E. coli*. Overall, it can be stated that due to the complex composition of our systems, the observed extraordinary data and phenomena should be subjected to further analysis in order to understand the exact processes behind these results.

These two series of experiments highlight that the excipients used in different liquid dosage forms are able to modify the cytotoxicity and antimicrobial activity of each other. By investigating such interactions, the irritant effect of the preparations (on the skin/mucosa) could be reduced and their microbiological protection could be increased. In the latter case, due to the beneficial effect of other potentiating excipients, the amount of preservative required is reduced, which would naturally result in smaller and less frequent side effects.

Do salts of carboxylic acids used as preservatives with different alkalis reduce cell viability to the same extent? No cytotoxicity was measured with the NR test for the different salts, than with the MTT test. In order to further understand the effect other compounds must be tested as well, which only differ in the cation (chlorides, sulfates, salts of simple carboxylic acids). If we see that compared to the sodium and potassium salts, the MTT assay always labels calcium salts incorrectly, toxic, then for the study of such chemicals MTT assay can not be used.

How is the cell damaging effect of sorbate esters differ from sorbic acid? The study that initiated **our research on sorbate esters** reported greater antimicrobial potential than sorbic acid for both ethyl and isopropyl sorbate, but we were also surprised by the results of time-kill experiments. The explanation for the outstanding activity and the enhanced cytotoxic effect observed during flow cytometric measurements in our hypothesis that the isopropyl sorbate has a special logP value. The lipophilicity required to cross the membrane and the water solubility required to reach the appropriate concentration are in the best proportions for this particular compound due to its relatively short carbon chain. To support our hypothesis, it is also necessary to study longer-chain derivatives, as we can already see that there is no increased activity with the shorter derivative, ethyl sorbate. Another possible explanation is the reduced efficacy of an efflux pump or enzyme (e.g., decarboxylase) present in both Caco-2 and microbial cells against the ester due to its different structure. Toxicity data missing from the original study were replaced by our own measurements. For sorbic acid, ethyl sorbate, and isopropyl sorbate, we cannot compare our results with existing publications, as these compounds have not yet been tested by these methods. Given the available data, it would be useful to explore the additional antimicrobial and biocompatibility profile of sorbate esters with

simple alkyl chain, looking for more potent compounds and/or subjecting isopropyl sorbate to further, more robust studies.

What biocompatibility interactions occur between essential oils and preservatives? The use of essential oils as preservatives is difficult due to their tendency to thermal degradation and volatility. This can be solved by their formulation as an emulsion, which provides adequate stability to the compounds responsible for antimicrobial activity. The three essential oils have different chemical compositions, which is well seen in the toxicity results. Due to the complex phytochemical composition of essential oils, only the essential oils themselves should be compared in terms of toxicity, not their individual components. The three essential oils have so far not been used in any studies where toxicity has been measured. The values in the safety data sheets are in exact contrast to the order of toxicity measured in cellular experiments, as the oral LD_{50} in rats is 2460 mg/kg for lemon essential oil and 6600 mg/kg for rosemary, although the species and route of administration are not indicated (12438 mg/kg). The reason for the difference is definitely caused by the substances in the emulsion medium and their different composition for each essential oil. However, since the three toxicity methods used showed the same order, it would be worthwhile to investigate additional toxicity in a model with a higher probative value instead of repeating. From an antimicrobial point of view, no synergistic effect was found, in which case emulsions supplemented with potassium sorbate would have been at least one order of magnitude more effective than potassium sorbate and emulsion alone. However, as no signs of synergism were found, it would be worthwhile to try another agent (like sodium benzoate) and if synergism is found, it is definitely worthwhile to perform a long-term stability study with gas chromatographic measurements, while monitoring the degradation and evaporation of the essential oil components. Synergism would allow a radical reduction of the concentration of both components, in the absence of it, their combined use there has no microbiological or toxicological benefits.

How different are the cell damaging effects of the constitutional isomer parabens? In our **last series of experiments**, we compared branched-chain parabens, which are rarely used. Various publications generally examine only the quaternary of methyl, ethyl, *n*-propyl, *n*-butyl paraben, neglecting additional branched, aromatic, or longer alkyl derivatives, so very few studies are available on the compounds we studied. It can be observed from the results that the derivatives containing 3 and 5 carbon atoms behave similarly in terms of both their water solubility and cytotoxicity, meaning that the branched derivatives have better water solubility and lower toxicity. In contrast, C4 esters showed very similar results. The difference between such even-odd-chain derivatives is known in the literature, which can be explained by the

different effect of secondary binding forces. In such homologous lines, differences in the physical properties of the compounds can be observed, and thus their biological effects are also different. However, this effect was found only in relation to water solubility and human cytotoxicity, with microbes showing different results for branched or unbranched derivatives. Our results may be explained by the different affinities (resulting from genetic polymorphism) of different human and microbial esterases for esters with different structures. For human microsome fractions, different metabolic activity has been demonstrated for unbranched and branched chain parabens. Bacterial esterases have also been shown to have similar variability and the presence of specific paraben degrading enzymes. However, all this requires further enzyme kinetic, molecular biological studies.

VI. Conclusion

During my PhD studies, I carried out the cytotoxicity and later biocompatibility analysis of liquid preservatives intended for oral application, looking for excipient-excipient interactions, examining the biocompatibility of preservatives related to their structure. The new scientific results are as follows:

1. Combining the MTT and NR cytotoxicity tests and the toxicological assessment of *G. mellonella* larvae, a rapid, parallelly executable, simple and inexpensive biocompatibility test protocol was created with predictive role for further experiments.
2. Excipient-excipient cytoprotective and antimicrobial interactions were discovered between parabens and cosolvent-surfactant systems/surfactant-mucus systems and parabens of which provide opportunities for further research relating their advantageous utilization.
3. First biocompatibility and antimicrobial activity comparison of sorbic acid and its potassium salt with different sorbate esters. Isopropyl sorbate showed outstanding bactericidal and fungicidal activity, which can lead to further studies. Our results provide a firm basis for these new experiments involving multiple species of pathogens and toxicity studies with vertebrates.
4. For the first time, the effects of even/odd and branched/open alkyl chains on different para-hydroxybenzoic acid esters were described regarding their respective water solubility, human cytotoxicity and antimicrobial activity, involving more constitutional isomers as previously seen.

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List of publications related to the dissertation

1. **Nemes, D.**, Kovács, R. L., Nagy, F., Tóth, Z., Herczegh, P., Borbás, A., Kelemen, V., Pfliegler, V. P., Rebenku, I., Hajdu, P., Fehér, P., Ujhelyi, Z., Fenyvesi, F., Váradi, J., Vecsernyés, M., Bácskay, I.: Comparative biocompatibility and antimicrobial studies of sorbic acid derivates.
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