UNIVERZA V LJUBLJANI
FAKULTETA ZA FARMACIJO

ALENKA STRUNA

TOKSIČNI UČINKI AFLATOKSINA B1, STERIGMATOCISTINA IN OKRATOKSINA A NA HUMANO KERATINOCITNO CELIČNO LINIJO HaCaT

TOXIC EFFECTS OF AFLATOXIN B1, STERIGMATOCYSTIN AND OCHRATOXIN A ON A HaCaT HUMAN KERATINOCYTE CELL LINE

UNIVERZITETNI ŠTUDIJSKI PROGRAM KOZMETOLOGIJA

Ljubljana, 2014

The practical part of this work and all analyses have been performed at the Department of Microbiology, the Faculty of Pharmacy and Biochemistry Zagreb, under the mentorship of Assoc. Prof. Matjaž Jeras, Ph.D., M.Pharm. and co-mentorship of Assoc. Prof. Maja Šegvić Klarić, Ph.D..

I would like to thank Assoc. Prof. Maja Šegvić Klarić, Ph.D. and the entire team at the Institute of Microbiology for the given opportunity and their generous help. I'm especially grateful to my mentor Assoc. Prof. Matjaž Jeras, Ph.D., M.Pharm. for his constant availability and support.

Declaration

I declare that I have elaborated this bachelor thesis independently, under the mentorship of Assoc. Prof. MATJAŽ JERAS, Ph.D., M.Pharm. and co-mentorship of Dr. Sc. Maja Šegvić Klarić.

Izjava

Izjavljam, da sem diplomsko nalogo izdelala samostojno, pod mentorstvom izr. prof. dr. Matjaža Jerasa mag.farm. in somentorstvom dr. sc. Maje Šegvić Klarić.

ABSTRACT

Mycotoxins are secondary metabolites of moulds, present all over the world. Due to their common whereabouts and considering the fact that skin is the biggest human organ, it is reasonable to check what would be the consequences for skin cells after contact. The aim of this work was therefore to examine the toxic effects of selected single and combined mycotoxins on human keratinocytes.

In real-life the most common multiple combinations of aflatoxin B_1, ochratoxin and sterigmatocystin can be in contact with human skin. Aflatoxin B_1 is the most frequent and has the highest carcinogenic and genotoxic potential. Ochratoxin has high skin permeability and high toxic potential, while sterigmatocystin is interesting, because it is a precursor of aflatoxin and is therefore suspected to have similar toxic activity. It is assumed that all of them are potential carcinogens and induce DNA damaging mechanisms.

Our hypothesis was that mixtures of these mycotoxins are more hazardous to skin cells, even in small doses, than single ones. To find that out, their single and combined cytotoxicities were tested *in vitro* on HaCaT human keratinocyte cell-line by using the classical MTT assay. We found that sterigmatocystin had the highest cytotoxic potential (IC_{50} = 44,4 μM), followed by ochratoxin (IC_{50} = 98,5 μM), while aflatoxin B_1 in all seven concentrations tested (1 – 150 μM) showed only weak cytotoxicity, never reaching the threshold (50% decrease in cell viability). Then we compared viabilities of cells exposed to various mixtures of two different toxins, combined in different concentrations and mostly observed that the cytotoxicity of mixtures deviated from expected effects, based on summing up the harmful potential of singular concentrations of individual toxin. The exposure to lower concentrations of mycotoxins (1 μM) in their tandem showed mostly no cytotoxic effect, but induced HaCaT proliferation instead. On the other hand, the higher concentrations (10 μM) of mycotoxins in different mixtures decreased metabolic activity of cells. The highest decrease in viability was observed with ochratoxin and sterigmatocystin mixtures, while those containing aflatoxin B_1 were less cytotoxic, although still exerting rather high negative influence on cell viability.

The hazards of toxins in general cannot be determined only by their cytotoxic potential. As those cell injuries caused by mycotoxins that do not cause immediate cell death could be even more dangerous, it would be important to explore also their other toxic effects on skin. Due to all these facts it is reasonable to avoid exposure to mycotoxins whenever is possible.

Keywords:

- Aflatoxin B_1
- Cytotoxicity
- Ochratoxin
- Skin
- Sterigmatocystin

POVZETEK

Mikotoksini so sekundarni metabolni proizvodi plesni, ki lahko kontaminirajo žita, rastlinska olja in rastlinske droge. Tovrstna kontaminacija se lahko pojavi že med rastlinsko rastjo ali pa med shranjevanjem surovin v neprimernih pogojih. Strukturno so si raznoliki. Večinoma imajo nizko molekulsko maso, so stabilni in lipofilni, saj so topni v oljih ter toksični že v pikomolarnih (pM) količinah. V človeški organizem lahko pridejo z vdihavanjem zraka, s hrano ali pa preko kože oz. transdermalno. Njihova prisotnost v telesu lahko vodi v poslabšano imunsko odpornost in genetske poškodbe celic ter posledično kancerogenezo, seveda v odvisnosti od časa izpostavljenosti, koncentracij mikotoksinov, morebitne sočasne prisotnosti drugih toksinov ter lastnosti vsakega posameznika.

Glede na to, da je koža največji človeški organ ter da so molekule mikotoksinov razmeroma majhne in lipofilne, kar jim omogoča prehod skozi kožo, je smotrno preveriti, kakšne vplive imajo te molekule na kožne celice. Mikotoksinom so sicer dermalno najbolj izpostavljeni delavci v predelavi rastlinskih surovin, prebivalci na območjih z veliko vlage, prav tako pa bi lahko prišli v stik z njimi preko kozmetičnih izdelkov, ki vsebujejo različne sestavine rastlinskega izvora, npr. koruzni škrob, mandljevo ali olivno olje in druge. Te so lahko kontaminirane, zaradi stabilnosti pa se v njih morda prisotni mikotoksini med procesiranjem ne uničijo. Upoštevati moramo tudi možnost, da je lahko v posamezni surovini sočasno prisotnih več mikotoksinov, zato moramo ob določanju tveganja predpostaviti tudi njihove interakcije in preveriti toksičnost takih kombinacij.

Najpogostejši in za dermalno izpostavljenost zanimivi mikotoksini so aflatoksin B_1, okratoksin in sterigmatocistin. Aflatoksin B_1 je eden najbolj razširjenih in najnevarnejših mikostoksinov. International Agency for Research on Cancer ga uvršča v skupino 1 glede na kancerogenost. Okratoksin A ima veliko sposobnost prehajanja skozi kožo, sterigmatocistin pa je strukturno podoben aflatoksinu, zato predpostavljajo, da ima njemu podobne učinke, ki so še vedno slabo raziskani.

Da bi določili citotoksične učinke omenjenih treh mikotoksinov, smo spremljali zmanjšanje preživetja epidermalnih kožnih celic po 24 h izpostavljanju različnim koncentracijam in kombinacijam toksinov. Vse raziskave smo izvedli *in vitro* na nesmrtni celični liniji človeških keratinocitov HaCaT. Te celice so sposobne hitre spontane transformacije in proliferacije in so zato dober model za testiranja *in vitro* ter

ekstrapolacijo rezultatov na razmere *in vivo*. Celične kulture smo izpostavili različnim koncentracijam posameznih toksinov in njihovih kombinacij ter določili tiste količine preiskovanih substanc, ki so povzročile 50 % upad metabolne aktivnosti celic. Dobljene rezultate smo primerjali s predvidenimi. Naša hipoteza je bila, da imata aflatoksin B_1 in sterigmatocistin zaradi strukturne podobnosti podoben citotoksičen učinek. Glede na razpoložljive informacije smo vedeli, da se okratoksin in aflatoksin B_1 lahko vežeta na makromolekule. Na osnovi tega smo sklepali, da se mikotoksini lahko povezujejo tudi med seboj in da bo posledično preživetje celic odstopalo od tistega, ki smo ga računsko določili s seštevkom citotoksičnih potencialov posamičnih testiranih substanc. Vrednotenje citotoksičnih vplivov smo izvedli s pomočjo kolorimetričnega testa MTT. Ta temelji na redukciji rumenega tetrazolijevega bromida v vijolični formazan, ki jo povzročijo metabolično aktivne celice.

Citotoksične potenciale posameznih mikotoksinov smo ovrednotili tako, da smo določili tiste koncentracije, ki so zmanjšale preživetje celic za 50 %. Ugotovili smo, da je imel največji citotoksični potencial sterigmatocistin (IC_{50} = 44.4 μM), sledil mu je okratoksin (IC_{50} = 98.5 μM), medtem, ko nobena od sedmih testiranih koncentracij aflatoksin B_1 (od 1 – 150 μM) ni povzročila 50 % upada živosti celic HaCaT. Citotoksično delovanje sterigmatocistina in okratoksina je bilo odvisno od uporabljene koncentracije, aflatoksin B_1 pa ni kazal tovrstne odvisnosti. Tandemne kombinacije mikotoksinov (aflatoksin B_1-okratoksin; aflatoksin B_1-sterigmatocistin in okratoksin-sterigmatocistin) so v nižjih koncentracijah (1 μM) povzročale celično proliferacijo. Citotoksično delovanje ostalih koncentracijskih kombinacij pa je večinoma statistično značilno odstopalo od predvidenih oziroma pričakovanih rezultatov, ki smo jih predpostavili s seštevanjem posameznih citotoksičnih učinkov iste koncentracije vsakega posameznega mikotoksina. Največji citotoksični vpliv so imele kombinacija okratoksin-sterigmatocistin. V kombinacijah, ki so vsebovale 10 μM okratoksina, smo zaznali sinergistično delovanje obeh mikotoksinov. Mešanica aflatoksin B_1-sterigmatocistin je v večini primerov kazala antagonističen vpliv. Predpostavljamo, da se je sterigmatocistin, ki je prekurzor aflatoksina B_1, med 24 urno inkubacijo v celicah dejansko pretvoril v aflatoksin B_1, s tem pa se je izrazito zmanjšalo njegovo citotoksično delovanje. Kombinacije okratoksina in aflatoksina B_1 so delovale sinergistično, še posebej v primerih, ko je bil okratoksin prisoten v višji koncentraciji. Po 24-urni prisotnosti v kulturah celic HaCaT je bila najbolj citotoksična kombinacija

okratoksin-sterigmatocistin, medtem ko so mešanice aflatoksin B_1-sterigmatocistin, aflatoksin B_1-okratoksin izkazovale manjši citotoksični potencial. Predvidevamo, da okratoksin poškoduje celice z indukcijo tvorbe reaktivnih kisikovih spojin, spremembami celične signalizacije in povzročitvijo poškodb DNA. Tudi aflatoksin B_1 naj bi po bioaktivaciji s celičnimi encimi povzročil nastanek reaktivnih kisikovih spojin in se vezal na DNA. Za sterigmatocistin pa predvidevajo, da povzroča lomljenje dvojne vijačnice DNA. Zavedati se moramo, da se tudi različne celice razlikujejo med seboj, npr. v prisotnostih in količinah encimov, različno izraženih popravljalnih mehanizmih DNA in sposobnostih samoobnavljanja, zato lahko pričakujemo različne učinke mikotoksinov na različne celice, ki jih uporabljamo za njihovo testiranje *in vitro*.

Če torej povzamemo, celokupne varnosti/nevarnosti mikotoksinov ne moremo opredeliti le z določanjem njihovega citotoksičnega potenciala, saj pogosto povzročijo celične poškodbe, ki so premajhne, da bi izzvale takojšnjo celično smrt. Ker so mikotoksini večinoma lipofilne molekule, lahko ob stiku s kožo preidejo v njene globlje plasti, kjer se nato vežejo na različne makromolekule in se tako v organizmu zadržijo daljši čas. Zato bi bilo nujno potrebno določiti tudi njihov genotoksični potencial in kancerogene učinke.

Ključne besede:

- aflatoksin B_1
- citotoksičnost
- koža
- okratoksin A
- sterigmatocistin

ABBREVIATIONS

AFB_1 - Aflatoxin B_1
AF - Aflatoxin
CYP- Cytochrome P_{450}
DDE - Daily Dermal Exposure
DMSO - Dimethylsulphoxide
DMEM - Dulbecco's Modification of Eagle's Medium
EFSA - European Food Safety Authority
FBS - Fetal Bovine Serum
GAP - Good Agricultural Practice
GHP - Good Harvesting Practice
GST - Glutation-S-Transferase
IARC- International Agency for Research on Cancer
LPO - Lipide Perokside Oxidation
MW - Molecular Weight
MT - Mycotoxin
NCRI - National Cancer Research Institute
OTA - Ochratoxin A
ROS - Reactive Oxigen Substance
SD - Standard Deviation
STC - Sterigmatocystin
SC - Stratum Corneum

1. INTRODUCTION

1.1 Mycotoxins (MTs)

Toxins are natural substances produced by plants, animals or microorganisms exerting negative effects on another organism (1). Mycotoxins are secondary metabolites produced by filamentous fungi. Humans are exposed to them accidentally. They can cause acute or chronic effects, depending on their concentration and duration of contact (1, 2). Although MTs are chemically and characteristically very heterogeneous substances, they are all toxic at very low concentrations. The symptoms after exposure depend on the type of MT and individual's characteristics, i.e. age, sex, health, genetics, life style (3). MTs can cause impairment of immune system, genotoxicity and promotion of cancerogenesis (4). As almost all fungi species are able to produce more than just one MT at the same time, they are quite often present in combinations that can act additively, synergistically, or antagonistically (1, 2, 3, 5). The presence of MTs is associated with various combinations of temperature, pH and humidity that determinate the expending of moulds and their MT production capabilities. Mycotoxins can be present in root crops, nuts, seeds and dairy products, and therefore also in commercial products, such as natural cosmetics, which usually contain unprocessed substances like corn starch, and various popular herbal drugs (1, 2, 5). Due to their co-appearance, an individual could be exposed to numerous different combinations of MTs via dermal contact, as well as respiratory and/or dietary intake (5). The most studied are effects following dietary and inhalation exposure of MTs. Due to their small molecular weights (MW), around 700 Da, oil solubility and other specific characteristics, it is necessary to explore their skin penetration ability, transition into the systemic circulation and effects on skin cells (1, 5). Skin exposure route is especially important for people who work in the processing of natural raw materials, where air and dust can contain MTs, as well as for consumers of natural cosmetics and people living in humid indoor places, favoring the growth of various types of fungi (5). The primary concern for dermal exposure would be the presence of aflatoxin B_1 (AFB_1), ochratoxin (OTA), and sterigmatocystin (STC), as they often co-occurre and exert high toxicity. AFB_1 is the most common and hazardous MT, OTA could be dangerous due to its high skin permeability potential, while STC is believed to be toxic because of its chemical similarity to AFB_1 (3, 5).

1.1.1 Aflatoxins (AFs) are produced by the *Aspergillus flavus* and *Aspergillus parasiticus* species (3). AFB_1 (Figure 1A) is the most common aflatoxin, has strong hepatotoxic effect and is also immunosuppressive, teratogenic, carcinogenic and genotoxic. The International Agency for Research on Cancer (IARC) assessed its carcinogenic and acute toxic potential and classified it as a member of the group 1. The highest limit for the AFB_1 content in EU for food is from 4.0 μg/kg to 15 μg/kg, depending on the product (6). The mechanism of AFB_1 toxic activity, following its cell entry, is mainly the formation of DNA adducts. It is metabolized into 8, 9-epoxide in endoplasmic reticulum by cytocrome P_{450} (CYP) enzymes (2). AFB_1 may cause chromosomal aberrations, sister chromatid exchange, unscheduled DNA synthesis and chromosomal strand break. In addition it can reduce number of immune cells such as T lymphocytes and macrophages and decrease complement activity. Its reactive metabolite (epoxide) mostly binds to DNA, RNA and proteins via guanine. This is then miscoded into tyrosine and by replications of DNA mutations leads to cancerogenesis (3, 5, 6).

1.1.2 Ochratoxin (Figure 1B) is mostly produced by *Penicillium verrucosum* and *Aspergillus species* (both pertaining to the *Circumdati* section), which occur commonly and can grow in wide range of conditions (7, 8). OTA is found in natural raw materials, ranging from 0.03 to 27.5 ppm (parts per million). Currently there is no unified limitation regarding its permissible quantities (3, 9) there are only some national regulations that contain their own recommendations (7). OTA and its mixtures are reported to affect blood coagulation and have genotoxic, embryotoxic, neurotoxic, teratogenic, carcinogenic, immunosuppressive and nephrotoxic effects (2). Due to its nephrotoxicity it has been implicated in the etiology of Balkan endemic nephropathy and associated urothelial tumours (2). According to the known data, IARC ranked it into the 2B category of substances, as a possible human carcinogen (3, 9). The mechanism of OTA toxicity is complex, consisting of many potential harmful pathways (Figure 2).

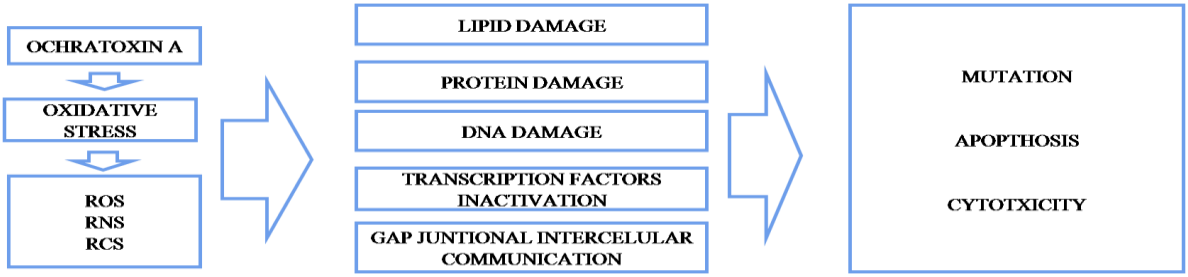

Figure 2: Schematic presentation of oxidative stress induced by OTA; adapted from (10).

1.1.3 Sterigmatocystin is a polyketide (Figure 1C) produced by *Aspergillus spp.* from the *Versicolores* section, including. *Aspergillus nidulans*, and 55 more related fungi of this species. The STC molecule is lipophylic and highly stable. Chemically it is similar to AFB_1 and some fungi can transform STC into AFB_1 (11). Studies in human adenocarcinoma, A549 human lung and Het-1A esophageal epithelial cells showed that STC is more cytotoxic than AFB_1, exhibiting mutagenic potential (12). Several EFSA studies assessed its carcinogenic potential to be lower than that of AFB_1 (13). According to *in vivo* and *in vitro* experiments on animals, it is suggested that STC may have immunomodulatory effects, is genotoxic and a potential human carcinogen. The IARC has categorized it into the 2B group of substances (11). STC is also inhibiting DNA synthesis (14). Its possible toxic mechanisms at the cell level could be the induction of DNA damage and DNA double strand breaks which cause necrosis. Currently there is no regulation regarding its occurrence.

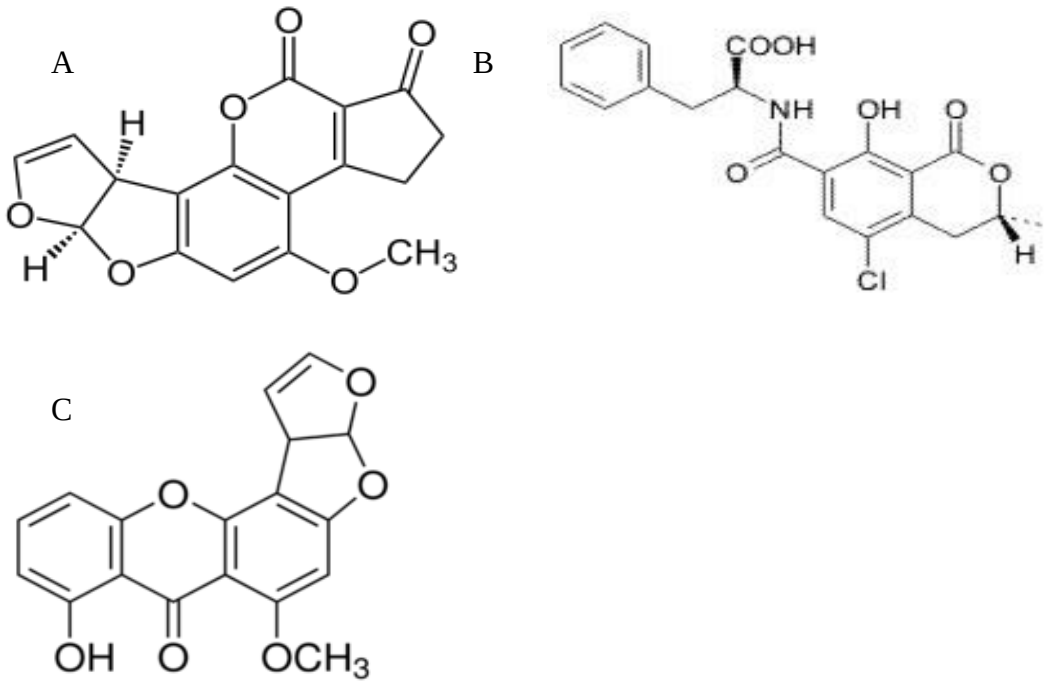

Figure 2: A - Aflatoxin B_1, $C_{17}H_{12}O_6$, MW = 312.27 g/mol;
B - Ochratoxin, $C_{20}H_{18}ClNO_6$, MW = 403.81 g/mol;
C - Sterigmatocystin, $C_{18}H_{12}O_6$, MW = 324.28 g/mol.

1.2 Structure and function of skin and MT penetration pathways

For compounds that may get in contact with the skin, the knowledge regarding their dermal absorption is crucial in order to evaluate the perils of systemic exposure and to elaborate risk assessments. Skin consists of three heterogeneous layers: epidermis, dermis and subcutis (Figure 3). Each of them has different anatomic and physiochemical properties. Skin is the main interface that separates body from the environment, controls water loss and thermoregulation and has important sensory functions. It is also preventing unwanted influences of potentially harmful exterior substances (15).

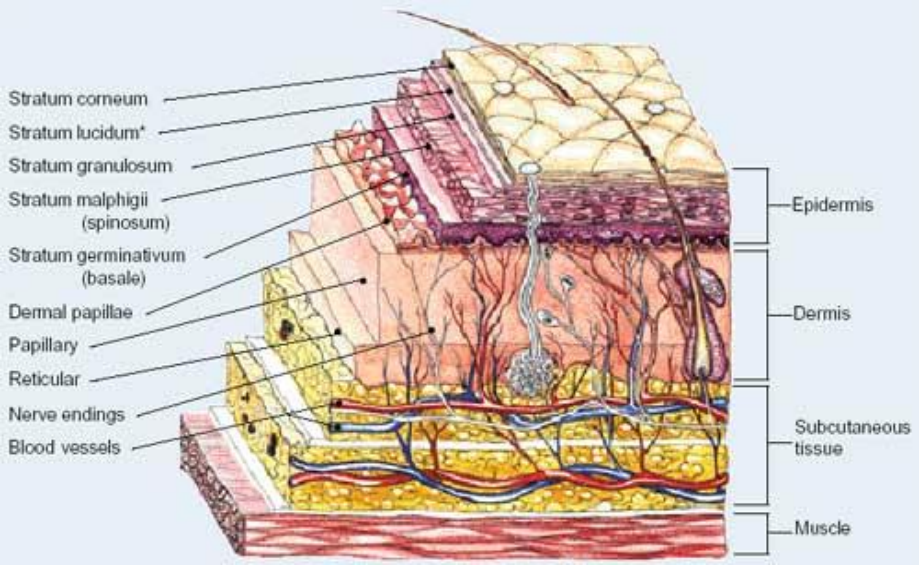

Figure 3: The anatomy of human skin (16).

The physical barrier of the skin is being further enhanced by its capabilities to gradually change pH and lipophilic characteristics that progressively pass into more hydrophilic areas. Structural and functional differences of skin layers and specific cells additionally help to prevent effects of potentially dangerous external substances. Keratinocytes represent the main hindrance for them, as they build epidermis in a form of blocks surrounded by lipid matrix. They descend from their precursors located at the basal layer, and during the process known as keratinization differentiate and migrate to the skin surface, where they finally slough off. While migrating to the outside part of the skin, these cells form epidermis, which undergoes several morphological and chemical changes, such as losing of cellular organelles, changing pH value and increasing lipophilicity. Anatomically the epidermis is divided into 5 sub-layers. Stratum corneum (SC), its outer layer that is 10 to 20 μm thick and contains about 20% of water has the strongest barrier

function for water-soluble chemicals. The inner sub-layers of epidermis are more hydrophilic. Dynamic changes and keratinocyte migration produce a protective self-renewing outer layer of the skin. However, the skin homeostasis can only be provided if there is a constant number of appropriate cells available and a proper hardness of adhesive junctions between them in place (15, 17, 18).

Even though the skin is a quite good protective barrier, dermal exposure presents an important potential entry zone. Actually, this is still mostly unexplored area in case of MTs, although they are small lipophilic molecules able to penetrate skin. There are few potential transdermal routes of entry, depending on MW and lipophilic/hydrophilic properties of substances, predicted by their partition coefficient -log P values, as well as on individual skin conditions, such as thickness of SC, skin hydration, presence of dermal injuries and diseases, ethnic differences, distribution of skin derivates, body temperature, number and activity of immune cells (19). One of the types of skin penetration is intercellular penetration, where a substance is able to enter through lipid layers surrounding corneocytes (terminally differentiated keratinocytes). The two other possibilities are via hair follicles and directly through viable cells. If the skin is intact, only highly lipophilic substances with MW lower than 700 Da can enter SC. After passing SC they are able to penetrate in deeper layers of the skin, depending on their log P values (5). Besides penetration, accumulation of substances in different layers may occur. While more lipophilic substances stay in SC and are removed during keratinization, hydrophilic substances penetrate deeper and must be eliminated by some other mechanism. Highly hydrophilic substances with small MW can reach systemic circulation exclusively by transport through hair follicles (20). Skin accumulated MTs can harm skin cells by binding to reactive sites of various biomacromolecules (5, 19).

In vitro experiment that was performed to determine the extent of penetrance of AFB_1 and OTA showed that the latter had the highest ability of permeation, measured by the permeability coefficient ($Kp = 8.20\times10^{-4}$cm/h) (5). The Kp values may diverge from other available data, depending on the solvent used. The extent of penetration through the skin also depends upon its hydration, condition and thickness. Twenty four hours after the exposure of the skin model to high local concentrations of AFB_1 and OTA, high concentrations of both toxins were still found in various skin layers. The results correlated with the obtained Kp values (Table I). According to amounts of MTs measured in each

layer it was concluded, that the skin acts like a reservoir (5, 19). A plausible explanation for such accumulation of AFB_1 and OTA may be that they can both bind to certain proteins present in skin. Even when MTs, contained for example in a cosmetic product, reach the area of skin toxication, their effects can still be modulated by a variety of factors, including nutrients, antioxidants, herbs, chemo-preventive agents, as well as food restriction, viral infections and genetic polymorphisms.

Table I: Partition coefficients -Log P, permeability rates - Kp and concentrations of AFB_1 and OTA, assayed 24 h after their application to an experimental skin model (5).

	MOLECULAR WEIGHT	**Log P**	**K_p(cm/h)**	**C_{24h}(mg/mL)**
AFB_1	312,27	1,16	2.11×10^{-4}	3.58±0.25
OTA	403,81	4,24	8.20×10^{-4}	1.89±0.33

1.3 Dermal exposure to cosmetics containing MTs

Herbal materials are often reported to be contaminated by MTs. Such contaminations can occur during plant growth, harvest, storage and processing. Herbal drugs are prone to MT contamination. They are often used as cosmetic ingredients, like almond oil, olive oil, and starch. This is especially important for countries with climatic conditions that are favorable for mould growth and toxin production. A lot of countries that are quite big exporters of natural raw materials, have often ill defined and regulated good harvesting practice (GHP), good agricultural practice (GAP), and bad monitoring of drug toxication. European Union (EU) has some regulations regarding the maximal allowed dose of AFB_1 in raw materials and recommendations for the presence of OTA in herbs, but it is not known if anybody is checking their contents in cosmetics. In case the natural cosmetic ingredients are contaminated, calculations can be made for the worst possible scenarios by using the following equation *1*:

$$DDE = \frac{[mycotoxin] \times K_p \times SA \times ED \times EF \times EV \times t_{event}}{BW \times AVs}$$

Equation *1*: Equation for calculating daily dermal exposures to MT; DDE – daily dermal exposures; Kp – permeability coefficient; SA – surface area; ED – exposure

duration; EF – exposure frequency; EV – event frequency; t_{event} – event duration; BW – body weight; AVs – averaging times (5).

Theoretical daily dermal exposures (DDE) were calculated for three randomly selected cosmetic products containing herbal ingredients by this formula. For each product the worst scenario of contamination was anticipated (Table II). The calculation took into account the concentration of natural ingredients contained in each particular product. Other variables used for calculations were adjustments for an average exposed individual and conditions: *surface area* = 0.7 m^2; *exposure duration* = 30 years; *exposure frequency* = 2x/week = 104 days/year; *event frequency* = 1 /day; *body weight* = 70 kg; *average years* = 70 years (5). Calculated DDE values were then compared to National Cancer Research Institute (NCRI) values, representing the exposure to MTs in ng/kg/day, associated with a risk level of 1 per 100 000 individuals exposed to genotoxic and carcinogenic MTs. The NCRI value for AFB_1 is 0.038 ng/(kg BW/day), and for OTA: 4 - 10 ng/(kg BW/day) (5).

Table II: Calculated DDE values for AFB_1 and OTA, theoretically contaminating three randomly selected products – the worst-case scenario (5).

INGREDIENT	**Theoretically contaminated ingredient**	**MT**	**Contamination of ingredient (ng/kg)**	**The quantity of ingredient in the product (ng/kg)**	**DDE (ng/kg)**
Almond oil	Californian almond oil	AFB_1	107	1700	**10,063**
		OTA			
Chamomile extract	Herbal drug from India	AFB_1	1180	5900	**0,555**
		OTA	290,8	1454	0,531-1,239
Oat extract	Cereals	AFB_1	66,7	2001	**0,0188**
		OTA	112	3360	1,228-2,865

The results show that MT contaminated cosmetic products could be hazardous to users. In the worst-case scenario, one of the three products could contain AFB_1 in amounts that exceed NCRI values (bolded numbers in table II). Even if the contaminated natural ingredients are being processed with heat, it only weakly decreases concentrations of MTs, as AFB_1, STC and OTA are rather thermically stable. On the other hand, high temperature decreases concentration of antioxidants and other positive acting ingredients, responsible

for the healing properties of the product. Therefore, natural oils used as ingredients are often unrefined. Another important fact is that AFB_1, OTA, and STC are oil soluble substances. Cosmetic products are formulated in such a way, that their sensory effects on skin are beneficial. As they interact directly with the skin, their formulations must contain oily components. Therefore, we can conclude that MTs are solvated in such vehicle, which is used to deliver active substances deeply in skin layers. As mentioned before, the vehicle may change Kp value of each particular substance. Besides desired active substances, MTs can also penetrate and affect skin. Physical pressure during cosmetic product application could additionally increase their penetration in deeper layers. One of inevitable scenarios is that even if the cosmetic product is intended for use on healthy skin, it is commonly applied on injured surfaces. Also, herbal drugs may be ingredients of dermal pharmaceutical products (21).

2. THE AIM OF THE WORK AND WORKING HYPOTHESES

The hazard of dermal exposure to MTs is poorly investigated, even though the skin is the biggest human organ, which directly interacts with environment. Knowing that AFB_1 is the most common and toxic MT, that STC has similar chemical structure as AFB_1 and that OTA has the highest permeability coefficient (Kp), we decided to investigate their *in vitro* cytotoxic potentials on human epidermal skin cells. We will determine effects of both, single and tandemly combined MTs, as they often co-occurre in natural products. The immortal human keratinocyte cells (HaCaT) will be chosen for testing cytotoxicity of different concentrations of AFB_1, OTA and STC and their tandem combinations in 24 h *in vitro*, using the classical MTT test and appropriate controls. Our additional aim will be to find out whether the tested MTs interact in synergistic, additive or antagonistic ways.

As AFB_1, OTA and STC use different mechanisms for harming cellular functions, we hypothesize that:

- the cytotoxic potential of tandem MT combinations will differ from those predicted by simply summing their single toxic potentials;
- the assessed cytotoxic potentials of MTs on HaCaT cell line will differ from those determined *in vitro* on other types of human cells, as reported in accessible literature data.

3. MATERIALS IN METHODS

3.1 The HaCaT cell line and its cultivation

The immortalized human keratinocytes cell line HaCaT was originally isolated from a histologically normal Caucasian woman. These cells are able to undergo quick spontaneous transformations and proliferation *in vitro* and are therefore a good model for studying toxic effects of various substances on skin and for extrapolating the results obtained to *in vivo* situation.

The HaCaT (Cell line services, Germany) cells used in our experiments were cultured in DMEM/Ham's F12 medium (Gibco, Invitrogen, Paisley), supplemented with 4 mM L-glutamine (Gibco,Invitrogen, Waltham) and 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Paisley). To prevent contamination, penicillin (2.5%) and streptomycin (2.5%) (Gibco, Invitrogen, Paisley) were added. The cells were growing in plastic cell-culture flasks (TPP, Trasadingen), with surface 75 cm^2 that provided their adhesion, in the incubator under standard conditions, providing constant temperature of 37 ^oC, 5% CO_2 and 95% humidity. When they reached 80 % confluency, they were passaged by tripsinization. All manipulations took place in aseptic conditions within a laminar flow box and were performed using the following sterile equipment.

Equipment:

- Centrifuge 5415R (Eppendorf, Germany)
- Electronic Microplate Reader (Labsystem iEMS, type 1404)
- Inverse microscope (Olympus IX51, USA)
- Laminar airflow (Klima Oprema, Samobor)
- Mehanical pipets 100-1000 µl, 10-100 µl, 5-10 µl, 0,1-3 µl (Biohit, Eppendorf, Germany)
- Microscope (Olympus BX40, USA)
- Multichanel pipet (8 channel) 5-100 µl (Biohit, Eppendorf, Germany)
- Vorteks (Techno Kartell TK35)

3.2 Mycotoxins

3.2.1 *Aflatoxin B1*

AFB_1 with the chemical formula $C_{17}H_{12}O_6$, has molecular weight of 312.27 g/mol. It was isolated from *Aspergillus flavus* (Sigma-Aldrich, Deisenhofen). We prepared a basic solution containig 0,01mol AFB_1/L diluted in 80% DMSO/ EtOH.

3.2.2 *Sterigmatocystin*

STC with the chemical formula $C_{18}H_{12}0_6$, has molecular weight of 324.28 g/mol. STC (Sigma-Aldrich, Deisenhofen) was diluted in DMSO to prepare basic solution containing 2.5 mg/mL (7709 μM).

3.2.3 *Ochratoxin*

OTA, with the chemical formula $C_{20}H_{18}ClNO_6$, has molecular weight of 403.81 g/mol. It was isolated from *Petromyces albertensis (Sigma-Aldrich, Deisenhofen)* and diluted in absolute EtOH to obtain a basic solution of 2.5 mg/mL (6191 μM).

3.3 Preparation of MT solutions for treatment of HaCaT cells

Appropriate aliquots of basic solutions of each MT were diluted with medium containing 1% of FBS, to obtain the following concentrations of AFB_1, OTA and STC, used for treating HaCaT cells: 0.1, 1, 10, 30, 50, 70, 100 and 150 μM. Additionally the following tandem mixtures of MTs in differently combined concentrations were prepared:

AFB_1 + OTA (1 μM +1 μM; 1 μM +10 μM; 10 μM +1 μM; 10 μM +10 μM)

AFB_1 + STC (1 μM +1 μM; 1 μM +10 μM; 10 μM +1 μM; 10 μM +10 μM)

OTA + STC (1 μM +1 μM; 1 μM +10 μM; 10 μM +1 μM; 10 μM +10 μM)

Negative controls consisted of exactly the same quantities of solvents that were used for the preparation of MT solutions, i.e. DMSO, EtOH and 80% DMSO/EtOH, devoid of MTs.

3.4 Testing of single and combined MT cytotoxicities with the MTT assay

The tetrazolium dye (MTT) based assay is a calorimetric method for determination of cell viability, using standard micro-plate absorbance readers. This kind of determination of cell growth rates is widely used in the testing of drugs and cytotoxic agents, as well as screening of other biologically active compounds. The assay is based on the reduction of yellow tetrazolium MTT in a purple formazan by metabolically active cells. The resulting intracellular purple formazan can be dissolved and spectrophotometrically quantified.

For our experiments we seeded 100 μL of HaCaT cell suspension, containing 5 x 10^5 cells/mL in medium containing 10% FBS, per well of a 96-well plate and incubated them for 24 h prior to the addition of various concentrations and combinations of MTs to be tested. This starting HaCaT concentration provided that the confluence of cells in each well after 24 h of incubation was 80%. Then we carefully removed the medium from each microculture and replaced it with appropriate solutions of single and combined MTs in medium containing 1% of FBS. This serum concentration still provides normal proliferation and growth of cells, but minimizes binding of tested MTs to serum proteins that could diminish their cytotoxicity. As already mentioned, we exposed HaCaT cells to various concentrations of OTA, AFB_1 and STC alone, as well as to their tandem combinations in at least quintuplicates for 24 hours. The percentages of living cells in negative controls, treated only with solvents in medium supplemented with 1% of FBS, were considered as 100% viability values. After that period, we removed the medium from each microculture and replaced it with 100 μL of 5 mg/mL MTT solution (Sigma, St. Louvis. MO, USA) in a serum-free medium. Following 3 hours of incubation, the microcultures were colored due to reduction of formazan by viable cells. Then we removed the MTT solution from each well and replaced it with 150 μL of DMSO/ Triton X-100 (Sigma-Aldrich, Deisenhofen, Germany) mixture in order to dissolve the intracellular formazan crystals. Spectrophotometric absorbance of each microculture was then measured at 570 nm. Viability of cells was calculated (Equlation *2*).

$$\text{Viability (\%)} = ((A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}})) \times 100$$

Equation *2*: Equation used for calculation for percentage of cell viability; A_{blank}-absorbance of empty wells; A_{sample} -absorbance of wells including cells treated with toxins; A_{control} - wells treated only with solvents.

3.5 Statistical analyses

We used the GraphPad® Prism v. 6 programme for all statistical analyses. The analysis of each MT cytotoxicity was performed using one-way ANOVA. Data are presented as means plus standard deviations (SDs) regarding to control group, for which ANOVA with multiple comparison procedure -Turkey test, was used. We also compared the measured or actual values with those that were calculated as expected (Equation *3* and *4*). They are also presented as means plus SD.

Expected viability (%) = % *(viability of cells after MT- 1 treatment)*+ % (*viability of cells after MT-2 treatment)* - 100% *(that express survival of control)*

Equation *3*: The calculation of expected cell viability.

$$SD\ (expected) = [(SD\ of\ cell\ viability\ upon\ treatment\ with\ MT\text{-}1)^2 + (SD\ of\ cell\ viability\ upon\ treatment\ with\ MT\text{-}2\ )^2]^{1/2}$$

Equation *4*: The calculation of expected standard deviations of cell viability.

Statistical significance of differences between expected and measured/actual values was further analysed by the unpaired t-test (Equation *5*)

$$t = \frac{Measured\ viability\ (\%) - Expected\ viability\ (\%)}{[SD\ (measured)^2 + SD\ (expected)^2]^{1/2}}$$

Equation *5*: Equation for determination of t value.

The $p \leq 0.05$ value was considered statistically significant. The results were interpreted as follows: (a) an additive effect was considered if the measured values were not significantly above or below the expected ones; (b) synergism was supposed, if the measured values were significantly below the expected ones; (c) an antagonistic effect was assigned, if the measured values were significantly above the expected ones.

4. RESULTS AND DISCUSSION

4.1 Individual cytotoxicities of AFB_1, OTA and STC on HaCaT cells

4.1.1 The cytotoxicity of OTA

While in low concentrations OTA stimulated cell proliferation, doses higher than 1 μM, decreased cell viability in a dose responding manner (Figure 4A). The IC_{50} was reached at the concentration of 94.39 μM. The results showing dose-depending cytotoxic effects of OTA, on adult T-cell leukemia KK-1 and human epithelioma HEP-2 cell lines have also been reported, clearly showing its different influences on different cell lines (22). While the observed IC_{50} in case of KK-1 cells was around 5 μM, it was around 70 μM for HEP-2 cells, and, as mentioned, 94,39 μM in case of our epidermal HaCaT cell line. This means that for each tissue-specific cell type its individual susceptibility to a particular MT should be determined (23). When mice were challenged *in vivo* with 20–80 μg of OTA/mouse for 12–72 h, increased apoptosis of dermal cells via mitochondrial pathway was observed. However, when in the same study smaller doses of 100 ng OTA/mouse were applied, increased concentrations of short-term markers of tumorigenesis were detected (23).

Therefore, for epidermal skin cells we assume that in lower concentrations OTA is inducing cell proliferation that could lead to skin tumor initiation and further carcinogenesis. This process is a consequence of OTA- induced oxidative stress and DNA damages (24). Higher doses, however, are much more cytotoxic, possibly due to potent additional interactions of OTA during ROS production. One possibility is the formation of DNA adducts. The known reactive positions within the OTA molecule are the C5-Cl atoms that bind directly to guanosine base in DNA thereby causing genotoxic injuries (7). Other toxic actions could be due to indirect induction of oxidative stress and apoptosis through its cascade activation (10). OTA can also disrupt phenylalanine metabolism that inhibits synthesis of cell widgets, reduces glyconeogenesis, and activates specific signaling pathways resulting in cell apoptosis (7, 10). There are also some reviews reporting about correlations between the exposure to OTA and the incidence of endemic neuropathy and urothelial neoplasia (2, 5, 11). Finally, there is not enough evidence to make definite conclusion about the hazardous/dangerous amounts of OTA, to which people can be dermally exposed. On the other hand, we currently have enough evidence about its dangerous effects to take care and to avoid it if possible.

4.1.2 The cytotoxicity of AFB_1

We found that ABF_1 is less cytotoxic for epidermal skin cells than OTA and STC (Figure 4B). Contrary to OTA and STC, AFB_1 at lower amounts did not induce cell proliferation. We could not confirm that AFB_1 acts in a dose independent manner. Its similar cytotoxic effects were found on Vero (kidney epithelial) cells that serve as a kind of standard in AFB_1 research (26). In our experiments the viability of the majority of HaCaT cells treated with AFB_1 was from 60-80%, not depending on the concentrations tested. The reason for this could be the expression of certain enzymes in the cell line. For example, CYP enzymes are detected in HaCaT cells which turn AFB_1 into epoxide, thereby preventing cytotoxicity of this MT, but then enhancing the mutagenicity (23). Higher levels of AFB_1 can damage cells intensively thereby inducing their death (in our case the cell viability was from 60 – 80%). The mechanism of AFB_1 action following its turnover by CYP is most probably binding to macromolecules and induction of ROS that in turn produce lipid peroxidation (LPO) which altogether leads to chromosomal damages. In cells, detoxication runs continuously via glutation-S-transferase (GST) activity. However, it was found that the GST system is present in skin in much smaller amounts than in e.g liver (25). Therefore, taking into account this finding, even if cytotoxicity of AFB_1 is not considered being extensive, that is when dermal exposure to lower amounts of this MT is constant, it can still be dangerous. Even when most of dermal cells are injured in such proportion or to such level that their recovery is still possible, the GST cannot remove reactive compounds completely. Consequently, considering the known accumulation of AFB_1 in lipophilic tissues, this may lead to its mutagenic and cancerogenic effects on cells.

4.1.3 The cytotoxicity of STC

We expected that the STC molecule, which is also a precursor of AFB_1 (Figure 5) will have similar cytotoxic potential as AFB_1 on HaCaT cells. However, also according to previous results obtained with human lung and esophageal epithelial cells, we found that STC has higher cytotoxic activity than AFB_1 (27) (Figure 4C). By applying same experimental conditions we showed a dose-dependent increase of cell mortality for STC comparing to AFB_1. At the concentration of 44.42 μM STC reduced cellular mitochondrial activity by 50%. At the cellular level it probably induces double strand breaks that inhibit DNA synthesis, thereby causing rather extensive cell death. On the other hand, the carcinogenic potential of STC assessed by EFSA is considered lower than that of AFB_1, which makes sense, because if cells die, they cannot stimulate growth of tumors anymore.

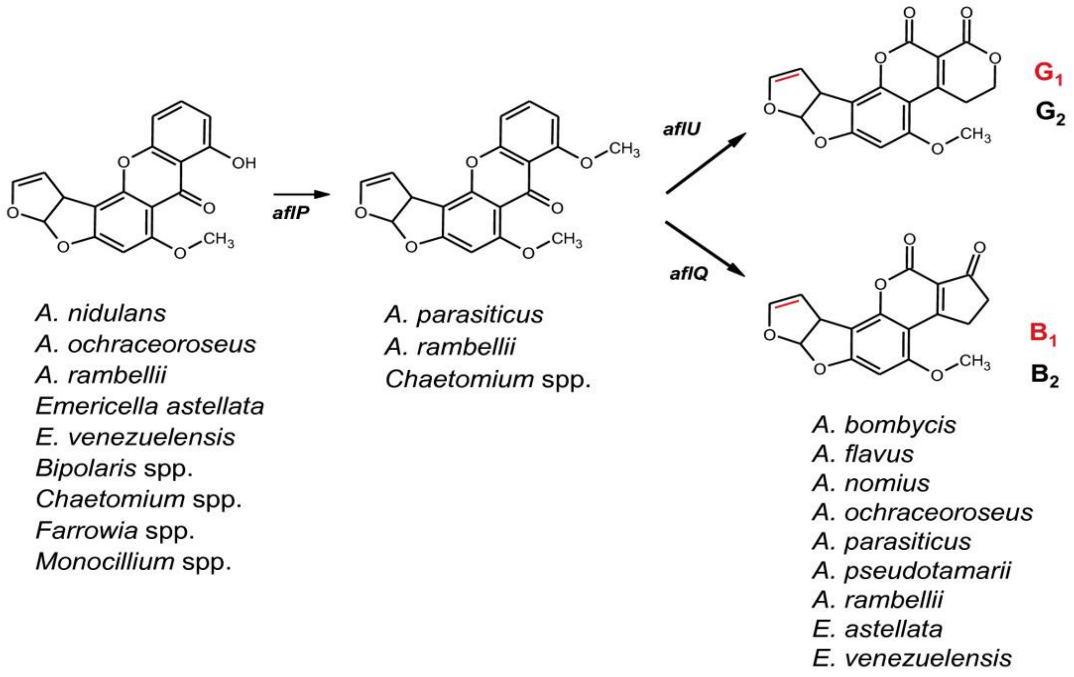

Figure 5: Transformation pathways of sterigmatocystin into various aflatoxins; adapted from (28).

In DDE-based scenarios for selected cosmetic products, the amounts of single mycotoxins that could be, in worst case, present in their ingredients are not significantly cytotoxic for skin. Besides that, some product's ingredients could have antioxidative properties which remove MT-induced reactive oxygen substances (ROS). Anyway, due to the fact that MTs

accumulate in skin layers and are suspected to exert carcinogenic activity, their continuous topical applications should better be avoided.

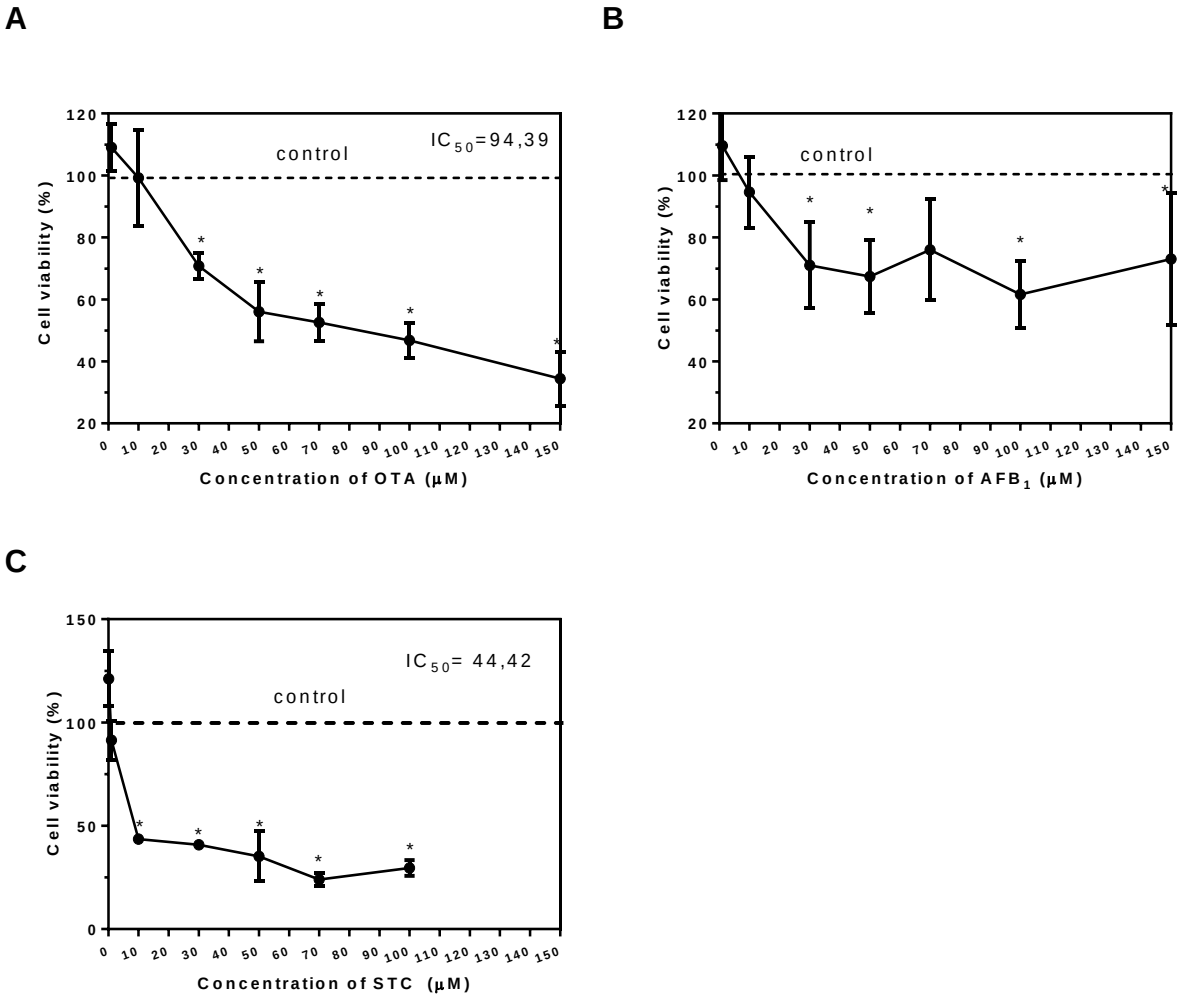

Figure 5: Cytotoxic effects on HaCaT cells after their 24 h exposure to: **A -** OTA (1, 10, 30, 50, 70, 100 and 150 µM); **B -** AFB_1 (1, 10, 30, 50, 70, 100 and 150 µM); **C -** STC (0.1, 1, 10, 30, 50, 70, 100 µM).

Data presented are expressed as mean percentage values regarding to controls ± SD. Control cultures were exposed to solvents without MTs (vehicle only), and their mean values considered as 100% cell viability, represented by dashed line. Asterisks (*) denote statistically significant differences ($p< 0,05$), as compared to control values. IC_{50} represents the concentration of a particular MT causing a 50% decline in metabolism (viability) of HaCaT cells, compared to control.

4.2 Cytotoxicity of tandemly combined MTs

4.2.1 AFB_1-OTA combinations

For AFB_1-OTA combinations we expected that their different quantitative relations would only have little proliferative or no effect. However, tandem mixtures containing 10 μM of OTA showed statistically significant synergistic effects in terms of decreasing cell viability, while those combinations that contained just a small concentration of OTA were not able to evoke significant cytotoxic effects (Figure 6A). AFB_1-OTA combinations have already been shown to have additive cytotoxic effects on Vero and HepG2 cells (7). On human epidermal HaCaT cells we observed the same effects only in tandem MT combinations with the smaller, 1 μM concentration of OTA. As already mentioned in AFB_1-OTA combinations, containing 10 μM of OTA, this particular MT synergistically interacted with AFB_1, resulting in a greater reduced cell metabolism than expected.

When we compared the effects of tested combinations of AFB_1 and OTA to those, measured for each individual toxin, we observed that only the presence of 10 μM OTA in mixtures caused synergistic cytotoxic effects, when compared to only OTA treatment. Also, regardless of the amount of AFB_1 used (1 or 10 μM), the difference was only significant when 10 μM OTA was present (Figure 7A). We can say that the main cytotoxic effect due to synergistic activation is because of the OTA presence. We have confirmed the hypothesis that AFB_1 and OTA compete for the same intracellular enzymes, and that in this competition OTA is obviously a winner, as its bioactivation is higher, and therefore its cytotoxic effects are stronger (7). When we tried to extrapolate from other findings whether such combinations have cancerogenic effects on skin, we were unsuccessful. For example it is known that combinations of OTA (1-20 μM) and AFB_1 (1-50 μM) induce more DNA damage, increase specific enzymes and decrease expression of anti-apoptotic factors in Vero cells (7). However, in contrast, OTA and AFB_1 were synergistically cytotoxic, and it was found that OTA reduces AFB_1-induced genetic changes in Hep G2 cells (7).

4.2.2 AFB_1-STC combinations

For AFB_1-STC tandem combinations, we expected that the cell mortality will be lower than predicted by summing up the individual cytotoxic effects of both individual MTs, because we hypothesised that STC can be bio-transformed to AFB_1 in skin cells. This MT however has no extensive cytotoxic effect in small doses. We expected that the effects of AFB_1-STC combinations will be similar to those obtained by 10-20 μM of AFB_1 (94-80% cell viability) (Figure 4B). Our tested combinations were found to have mostly antagonistic effects regarding decreasing cell viability (Figure 6B). AFB_1-STC combinations containing small concentrations of both MTs (1 + 1 μM) were expected to have small proliferative influence on cells but in reality it was quite high, so we can hypothesize that it poses a certain risk for carcinogenesis. We also observed statistically significant antagonistic effects of tandem combinations containing the higher of the two tested concentrations of STC, i.e. AFB_1-STC (1 + 10 μM; 10 + 10 μM), while in less concentrated mixtures (1 + 1 μM) both MTs exert kind of additive interactions. Because the combination AFB_1-STC (10 + 1 μM) did not induce statistically significant difference between the observed and expected values, as other combinations did, we conclude that by an unknown mechanism, STC is more important for the observed STC-AFB_1 interactions.

We found that mixtures containing the higher concentration of STC, caused a significantly lower decrease in cellular metabolic activity, as compared to appropriate concentrations of only AFB_1 and a significantly higher decrease compared to those of only STC (Figure 7B). The transformation of STC during 24 h probably goes on to a limited extent. Therefore, in mixtures with 10 μM of STC, more STC is left untransformed than in those starting with just 1 μM. This amount of untouched STC can then directly kill the cells. Comparing to single AFB_1, in combinations of two toxins, containing the smaller concentration of AFB_1 (1 μM), increased viability of cells was found out. We also suspect that during STC-AFB_1 treatment not only STC bio-transformation in HaCaT cells goes on, but also both types of MT molecules are able to interact between themselves. In mixtures containing small concentrations of both MTs, these amounts of AFB_1 and STC do not interact so easily and therefore they act more or less according to their own modes, Additionally, we must also take into account that a certain amount of STC is bio-transformed and therefore the results can be significantly different, but nevertheless shows a certain degree of antagonistic cytotoxicity. We suspect that even if the cytotoxic effect of this particular tandem MT

combination is decreased, there is still DNA damage present. Although it is not extensive enough to kill the damaged cells, it can be inherited and could lead to skin carcinomas.

4.2.3 OTA-STC combinations

We were aware from previous studies that STC alone acts totally different than OTA in causing cell apoptosis and necrosis. Now we know that also the combined cytotoxicity of OTA-STC mixtures (Figure 6C) is different from that of OTA-AFB_1 (Figure 7A). (When applied 1 μM + 1 μM combination, cytotoxic effects of OTA and STC are additive and they do not deviate from effects detected with these toxins applied alone. Other OTA-STC combinations act hardly synergistic, as compared to OTA alone (Figure 7C). However, when compared to only STC treatment, certain OTA-STC mixtures (10 + 10 μM and 10 +1 μM) caused decreased HaCaT cell viability, while the others (1 + 1 μM and 1 + 10 μM) were able to increase it. When comparing the expected and the actual measured results, we observed antagonistic effects with the OTA-STC mixture containing the lower tested concentration of OTA, while tandem MT combinations (10 μM OTA + 10 μM STC and 1μM OTA +1 μM STC) evoked synergistic cytotoxicity (Figure 6C).

When taking into account only percentages of HaCaT epidermal cell survival, we can conclude that the tandem combinations of OTA- STC showed the highest cytotoxic hazard for skin, followed by the mixtures of AFB_1-STC and the less cytotoxic combinations of AFB_1-OTA, However, we are well aware of the fact that these limited findings do not show a complete danger that MTs pose to the human skin.

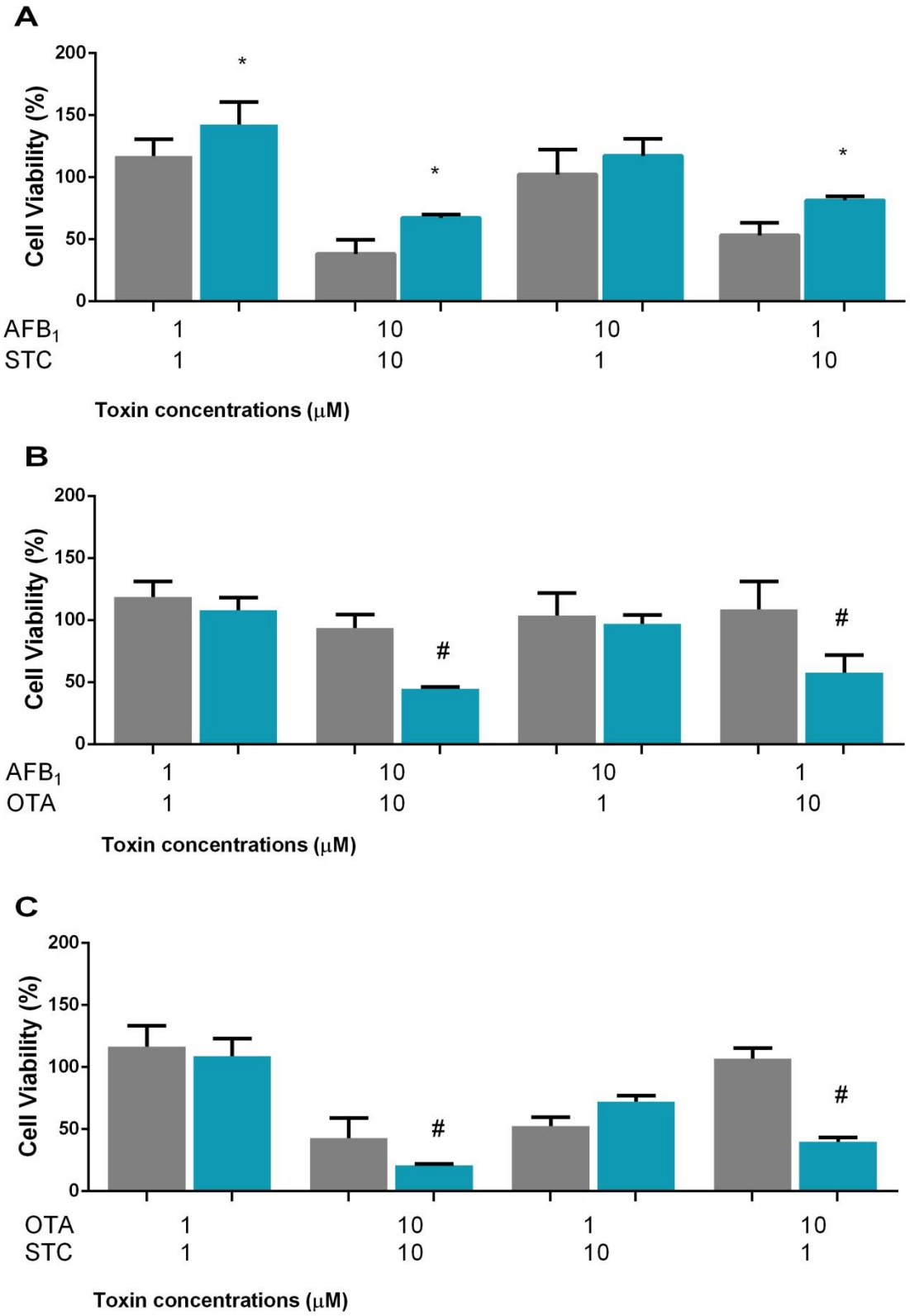

Figure 6: Cell viability of HaCaT cells following their 24 h *in vitro* treatment with different tandem MT mixtures in variously combined concentrations. The grey bars represent expected, and the blue bars measured values. Asterisks (*) denote statistically significant antagonistic

differences of measured values in comparison to expected values, while the # sign denotes statistically significant synergistic effects of measured values in comparison expected values (both, $p \leq 0,05$). The unsigned bars show no significant differences between measured and expected values, which mean that in this case the MT effects are mostly additive.

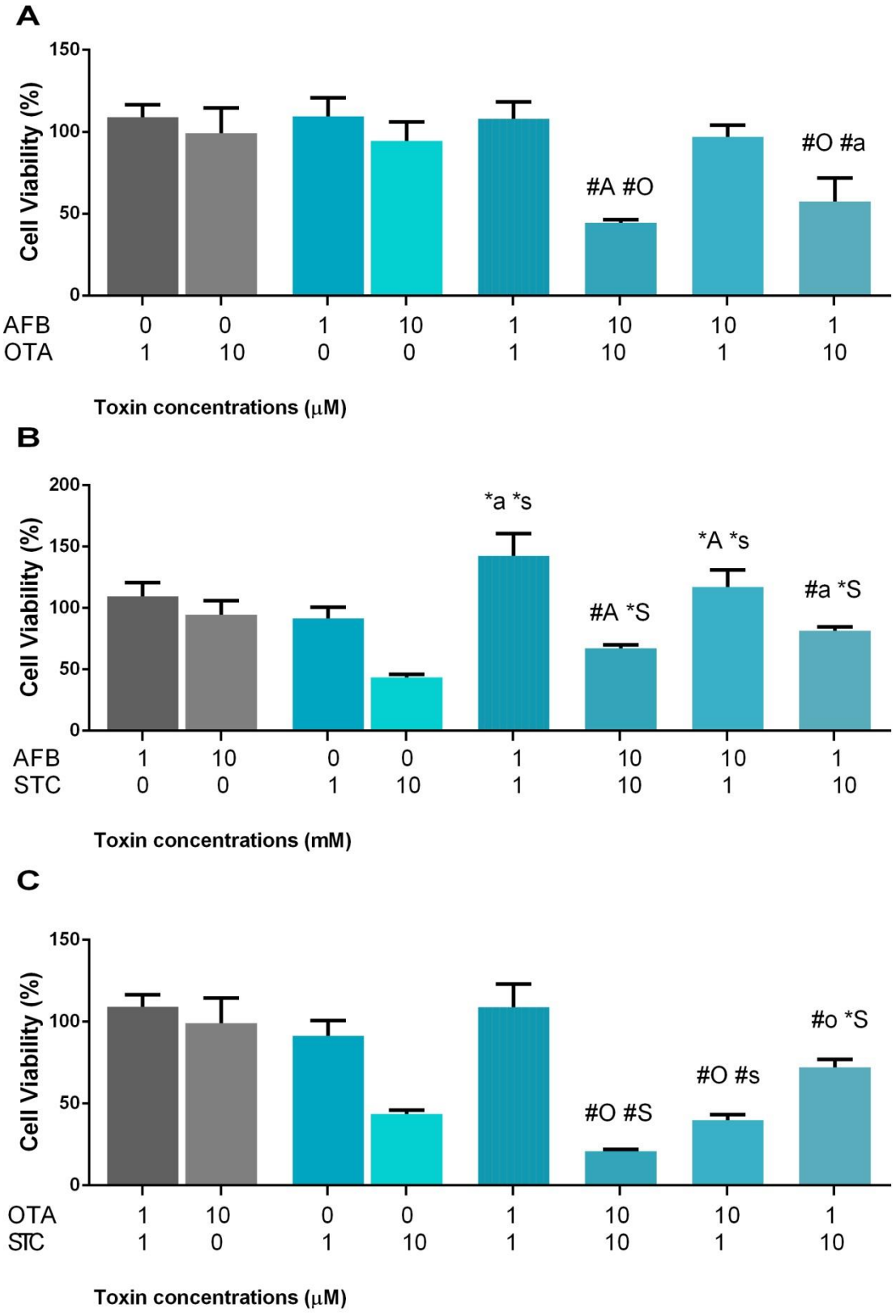

Figure 7: The measured cytotoxic effects of single MTs on HaCaT cells compared to those, evoked by different tandem MT combinations, containing that particular MT. Bars show percentage of cell viability following 24 h of MT treatment. The # sign denotes

statistically significant synergistic, while the asterisk (*) represents statistically significant antagonistic effects in comparison to single toxin cytotoxic potential ($p \leq 0,05$). Combined signs * or # with O denote tandem MT combinations containing 10 μM of OTA with statistically significant antagonistic or synergistic effects in comparison to those obtained by the same concentration of OTA only treatment. The sign o represents 1 μM of OTA, the sign A 10 μM of AFB_1, the sign a 1 μM of AFB_1, the sign S 10 μM of STC and the sign s 1 μM of STC.

5. CONCLUSION

AFB_1, OTA and STC are widespread, commonly in co-occurring MTs with cytotoxic and mutagenic potential to which we can be dermally exposed. The aim of our work was to assess their single and tandemly-combined *in vitro* cytotoxic effects on human HaCaT epidermal cell line. While determination of toxic effects and mechanisms of actions for single MTs can be rather easily determined, this is far more complex and demanding in case of their various combinations. These however should be explored as it is clear that MTs can either naturally coexist in plants where they are produced by different molds, but can also be mixed together during manufacturing of natural raw substances. Due to their different chemical structures, these toxic molecules not only impact the cells they come in contact in different ways, but they also interact between themselves. Therefore we decided to explore also the cytotoxic effects of various tandem mixtures of AFB_1, OTA and STC, combined in different concentrations (1 and 10 μM). We observed that cytotoxic effects of these mixtures deviate from the expected ones that we have calculated by simply summing up the effects of single MTs. We found that the cytotoxic hazard for skin exposure to single MTs is as follows: STC > OTA > AFB_1. However taking into account current regulations such single MT cytotoxicity regarding dermal exposure is not too problematic.

When assessing their tandemly combined effects, however, we noticed that they mostly exerted synergistic cytotoxic potential. In small concentrations all tested MT combinations showed cell proliferation effects. The hazardous effects of all other tandem mixtures were dependent on the combination of the two mixed MTs, and were found to be as folows: OTA-STC > AFB_1-STC > AFB_1-OTA. However if we would like to determine the total toxicity of these combinations, we should look also to other effects not only just their cytotoxic potential. When exposure to MTs is constant we must pay more attention to their single and combined genotoxic effects, as well as mechanisms of interactions. This should be investigated in future, as it is very plausible that the results of such approach will completely change our view regarding the hazards of dermal exposure to mycotoxins. Within this concept it would be quite appropriate to start testing natural ingredients of various cosmetic preparations for at least the most common toxins.

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