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PREUČEVANJE VPLIVA AVOBENZONA NA EKSPRESIJO RACK1 IN SPROŠČANJE CITOKINOV IZ MONOCITNIH CELIC THP-1

THE INFLUENCE OF AVOBENZONE ON RACK1 EXPRESSION AND CYTOKINE RELEASE FROM THP-1 MONOCYTE CELLS

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STATEMENT

I hereby declare that this Master's thesis was done by me under supervision of the mentor prof. dr. Marija Sollner Dolenc and the co-mentor prof. dr. Emanuela Corsini.

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ABSTRACT

Avobenzone, a derivative of dibenzoylmethane, is an organic ultraviolet filter widely used in personal care products such as sunscreens and it is also found as a contaminant in the environment. Extremely worrying is the fact that it may possess endocrine disrupting properties. Endocrine disrupters are defined as exogenous substances with the ability of action on the hormonal system, where they cause side effects, affecting organism by itself or its progeny. Studies have already stated that avobenzone has affinity towards binding to hormone receptors and discovered estrogenic, anti-androgen and glucocorticoid activities. As the immune system is indirectly regulated via hormones, this raises a suspicion that it is also affected by avobenzone. The immune system is crucial to the subject defence and survival; any malfunction may have fatal consequences.

Investigating avobenzone effects on the immune system, *in vitro* testing was performed on the monocyte cell line THP-1 in operating range concentrations from 2 pM to 2 nM. Cell viability was assessed using LDH leaking. Potential immune modulation was inspected through RACK1 production and cytokines release. RACK1 is an anchoring protein which activates PKC $ß_{II}$, a kinase included in numerous signalling pathways of the immune cells. It is encoded by GNB2L1 gene, the expression of which was investigated using polymerase chain reaction. Moreover, Western blot methods were applied to assess RACK1 protein production. Messenger molecules of the immune cells, TNF-α and IL-8, were examined with LPS-induced cytokine release followed by ELISA assay. In the research avobenzone did not affect cell viability. The decrease of RACK1 gene and protein expression was modest, inhibited for about 10 – 20% of the production following avobenzone exposure. The cytokines displayed an 18 – 41% reduction compared to the control in TNF-α samples and an about 17 – 35% reduction in IL-8 samples. The results were statistically significant, and we can therefore conclude that avobenzone affects RACK1 production, as well as LPS-induced TNF-α and IL-8 release. Reduction of these responses displayed that avobenzone causes suppression of the immune system. It is reasonable to speculate that these pathways are not only mechanisms involved in AVB-induced immune modulation. To fully confirm the antagonistic effect and to accurately determine the mechanism of action, additional *in vitro* and *in vivo* studies in accordance with the OECD principles are required. To complete the risk assessment of avobenzone, the level of exposure should be defined more precisely as well as joint effects along with the substances in environmental mixtures.

Key words: avobenzone, endocrine disrupting chemicals; THP-1 monocyte cell line, RACK1, cytokines

EXTENDED ABSTRACT IN SLOVENE

Razširjen povzetek v slovenskem jeziku

Napredek človeštva je poleg številnih izumov v vsakdanje življenje prinesel tudi nove produkte in kemikalije. Zaskrbljujoče je dejstvo, da nekatere izmed njih vplivajo na endokrini sistem ter druga tkiva, ki so regulirana preko hormonov. V splošnem bi hormonski motilec opredelili kot telesu tujo snov ali spojino s sposobnostjo delovanja na hormonski sistem, kjer lahko povzroči stranske učinke, ki vplivajo na sam organizem ali njegovo potomstvo. Hormonski motilci so strukturno zelo raznolika skupina. So relativno majhne molekule, ki pogosto vsebujejo halogene skupine ter fenolni skelet, ki oponaša zgradbo steroidnih hormonov. Sem sodijo številne kemikalije, ki jih uporabljamo v proizvodnji plastike, kot industrijska topila, barve, zdravila, pesticide in fungicide, pa tudi naravne snovi in spojine, na primer fitoestrogeni. Motijo lahko sintezo hormona, njegovo sproščanje, delovanje, metabolizem ter izločanje. Drug način delovanja je tekmovanje za receptorska mesta in vpliv na kompleks hormon-receptor. Najbolj ranljivo obdobje je čas razvoja zarodka ter zgodnje otroštvo, ko se tkiva šele razvijajo ter lahko že kratkotrajna izpostavljenost pusti trajne posledice. Ljudje pridemo v stik z endokrinimi motilci prek potrošniških dobrin, kontaminiranega zraka in vode. Izpostavljene so jim tudi živali, zlasti (ob)vodni organizmi, pri katerih so prav tako zaznali vpliv okoljskih onesnaževal na njihov razvoj, reprodukcijski ter imunski sistem. Tudi v produktih za osebno nego najdemo učinkovine s sumom na hormonsko delovanje, primer so ultravijolični filtri v sončnih kremah, ki naj bi ščitili pred negativnimi vplivi sonca. Sem sodi tudi avobenzon, derivat dibenzoil metana, ki ga kot organski filter uporabljamo za zaščito pred UVA sevanjem. V predhodnih raziskavah so že odkrili afiniteto do vezave na hormonske receptorje ter določili estrogeno, antiandrogeno ter glukokortikoidno delovanje. Zaradi teh lastnosti se pojavlja sum vpliva na imunski sistem, ki je posredno reguliran preko hormonov, in katerega smo se odločili preveriti.

Imunski sistem je mreža organov, tkiv in celic, katerih skupni namen je obramba organizma pred vdorom patogenov. Je ključnega pomena za preživetje osebka, njegovo nepravilno delovanje ima lahko usodne posledice, bodisi zaradi nezadostnega odziva na infekcije ali pa preveč burnega odziva, ki se kaže kot alergije in avtoimune bolezni. Delimo ga na naravni, ki deluje nespecifično, ter pridobljeni imunski odziv, ki je sicer počasnejši, vendar specifičen ter ohrani imunski spomin na vsiljivca. Celice imunskega sistema se medsebojno sporazumevajo in uravnavajo delovanje preko sproščanja signalnih molekul, imenovanih citokini. Pomembno vlogo pri prenosu informacij imajo tudi kinaze C, ki so vključene v številne signalne poti celic

imunskega ter drugih sistemov. Protein kinaza PKC $ß_{II}$ ima poseben pomen pri imunskem odzivu, diferenciaciji celic ter signalnih poteh za produkcijo citokinov. Aktivacijo usmerja receptor za aktivacijo C-kinaze 1 (RACK1), sidrajoči protein, ki stabilizira njeno aktivno konformacijo in stimulira prenos bližje k substratom.

Učinek avobenzona na delovanje imunskega sistema smo raziskovali prek citokinov TNF-α in IL-8, ter nastajanja RACK1 proteina. *In vitro* testiranja smo izvedli na humani monocitni celični liniji THP-1. Pri načrtu dela smo se oprli na predhodne raziskave in izhajali iz koncentracij, ki so sprožile polovico maksimalnega odziva na hormonskih receptorjih. Tako smo se odločili za uporabo koncentracij v obsegu od 2 pM do 2 nM. Glede na predhodne teste in nizke koncentracije spojine, ki smo jih želeli uporabiti, smo sklepali, da avobenzon v danih koncentracijah (2 pM, 20 pM, 200 pM ter 2 nM) ne bo citotoksično vplival na THP-1 celice, vendar smo se dano hipotezo odločili preveriti z LDH testom, kjer smo izpostavljene celice primerjali s kontrolnim vzorcem. Test temelji na kolorimetrijski zaznavi količine laktat-dehidrogenaze, ki se v medij sprošča iz celic s poškodovano membrano. Rezultati so potrdili našo domnevo, saj nobena od meritev živosti celic ni bila značilno drugačna od kontrole. Nadalje smo celicam, izpostavljenim avobenzonu, ugotavljali nivo sproščanja RACK1 proteina ter ekspresije gena GNB2L1, ki ta protein kodira. Avobenzon je pokazal v predhodni raziskavi na Fakulteti za farmacijo v Ljubljani glukokortikoidno ter antiandrogeno delovanje, iz česar smo sklepali, da bo imel na imunski sistem zaviralne učinke. To bi v preiskavi zaznali kot nižjo stopnjo izražanja RACK1. Po izpostavitvi THP-1 celic avobenzonu smo iz vzorcev izolirali RNA. Ekspresijo gena GNB2L1 smo proučevali preko verižne reakcije s polimerazo, kjer smo v prvem koraku nestabilno in občutljivo mRNA z retrotranskripcijo pretvorili v bolj stabilno cDNA molekulo. Količino izražanja gena za RACK1 smo ugotovili preko polimerazne verižne reakcije v realnem času, jo normalizirali z endogeno kontrolo, ribosomalno RNA 18S, ter izrazili kot relativno koncentracijo glede na kontrolni vzorec. Tudi za ugotavljanje nastajanja proteina smo celice izpostavili avobenzonu, nato pa iz celične suspenzije izolirali proteine. Prek Bradfordove metode smo ugotovili celokupno koncentracijo proteinov v vzorcih, da smo jih lahko v enaki količini nanesli na acetilamidni gel ter izvedli gelsko elektroforezo, kjer so se proteini ločili na podlagi velikosti. Proteine smo prenesli iz gela na membrano in jo inkubirali s specifičnimi protitelesi za ugotavljanje proteina RACK1 ter ß-aktina kot endogene kontrole za normalizacijo rezultata. Proteine smo zaznali preko kemiluminiscentne reakcije ter dobljene rezultate denzitometrijsko analizirali in jih predstavili kot relativno koncentracijo glede na kontrolo. Vse omenjene postopke smo izvedli v treh neodvisnih ponovitvah in za statistični izračun uporabili Dunnettov test večkratne primerjave.

Avobenzon je v testih znižal izražanje gena GNB2L1 v obsegu 9 – 20% glede na kontrolo. Tudi pri meritvah produkcije proteina RACK1 smo prišli do podobnih rezultatov, in sicer je zavrl nastajanje v obsegu od 13 do 21%. Zadnji del eksperimentov je obsegal ugotavljanje sproščanja proinflamatornih citokinov TNF-α in IL-8. V celični kulturi smo z lipopolisaharidom sprožili imunski odziv nato pa preiskovali, če je avobenzon v vzorcih zavrl sproščanje citokinov glede na kontrolo, kjer je bil prisoten le lipopolisaharid. Meritve smo opravili s specifičnimi ELISA testi ter predstavili kot relativne vrednosti glede na kontrolo. Vse eksperimente smo izvedli v treh neodvisnih ponovitvah ter statistično analizirali z Dunnettovim testom. Pri testih sproščanja citokinov se je zaviranje imunskega odziva izrazilo v večjem merilu, z značilnimi rezultati. Sproščanje citokina TNF-α se je zmanjšalo v obsegu od 20 do 40% glede na kontrolo; višje koncentracije avobenzona so bolj zavrle produkcijo citokina. Enake rezultate smo dobili tudi pri IL-8, kjer se je njegovo sproščanje zmanjšalo v obsegu 20 – 35% glede na kontrolo.

Na podlagi rezultatov testiranj lahko zaključimo, da avobenzon v danih koncentracijah ne deluje citotoksično na THP-1 celice. Produkcijo gena ter proteina RACK1 zmerno, vendar statistično značilno zavira. Prav tako tudi statistično značilno zavira s stimulusom izzvano sproščanje citokinov TNF-α in IL-8, iz česar lahko sklepamo, da avobenzon modulira imunski sistem z antagonističnim učinkom na njegovo delovanje.

Ob pregledu literature smo ugotovili, da smo preko okolja posredno najverjetneje izpostavljeni pM ter nM koncentracijam, ki smo jih v preiskavi tudi sami uporabili. Absorpcija avobenzona preko kože je glede na *in vitro* ter *in vivo* raziskave dokaj majhna, večina se ga absorbira v zgornjo plast kože, v dermisu ter receptorski tekočini/serumu pa ga niso zaznali. So pa opazili sposobnost bioakumulacije v maščobnem tkivu rib, kar pripisujejo njegovi lipofilni naravi. To nakazuje na sposobnost prehajanja in biokoncentracije preko prehranjevalne verige.

Avobenzon je izkazal antagonistično delovanje na RACK1, TNF-α ter IL-8, sklepamo pa lahko, da ima verjetno vpliv tudi na druge komponente imunskega sistema.. Da bi potrdili antagonističen učinek na sesalski imunski sistem ter točno ugotovili mehanizme delovanja, bi bile nujne dodatne *in vitro* ter *in vivo* raziskave v skladu z OECD načeli. Za popolno oceno tveganja bi morali natančneje ugotoviti nivo izpostavljenosti v okolju ter oceniti njegov učinek na organizem v kombinaciji z drugimi hormonskimi motilci v okolju, da bi oceno čim bolj približali realnemu stanju.

Ključne besede: avobenzon, hormonskimi motilci, monocitna THP-1 celična linija, RACK1, citokini

ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
APC	Antigen presenting cell
APS	Ammonium persulfate
AR	Androgen receptor
AVB	Avobenzone
BSA	Bovine serum albumin
CK	Cytokine
Ct	Threshold cycle
DAG	Diacilglycerol
DC	Dendritic cell
DDT	Dichlorodiphenyltrichloroethane
DEPC water	Diethylpyrocarbonate water
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
dT	Deoxythymine primer
EC_{50}	Half maximal effective concentration
ED	Endocrine disruptor
EDC	Endocrine disrupting chemical
ER	Estrogen receptor
FCS/FBS	Fetal calf/bovine serum
FDA	Food and Drug Administration
GM-CFS	Granulocyte macrophage colony-stimulating factor
GNB2L1	Guanine nucleotide-binding protein subunit beta-2-like 1
GR	Glucocorticoid receptor
HRP	Horseradish peroxide
IC_{50}	Half maximal inhibitory concentration
IFN-α	Interferon alpha
Ig	Immunoglobulin
IL	Interleukin
IPSC	International Programme of Chemical Safety
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

n/a	Not applicable
MHC	Major histocompatibility
NK cell	Natural killer cell
OECD	The Organisation for Economic Co-operation and Development
PBB	Polybrominated biphenyl
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PPCP	Pharmaceutical and Personal Care Products
PVDF	Polyvinylidene difluoride
qPCR	Real-time polymerase chain reaction
QSAR	Quantitative structure–activity relationship
RACK1	Receptor for activated C-kinase 1
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Retrotranscription polymerase chain reaction
SDS	Sodium dodecyle sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor α
TR	Thyroid receptor
T_C or CD8+	Cytotoxic T cell
T_H or CD4+	Helper T cell
WB	Western blot
WWTP	Wastewater-treatment plant

1 INTRODUCTION

1.1 ENDOCRINE DISRUPTING CHEMICALS (EDCs)

1.1.1 Classification

The progress of humanity beside a number of inventions in everyday life has also brought numerous new products and chemicals. A worrying fact is that they encompass contaminants which adversely affect human and animal health. Substances with the ability to interfere with the endocrine system, called endocrine disrupting compounds (EDCs), are a highly important member of this group. (1) (2) (3) More specifically, according to the International Programme of Chemical Safety (IPCS), an endocrine disruptor (ED) is each molecule defined as »an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.« (1) (4)

Endocrine disrupting compounds are a very heterogeneous group. It includes many synthetic chemicals, which are used as plastics (bisphenol A), plasticizers (phthalates), industrial solvents, paints, lubricants and their byproducts (PCBs, PBBs, dioxins), pharmaceuticals (contraceptives, diethylstilbestrol), pesticides (DDT) and fungicides (vinclozolin). Natural chemicals such as phytoestrogens, extracts from various plants or fungi also act as endocrine disruptors. (3) (5) (6) EDCs are very diverse and do not share any structural similarity, except for their small molecular mass (< 1000 Daltons). In broader terms, they often contain halogen group substitutions (e.g. Cl, Br) and have phenolic moiety, which is suspected to mimic natural steroid hormones. Thus, it is very difficult to predict if the compound may possess endocrine-disrupting features only on the basis of its structure. (5)

1.1.2 Basics of the endocrine system, EDCs action and humans' health

In a human body there are numerous hormones and hormone-related molecules (i.e. neurotransmitters and cytokines) which communicate between tissues and organs in order to regulate physiological and behavioural activities. A hormone is a signal molecule produced by an endocrine gland that travels through the circulatory system to distant cells where it produces effects via signalling pathways usually involving a hormone receptor. (1) EDCs can affect a specific protein that controls a part of hormone synthesis, release, transport, effect, metabolism and excretion. On the other hand, some of these natural substances and xenobiotics disrupt the hormone action by interfering the hormone-receptor complex (Figure 1). (1) (4)

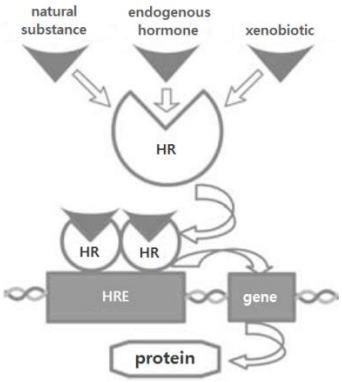

Figure 1: Competition for the receptor binding. The ligand-receptor complex interacts with the hormone responsive element (HRE) which through the response gene activates a hormone-dependent production of proteins. (1) (4)

Receptor-binding compounds arouse a cascade of events regulated by the receptor. The competing compounds which imitate the hormone effect are called agonists; those which block the receptor are antagonists. The prosperity of ligand replacement on the receptor depends on their affinity and concentration. (4) EDs may also interfere with signalling cascades after steroid-binding and co-activators or co-repressors at the initiation of DNA transcription. (3) Diverse actions of EDCs likely include all hormonal systems, from those that control reproductive organs to the ones which regulate various tissues of other systems. (1) Therefore, beside the reproductive system, EDCs affect the adrenal glands, the cardiovascular system, the liver, the kidneys, the central nervous system, the immune system and the bones (Figure 2). (4)

Hormone action can be affected any time during the lifetime. Especially vulnerable are the embryo, fetus, infancy periods, the early childhood, the puberty and the menopause. The exposure timing is the key factor to set up a human disease. In contrast to the reversible changes in adults, in the young age, when structural and functional events are most sensitive and rapid, even a transient exposure to EDCs can cause permanent consequences that manifest during growing-up and later in life, or even after generations. (1) (2) (5) (7)

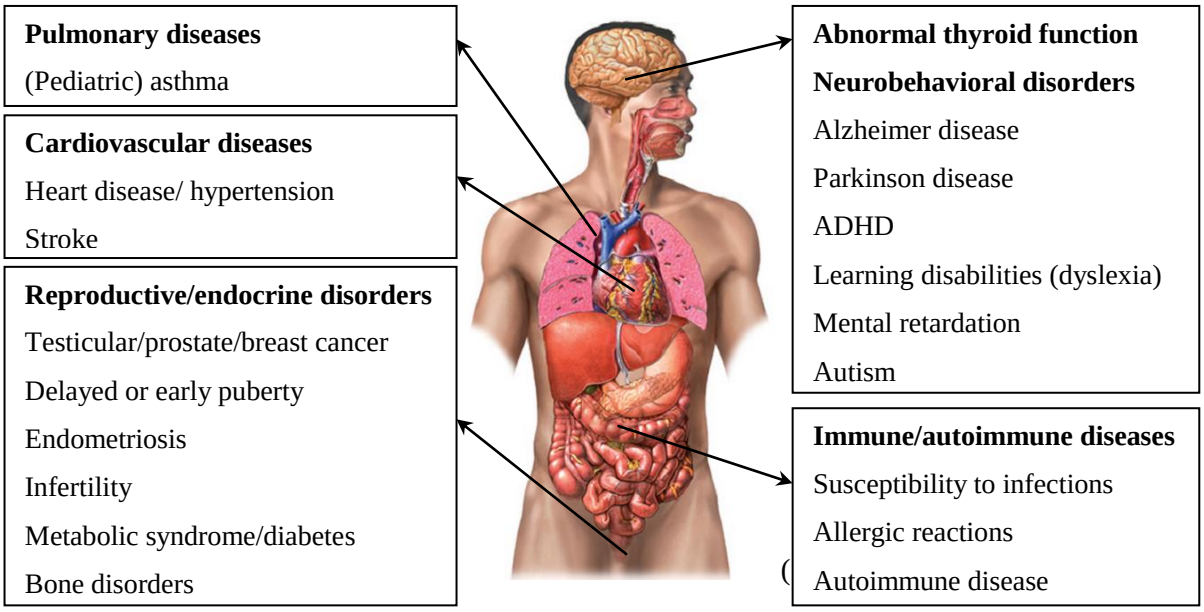

Figure 2: Diseases induced by being exposed to EDCs during development, evidence based on animal models and human studies (1) (5) (8)

1.1.3 Human and wildlife exposure to endocrine disrupting chemicals

The human population is exposed to EDCs through water, air, food, personal care products and a variety of consumer products. Measurements of multiple environment contaminant levels in bodies of people from different countries manifested that all humans are exposed to a certain level of synthetic chemicals. (9) Endocrine disruptors are released in the environment through emissions to the atmosphere, rivers, lakes and oceans, and through the sewage system with treated or untreated wastewaters. Infants and children can suffer a greater exposure to ECDs due to hand-to-mouth activities and breast and formula feeding, where a higher concentration was observed. (1) (5) EDCs in wildlife and humans vary by location; they are higher in urban areas or near industrial areas, but they can even be found at remote places. Many parallels between the increasing incidence of some human diseases compared to the wildlife have been found while monitoring the populations. Numerous observations indicate that chemical exposure considerably deteriorates the health of wildlife species. Mechanisms underlying these pathological changes and final health outcomes with animals are often quite similar to those with humans. (1)

1.1.4 Important issues of mechanisms of EDCs action

When evaluating risk assessment, some key characteristics should be considered. First, as described above, ED exposure is **timing and life stage** dependent, with the most devastating consequences at the developmental exposure. (2) Endogenous hormones act at extremely low concentrations, typically in nanomolar to picomolar range, or even lower. EDCs can act at the same low ranges as well. Furthermore, they indicate **low-dose effects**, which cannot be predicted regarding to the effects at higher doses and can be even more potent. (10) Another concept in EDCs studies are **nonmonotonic dose-response curves**, defined as nonlinear dependence between the dose and the effect, where the slope of the response curve changes direction at least at one point in the tested dose range and creates an inverted U or U-shaped curve. (2) (5) (10) Moreover, a single chemical can have **multiple mechanisms of action** or even **epigenetic effects**. (5) (9) Humans and wildlife are usually exposed to multiple environmental contaminants. **The mixtures** can influence on a disease through different mechanisms of action and may have additive or synergistic effects, arousing action even if each chemical is present in concentrations below their individual limits to induce effect. (2) (5) Due to their lipophilicity they can undergo **bioaccumulation** in adipose tissues and move up through the food chain. Moreover, EDCs may not have impact only on the exposed individual, but also further generations (**transgenerational effect**). (5)

1.1.5 OECD test strategies to assess human hazard

Due to their threats in human and wildlife health, scientists have taken a closer look to assess EDCs health outcome. EU, OECD, the USA and Japan have established testing programs and approaches to assess the safety of chemicals with potential endocrine disruption properties. (11) Specific guidelines need to be settled for a systematic organization and analyses of chemicals that may impose risk. Testing strategies usually involve four stages: hazard characterization, exposure assessment, dose-response assessment and risk characterization. (12) The choice of appropriate test approaches and their development to be able to identify and characterize possible endocrine disruptors is complicated due to their atypical characteristics as nonmonotonic responses, numerous mechanisms producing various responses, very low doses, etc. (13)

Table 1: OECD framework approach for the assessment and testing of EDCs (11) (14)

Level		Examples of toxicology methods
1	Sorting and prioritisation • Using existing data	Physical and chemical properties (MW, stability, reactivity, biodegradability) Environmental/human exposure (release, use patterns) Toxicological data about possible hazard
2	*In vitro* screening assays • Providing mechanistic data, structure activity relationship	Receptor binding affinity (ER, AR, GR, TR) Aromatase/Steroidgenesis (*in vitro*) Fish hepatocyte vitellogenin assay (estrogenic) Activation of transcription QSARs High throughput prescreens
3	*In vivo* assays • Providing data about single, specific endocrine effects	Uterotrophic assay, fish vitellogenin assay (estrogenic) Hershberger assay (androgenic) Non-receptor mediated hormone effects
4	*In vivo* assays • Providing data about multiple endocrine effects	Enhanced OECD 407 (endocrine mechanisms) Pubertal assay (male/female), adult intact male assay Fish gonadal histopathological assay Frog metamorphosis assay
5	*In vivo* assays • Providing data of effects from endocrine and other mechanisms	1-/2-generation mammalian assay Reproductive screening Partial/full life cycle assay – development and reproduction (fish, amphibians, birds, invertebrates)

1.2 ULTRAVIOLET FILTERS

Pharmaceutical and Personal Care Products (PPCPs) are one of the contaminant groups we are exposed to via their use or as pollutants in the environment. (15) (16) Finding indicate that some UV filters also have a potential of endocrine-disrupting properties and possess an ability to affect reproduction of several species. Although concentrations, measured in the environment are quite low, multicomponent mixtures with additive or synergetic effects should be considered. (17) (18) (19)

The growth of public awareness of negative UV radiation consequences, such as sunburns, photo-ageing and skin cancer, have increased the usage of UV protection products containing UV filters. UVA (315 – 400 nm) and UVB (280 – 315 nm) radiation can reach the Earth surface, while UVC (200- 280 nm) is absorbed by the ozone in the stratosphere. Therefore, UV filters are designed to protect us against UVA and UVB ranges. Depending on their mechanism of action they are divided into organic (chemical) absorbers and inorganic (physical) blockers. Organic UV filters absorb UV radiation and pass it to an excited state of molecule, while inorganic filters reflect and scatter UV radiation. (17) (20) (21) Beside extensive UV filters use in cosmetic products like sunscreen lotions and facial makeup for many decades, they are also incorporated in a number of industrial products, including plastics, rubber, paint, in order to minimize the effects of light. (20) (22)

In the European Union UV filters currently allowed in cosmetic products are defined in the Regulation (EC) No. 1223/2009 (Annex VI, List of UV filters allowed in cosmetic products). The list contains the substance identification and the maximum concentration of ready-to-use preparations for each filter. (23)

The chemical structure of organic UV filters typically contains an aromatic moiety along with an unsaturated side-chain. Because many of them have a high lipophilicity (log K_{ow} 4 - 8) and a poor biodegradability, UV filters can persist in the environment and accumulate in the sediments and biota. (17) (18) They find a way into the environment through industrial wastewaters, landfills, bathing/swimming venues, sewage networks and the discharge from wastewater-treatment plants (WWTPs). (18) Moreover, *in vitro* and *in vivo* studies have presented, that certain UV filters have the ability to absorb through human skin following topical application. (24) *In vitro* percutaneous absorption research shows different results, depending on the UV filter, the skin model and the analytical technique. In the human skin they absorb in the range 0 - 5.8% of the applied dose and in the animal models from 0 to 7.6%. (25)

1.3 AVOBENZONE

1.3.1 Use and properties

An ideal sunscreen should provide protection against UVA and UVB light ranges. (26) While a broad choice of UVB filters can be found on the market, truly appropriate UVA filters are rare. Avobenzone (AVB) is one of the most efficient UVA filters, present in numerous skin care products. (27) Chemically it belongs to the group of dibenzoylmethane derivatives with a conjugated π-electron system (cromophore), which is responsible for the absorption of UV radiation. (26) (20) However, its main drawback is through the UV irradiation caused photodegradation and photoisomerization, resulting in a loss of UVA protecting features. (27) (28) In 1978, it was approved for use in Europe and with a ten-year delay also in the United States (by the US FDA). The maximum permitted concentration of AVB in sunscreens is 5% in Europe and Australia, 3% in the USA and 10% in Japan. (26)

In solutions, it exists in an equilibrium mixture of enol- and keto-tautumeric form. Because of higher stability, it dominates in chelated enol-form, which absorbs in the UVA region with wavelengths ranging from 350 to 365 nm. Non-chelated enol-forms are also present, but they have a transient existence (Appendix, Figure 21). After exposure to UV light, it extensively converts to ß-diketone tautomer, which absorbs in the UVC region (260 - 280 nm), and acceleratedly degrades. Its photostability mechanisms also highly depend on the polarity and proticity of the solvent and on the presence of other solutes. (29) (30)

(More avobenzone properties are presented in Appendix, Table 12.)

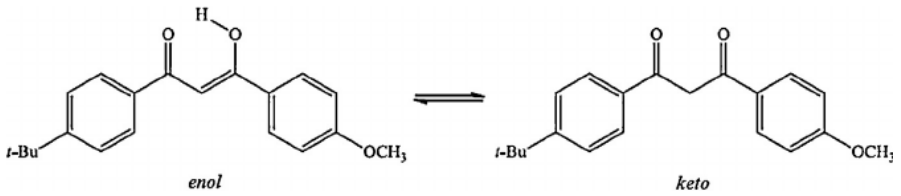

Figure 3: Keto-enol tautomerism (31)

1.3.2 Toxicokinetics and the system exposure

Photochemical intermediates or stable photoproducts can display photosensitizing effects. AVB interactions with skin components and phototoxic abilities have been evidenced through cytotoxicity to human keratinocytes, oxidative modification of albumin, lipid peroxidation and damages of plasmid DNA strands. (27) Decreasing effectiveness, phototoxicity and photoallergy reactions have been the reason to incorporate stabilizers like octocrylene. They quench the diketone triplet state and prevent photofragmentation due to Norrish Type I reaction. (27) (32)

Considering that AVB and other UV filters are extensively used and applied to skin, it is essential to study their transformation products and the penetration through the skin. (20) As numerous research have presented, AVB is persistent enough to enter into ecosystems where it can be mostly detected in ng/L ranges, depending on the area, the season, the population density and the analytical method sensitivity (Appendix, Table 14). (19) (20) (22) (30) Avobenzone is susceptible to sunlight, its degradation also happens in chlorine media, such as swimming pools. In a typical disinfection process of aquatic chlorination and UV-C irradiation, 25 transformation by-products were identified (Appendix, Figure 23). The toxicity of the majority of these products remains unknown, whereas some are known to have toxic properties. (30)

1.3.3 Toxicodynamics of avobenzone

Avobenzone is suspected to act as an endocrine disruptor to various hormonal systems and some researchers have already investigated its interference to different hormone receptors.

1.3.3.1 Endocrine disruption

Estrogenic activity following exposure to avobenzone was investigated through *in vitro* and *in vivo* studies. At first, they tested AVB in a concentration range 0.1 – 100 μM on HEK293 cell line, which expresses receptors hERα and hERβ separately. It displayed some estrogenic activity on both receptors, but the response was quite weak. They continued testing using *in vivo* assay in zebra fish, but 10 μM AVB did not show effect on estrogen receptors. (33) In another research inspecting agonism on HEK293 cell line, AVB had no effect on ERβ, but on the contrary, it affected hERα (in concentrations above 1 μM, plateau level 37%). (34) Action on the estrogen system was also examined on MCF-7 cells (35), recombinant yeast expressing the rainbow trout ERα (36), and *in vivo*, using uterotrophic assay in immature rats (35), but none of these tests showed any effect.

Androgenic and antiandrogenic action was investigated on human breast carcinoma cell line MDA-kb2. Two cases of research failed to demonstrate the antiandrogenic effect (35) (37), but on the contrary, in another research they determined antiandrogenic activity with IC_{50} 20 pM. (38) In U2-OS cells, it displayed antiandrogenic activity (extrapolation outside testing dose range gave result IC_{50} 11 μM). On the same cell line they also examined effect on **progesterone receptor**, but AVB exhibited no influence in range to μM values. (34) On MDA-kb2 line, they also examined influence on **glucocorticoid receptor**, which resulted in agonistic response with EC_{50} 2.66 x 10^{-9} μM. (38)

1.3.3.2 Immune disruption

Except for photosensitizing effects of AVB following UV exposure, the impact of this chemical on the immune system is still a relatively unchartered territory. In THP-1 monocytic cell line they investigated how well ZnO nanoparticles and some organic UV filters are tolerated. They examined AVB in range from 1 to 250 µg/mL (3.22 - 805.4 µM). In these concentrations, AVB did not reach EC_{50} value for cytotoxicity. IL-8 and IL-1ß were measured in THP-1 monocytes and macrophages using ELISA essay. In this concentration range, most organic filters, including AVB, significantly increased cytokine production in comparison to the control sample without any treatment. (39)

1.4 THE IMMUNE SYSTEM

The immune system is a network of cells, tissues and organs, whose collective aim is to defend the body against the invasion of foreign invaders, including viruses, bacteria, fungi, parasites, as well as macromolecules such as proteins and polysaccharides. (40) (41) It is vital for the human survival. When a malfunction occurs, it can be fatal, not only due to the absence of fighting the infections, but also due to the ability to react exaggeratedly and attack its own body (hypersensitivity and autoimmune diseases). (41) (42) (43) Simply defined, immunity is the capability of the immune system to provide sufficient defence mechanisms to avoid and/or resist potentially harmful pathogens. (43) Immune responses are divided into innate and adaptive immunity, the components of which collaborate mutually.

Innate immunity (also native or natural immunity) consists of cellular and biochemical defence mechanisms, which are the first line of defence, ready for a rapid response against invading pathogens. (41) They are also responsible for the initiation of the adaptive immune responses. These mechanisms recognize conserved features of pathogens, such as many types of molecules on the microbial surfaces and virus double-stranded RNA, but do not have a specific response to a particular pathogen in a manner of adaptive immune responses. (44) Innate immunity is consists of:

I. Physical and chemical barriers, such as epithelia and surface antimicrobial substances;
II. Phagocytic cells (neutrophiles, macrophages), and NK cells;
III. Plasma proteins, which include mediators of the complement system;
IV. Cytokines, proteins that regulate cell activity of the immune system. (41)

If the natural immune response is outrun by an infection, it stimulates an **adaptive (also called specific) immune response**. The adaptive immunity characteristics are the exquisite

specificity to determine foreign substances or microbes, produce large number of antibodies and effector cells specific for a particular pathogen, and provide a long-term immunological memory. It enhances protective mechanisms of innate immunity, direct immune cells to those cells and tissues that contain antigen, and allow them to more easily remove the invader. (41) (42) (45) Because these responses are destructive, it is very important that they elicit just in contact with foreign substances called antigenes, not with molecules of the host itself. The adaptive immunity is carried out by white blood cells called lymphocytes, and their products. (44) There are two types of such responses, called humoral immunity and cell-mediated immunity. They are mediated by different classes of lymphocytes - B cells and T cells, and eliminate microbes in different ways. In humoral immune response, B lymphocytes are activated to produce antibodies, which are proteins called immunoglobulins (Ig). Humoral immunity promotes the destruction of extracellular microbes, microbial toxins and viruses. The antibodies circulate in the blood and bind specifically to the antigen that stimulated their production. Antibody binding deactivates the invading pathogen by disabling their binding to the receptors of the host cells and also targets them for elimination by various effector mechanisms such as phagocytic cells. Cell-mediated immunity is carried out by T lymphocytes. Intracellular microbes (viruses, some bacteria) proliferate inside host cells, where they are inaccessible to circulating immunoglobulins. The activated T cells react directly against the foreign protein that is presented in the MHC-associated antigen complex on the surface of the infected host cell. T-cells kill infected host cells ($CD8^+$ lymphocytes) or produce signal molecules that stimulate B cell growth, their differentiation, and activate macrophages to destroy invaders within their phagosomes ($CD4^+$ lymphocytes). (41) (44)

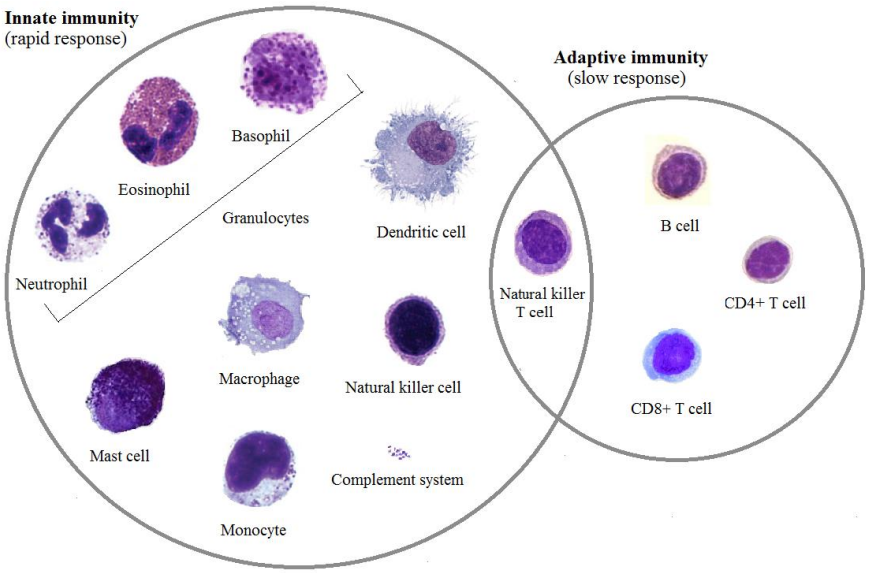

Figure 4: Structural members of the innate and the adaptive immunity

1.4.1 Cells and mediators of the immune system

The immune system is supplied with a huge number of cells, which are developed from immature stem cells as a response to different cytokines and other chemical signals. (40) The white blood cells are grouped into three main categories: lymphocytes, granulocytes and monocytes. (44) Some immune cells confront all intruders, whereas others clash with highly specific targets. To work effectively, most of the immune cells need a mutual cooperation. They communicate by direct physical contact, or by releasing chemical messengers. (40)

1.4.1.1 Phagocytes

Phagocytes are the white blood cells with the ability to swallow and break down foreign particles or microbes. (44) Monocytes are phagocytes that circulate in the bloodstream. Once they migrate into tissues, monocytes develop into macrophages, which in cooperation with neutrophils perform the main part of phagocytic activities. (40) (44) They both contain specialized lysosomes which fuse into the newly created phagocytic vesicles (phagosomes) and destroy foreign particles with highly reactive molecules of superoxide (O_2^-), hypochlorite (OCl^-) and a mixture of lysosomal hydrolases. Unlike the neutrophils, macrophages are longer-lived and larger, therefore they are able to digest larger organisms, such as protozoa, and are responsible for the identification and destruction of damaged or dead cells. (44) Mononuclear phagocytes also bear the role of APCs in the adaptive immune response. (41)

1.4.1.2 Granulocytes

Granulocytes (polymorphonuclear leukocytes) contain numerous cytoplasmic secretory granules containing reactive substances that kill microbes and enhance inflammation. (42) There are three types of granulocytes which have a relatively short life span and an increased production during the immune response when they migrate from blood to the areas of inflammation or infection. Neutrophiles are phagocytic cells which are the most numerous and the most important component of the innate immunity. (46) Eosinophils are the second most common type of granulocytes, which are important in the defence against parasitic infections and modulate allergic inflammatory responses. (42) (44) Basophils are inflammatory granulocytes which release histamine and direct inflammatory reaction. After contact of the antigen with the attached IgE, basophils degranulate and release the content of granules into the neighbourhood, causing an immediate hypersensitivity reaction which is perceived as allergy symptoms. (44) (45)

1.4.1.3 Mast cells

Mast cells are crucial for the inflammation, and have the same structural and functional similarities as basophils. They cannot be found in the blood, rather, progenitor stem cells travel to the peripheral tissues where they transform into mature mast cells. Mast cells can be found in skin, lungs, and linings of the nose and the intestinal tract. (40) (45)

1.4.1.4 Lymphocytes

We know two main classes of lymphocytes, T cells and B cells. B cells secrete five different types of antibodies (IgG, IgM, IgD, IgE, IgA), which circulate in the blood and attack target cells. Each B lymphocyte specifically makes just one type of antibodies. (40) Following the primary infection, memory B cells release large amounts of antibodies in case of re-infection into the bloodstream. T cells, the other class, contribute to body defence in two ways; by regulating the immune response with helper T cells (T_H or CD4+ cells) or with a direct attack on the infected cells with cytotoxic T cells (T_C or CD8+ cells). (45) Their specific modes of action are described in detail under adaptive immunity at the start of Chapter 1.4. Natural killer (NK) cells are another type of lethal T lymphocytes. Like T_C cells, NK cells also attack intruders and destroy them using granules, which contain potent chemicals. The only difference is in T_C cells reacting to MHC-associated antigen complexes in contrast to NK cells, which attack cells lacking self-MHC molecules. This feature allows NK cells a potential to recognize a wide range of foreign cells. (40)

1.4.1.5 Specialized antigen presenting cells

APCs include dendritic cells (DCs), mononuclear phagocites and B cells. (45) DCs are migratory cells and have similar properties as macrophages. (40) (44) Their role is to present antigen on the surface with the help of the major histocompatibility (MHC) complex. T_C cells recognize the antigen and destroy the invader. (45)

1.4.1.6 Complement system

The complement system consists of about 30 serum proteins which help remove the microorganisms or damaged cells, and control the inflammation. It is a part of the innate immune system, but also can be recruited by the adaptive immune reaction. The activation of the complement system is a cascade reaction, which results in peptides with multiple effects such as opsonization, chemotaxis, release of inflammatory mediators from mast cells, increased blood flow and capillary permeability. The system can be activated via alternative pathway, which is non-specific and an innate response. This phenomenon is called opsonization and is operated in a way that micro-complement molecules cover the

microorganism and enable phagocytes to digest it. The complement also can be activated by antibodies on the cell surface, which is called a classical activation pathway and is a part of the specific immune response. (45)

1.4.1.7 Cytokines

Cells of the immune system mutually communicate by releasing and responding to messengers called cytokines. (40) Cytokines are small (~25 kDa) proteins secreted by various cells and arouse responses through binding to specific receptors. (41) (46) (47) The nomenclature is often based on their cellular sources or roles, so the following groups exist: monokines (CKs produced by mononuclear phagocytes), lymphokines (CKs produced by lymphocytes), interleukins (CKs produced by one leukocyte and acting on other leukocytes), chemokines (CKs with chemotactic activities), and interferons (CKs which interfere with viral infections). With the development of science methods it became clear that the same protein may be synthesized by a variety of cells and it also acts on various cells, therefore, the general name cytokines is the preferred term for these mediators. (41) (47)

Cytokines actions can be local or systemic and can regulate both, the innate and the adaptive immunities. They have an impact on the cells, that produce them (autocrine action) or on cells nearby (paracrine action). When they are produced in higher amounts, they can enter the circulation and affect distant tissues (endocrine action). (41) (47) Cytokines production is a result of cell activation, their synthesis is transient and they are rapidly secreted to the mediate response. Pleiotropism, e.g. the ability that one cytokine affect different cell types, is very common. They also indicate redundancy, which is the feature that multiple cytokines can affect the same biological response and consequently compensate single cytokine mutation. Regarding mutual effects, two cytokines may act synergistically or antagonistically to each other's actions. (41) Cytokines are produced by many cell types, but they are predominantly secreted by T helper cells and macrophages. (47) The cytokines synthesized in macrophages in response to invaders are a group of structurally diverse molecules and include TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-12 (IL-12). (46)

In our experiments, two cytokines, IL-8 and TNF-α were examined. Interleukin-8 is a member of the chemokine family. As other chemokines, it regulates inflammatory reactions through the recruitment of immune cells to the sites of infection. To control the nature of inflammation, different chemokines attract different cells, and thus IL-8 Tumor preferentially acts on neutrophils and regulates their migration from blood to tissues. (41)

necrosis factor α, also known as cachectin, is a proinflammatory cytokine, mostly secreted from macrophages, although T cells, NK cells and mast cells also produce it. (41) (47) TNF-α is an endogenous pyrogen, inducing fever via hypothalamus. Additionally, it causes local heat, redness, swelling and pain in increased concentrations in a limited area. It stimulates the recruitment of neutrophils and increases their adhesiveness to vascular endothelial cells, which are also stimulated by TNF-α to express new adhesion molecules. Moreover, TNF-α arouses production of chemokines, IL-1, IL-6, and TNF-α itself via macrophages stimulation. (48)

1.4.2 Protein kinases C (PKCs)

Different research have shown that protein kinase C (PKC) have an important role in immune cell activation. PKCs are phospholipid-dependent serine threonine kinases, which mediate numerous physiological processes, including signal transduction of neurotransmitters, hormones and cytokines, gene expression, cell growth and differentiation, etc. (49) (50) After the activation, these enzymes perform a translocation from the cytosol to the membrane. A family of 12 isoenzymes is divided by their mode of activation into three major classes: classical PKCs (α, β_I, β_{II} and γ), novel PKCs (δ, ε, η and θ), atypical PKCs (ξ and ι/λ isoforms). The occurrence of each isomer differs among tissues, and specific PKCs affect specific biological pathways and cellular signals to mediate the cell proliferation and differentiation (Appendix, Figure 24). (50) (51) According to research, PKC ß is involved in immune responses, possessing a central role in the activation of immune cells. (52) It was presented that PKC ß cooperates in signal transduction pathways to activate dendritic cells after the exposure to allergens. More specific, it was a critical signalling component of allergen induced IL-8 and CD86 up-regulation in investigation on human promyelocytic cell line THP-1 and dendritic cells. (51) Moreover, it mediates immunity; its attendance was demonstrated in IL-2 transcription and secretion, IL-2R regulation in T-cells and activation of NF-κB. PKC ß consists of PKC $ß_I$ and PKC $ß_{II}$ - two variants, which originate from same gene and are different only in sequence of the last 50 amino acids. (49) It has been demonstrated that during differentiation-inducing stimuli on dendritic cells, only PKC $ß_{II}$ was constantly stimulated and have been associated with DC surface markers up-regulation (MHC1, MHC2, CD11c, CD40, CD80, CD83 and CD86), induction of expression of c-Rel (NF-κB family member) and stimulation of allogeneic T-cell proliferation. (50)

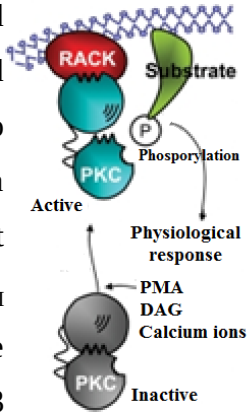

Figure 5: PKC activation (53)

1.4.2.1 Scaffold protein RACK1

To complete their role, activated PKCs redistribute from cytosol to the membrane compartments. This process is mediated by a WD repeat protein family, named receptors for the activated C-kinase (RACKs). (51) The best characterized member of this family is RACK1, 36-kDa protein, highly conserved throughout evolution, with a seven WD repeat-bladed propeller structure. The preferred binding partners are PKC $ß_{II}$ and PKC ε. In order to activate the signalling cascades, RACK1 stabilizes their active conformation and consequently prevents premature degradation, stimulating their translocation nearer to the specific substrates, involved in the defined pathways. (50) (51) (52) Further evidence which is linking RACK1 to the immune system, is its effect on interferon and other cytokine receptors. Research demonstrated that RACK1 may modulate IL-3, IL-5 and GM-CFS receptor signalling pathways. It was also linked to IFN type I receptor activation, which through activation of Janus kinases 1 and 2 leads to tyrosine phosphorylation of Stat1 and Stat2 transcription activators. Furthermore, it has been reported that RACK1 acts as an adaptor that links nonphosphorylated Stat1 to the IFNα receptor. (54)

In general, RACK1 is a scaffolding protein, ubiquitously expressed in our body, which interacts with a wide range of proteins, mediates diverse signalling pathways and biological functions. (54) (55) The general function of RACK1 and other anchoring proteins is to organize proteins into signalling networks. Several scaffolding proteins can function within a singular signalling cascade, which is usually tightly controlled. Therefore, even a slight deviation from their optimum expression level is a serious danger to cause severe change in the signalling pathways. (55)

1.4.3 Immunomodulatory effects of EDCs

In addition to interfering with endocrine, nervous and reproductive systems, endocrine disrupting chemicals can also affect the immune system. (56) The endogenous sex steroids are highly important as immune the system regulators and EDCs can mimic or antagonise them. The immune cells express hormone receptors on their surfaces, so androgens, estrogens, progestogens and other hormones can modulate their diverse actions, depending on the cell type and the signalling pathways. If we limit research results on the impact of hormones on monocytes and macrophages, the results as follows. In monocytes, estradiol reduces IL-8 production and inhibits chemotaxis through the chemotactic protein MCP-1. Progesterone has various effects, inducing or repressing cytokines release, depending on stimuli. Sex steroids also influence the macrophage activity. DHT reduces the secretion of

TNF-α from splenic macrophages, while it induces it in Kupffer cells. Testosterone increases ROS production in macrophages. Progesterone stimulates TNF-α, IL-1ß and chemokine MIP-2 secretion, while estradiol suppresses them and also reduces the secretion of IL-1α, IL-6 and the transcription factor NF-κB with inflammation properties. (57) In monocytic THP-1 cells, cortisol and synthetic corticosteroids partially suppressed RACK1 production and reduced cytokines TNF-α, IL-8 release. (58)

In general, immunomodulatory effects caused by EDCs are divided into three main groups: 1) immunity reduction 2) induction of autoimmune diseases, and 3) induction of allergies and inflammation.

Studies revealed that some EDCs, e.g. BPA and triclosan, affect the immune response through a decrease of antiviral immunity, modulation of cytokine release, and lower cell differentiation and survival. They may suppress through T_H1 (reduction of intracellular pathogens) and T_H2 (decrease of parasitic and bacterial infections) caused immunity. For certain chemicals (butyl benzyl phthalate, nitrofen) indications were found that they inhibit LPS-induced activation of macrophages via the suppression of TNF-α.

An epidemiologic survey demonstrated a possible link between the contaminants and the emergence of allergies. Exposure to compounds like polyvinyl chloride (PVC) correlates with rapid increases of several chronic inflammatory diseases such as pediatric asthma and allergic rhinitis. One of the possible mechanisms is modulation of antigen presenting cells (e.g. dendritic cells) which further guide the T_H2 polarization and lead to an exacerbation of airway inflammatory processes. In addition, multiplication of immunoglobulins (IgG1, IgE) occurs due to the increase of T_H2 cytokines as IL-4, which may augment allergic responses. The hypothesis that environmental contaminants contribute to the development of autoimmune diseases is also confirmed by the parallel trends comparing increased use of plastics as well as an increased incidence of diseases like diabetes type 1, rheumatoid arthritis, systemic lupus erythematosus and thyroiditis.

Chronic inflammation diseases indicate the over expression of inflammation-related genes. EDCs may have the ability to alter the DNA methylation, and these epigenetic modifications are expressed in the further gene transcriptions. (56)

2 RESEARCH AIM AND OBJECTIVES

We are all exposed to many chemicals daily, and the list of substances used the industry, households and cosmetic products increases every year. Some of them have a potential impact on the endocrine system, including avobenzone (AVB), a UVA filter which can be found in sunscreens and other cosmetic products. Previous studies have shown impact of AVB on estrogen, androgen and glucocorticoid receptors. As the immune response is also indirectly regulated through steroid hormone receptors, this raises suspicion about the possibility that AVB may affect the immune system.

In this Master's thesis we investigate the influence of AVB on the immune system. Experiments will be performed *in vitro* using the human monocytic cell line THP-1. AVB concentrations will be selected based on preliminary information on the IC_{50} and EC_{50} of AVB on the androgen and glucocorticoid receptors. (38) Therefore, concentrations ranging from 2 pM to 2 nM will be used, which are operating range concentrations of endogenous hormones. The entire work plan is presented in Figure 6.

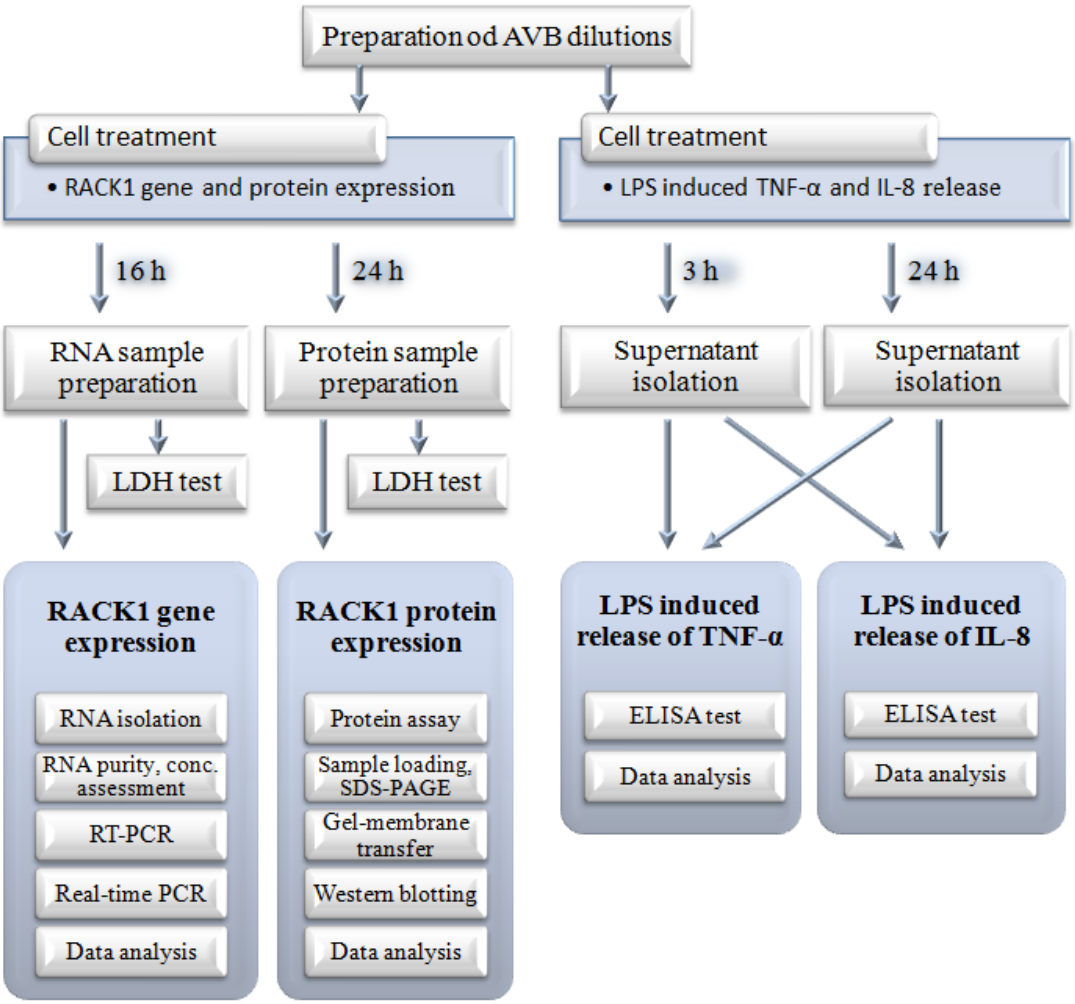

Figure 6: The research plan and working scheme

Experiments will be divided into three main parts, namely 1) the verification of cell viability after exposure to AVB, 2) RACK1 gene expression and protein production, and 3) extent of cytokines TNF-α and IL-8 release.

First, using LDH leakage, the cytotoxicity of AVB following 16- and 24-hour incubation will be assessed. Then, the expression of RACK1 both at the mRNA and protein level will be assessed as an indicator of potential modulation of the immune response. Cells will be treated for 16 hours for the mRNA expression of the GNB2L1 gene, which encodes RACK1. The analysis will be carried out by RT-qPCR. SDS-PAGE and Western blotting will be used to assess the level of expression of RACK1 at the protein level after a 24-hour treatment. Within the last set of experiments, the immunomodulatory effects of AVB will be evaluated by assessing its effect on LPS-induced cytokine production. The release of the proinflammatory cytokines TNF-α and IL-8 will be evaluated by specific ELISA.

With these experiments, we aim to confirm or disprove the following hypotheses:

1) AVB at the tested concentrations is nontoxic for THP-1 cells. After the treatment with AVB, cell viability higher than 80% in comparison to the control is expected.
2) According to the agonism on the glucocorticoid receptor and the antiandrogenic activity proved in a previous investigation (38), an antagonistic effect of AVB on the immune system activation is foreseen. As the parameters investigated are under the control of RACK1 and PKC activation, an influence of AVB on this pathway is expected. In analogy to what has been previously shown with glucocorticoids (58), a reduction of the expression of RACK1 is expected.
3) Due to a reduction in the PKC pathway, a suppression of the immune response is suspected, with a decrease in LPS-induced proinflammatory cytokines IL-8 and TNF-α release.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Preparation of avobenzone solutions

To determine the effect of avobenzone on the immune system, THP-1 cells are treated with 4 different concentrations of avobenzone, distinguished one from another by a concentration factor 10. As the starting point an inhibitory concentration with 50% max effect on androgen receptor (IC_{50} AR) is taken, which is 20 pM and was established in a previous study investigating the impact of AVB on AR and GR. (38) Based on concentration 20 pM, four final concentrations are determined: 2 nM (100x IC_{50}), 200 pM (10x IC_{50}), 20 pM (1x IC_{50}) and 2 pM (0.1x IC_{50}). DMSO is used as a solvent and a control solution. Preparing solutions for experiments, it is necessary to consider the maximum allowed DMSO concentration in cell suspensions for treatments, which is 0.2%. Otherwise, DMSO may affect measurements.

Depending on the final volume of the treated cell suspensions, an appropriate AVB stock solution is prepared and further dilutions are performed as shown in Figure 7. Prior to each treatment, a new serial dilution is prepared from the stock solution.

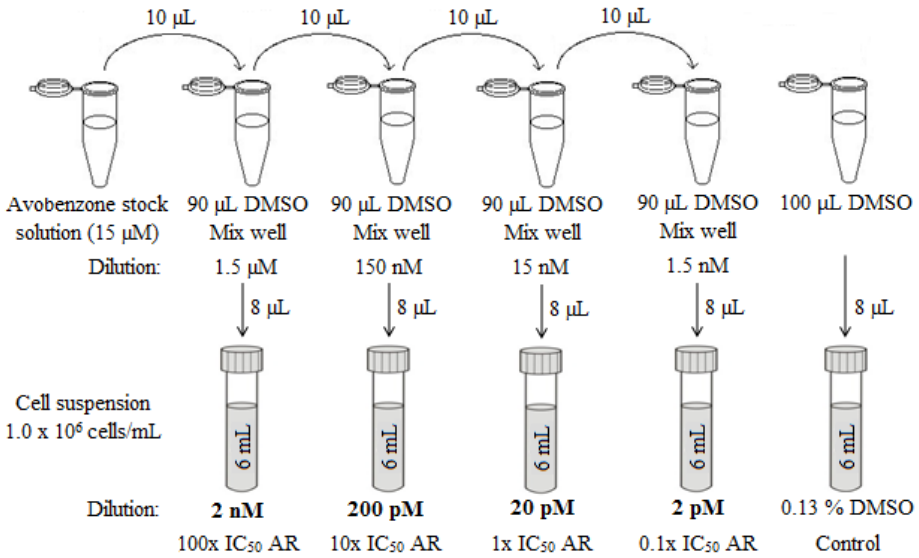

Figure 7: Serial dilutions from AVB stock solution for THP-1 cell treatment

3.1.2 Cell line THP-1

THP-1 cells were received from Istituto Zooprofilattico di Brescia (Brescia, Italy).

The human acute monocytic leukemia THP-1 is the most common cell line utilized as an *in vitro* model of human monocytes and macrophages to study their differentiation, function and consequently immune responses. (59) It was derived from the blood of a 1-year old male suffering from acute monocytic leukemia. (60) THP-1 cells resemble native monocytes and monocyte-derived macrophages in morphological and functional properties, including differentiation markers. They are useful to examine inflammatory responses mediated by LPS and/or drug exposure. (61) THP-1 line is highly plastic, sensitive to many stimuli, and can therefore be polarized into multiple lineages. (59)

Table 2: Advantages and disadvantages of THP-1 cell line (61)

Advantages (+)	**Disadvantages (-)**
• Lower financial expenses and no ethical constrains compare to *in vivo* experiments • Average doubling time is around 35 to 50 h • Cell line can be cultured *in vitro* up to passage 25 (approx. 3 months) without changes of cell sensitivity and activity • Availability; cells can be stored for longer time periods and by following appropriate protocol, they can be recovered without any obvious effect on their features and viability • Findings are reproducible because of homogenous genetic background, that minimizes the degree of variability	• Malignant background and cultivation under controlled conditions might result in different sensitivities and responses compared to normal somatic cells in their natural environment • Possibly relevant interactions between target cells and surrounding cells cannot be easy mimicked as in natural tissues

3.1.3 Laboratory equipment

Table 3: List of equipment used in the laboratory

Equipment	Manufacturer	Use and additional information
Cell incubator	Thermo Fisher Scientific, Waltham, MA, USA.	Cell cultivation (37 °C, 5% CO_2)
Laminar flow hood	Gelarie, Sydney, Australia	Cell cultivation and treatments
Fridge, freezer	n/a	Samples, reagents storage
Multiwell plate reader (VMax Kinetic ELISA Microplate Reader)	Molecular Devices, LLC., CA, USA	LDH test, Protein Assay, ELISA
UV/Visible Spectro-photometer (NanoVue)	GE Healthcare, Illinois, USA	RNA isolation
Thermal Cycler Gene Amp PCR System 2700	Thermo Fisher Scientific, Waltham, MA, USA	RT-PCR
Real-time instrument ABI PRISM® 7000 Sequence Detection System	Thermo Fisher Scientific, Waltham, MA, USA	Real-time PCR
Digital imager ChemiDoc MP Imaging System	Bio-Rad, Richmond, CA, USA	WB analysis
Benchtop centrifuge (Hareo Biofuge model)	Thermo Fisher Scientific, Waltham, MA, USA.	Cell treatments
Centifuge	Eppendorf, Hamburg, Germany	RNA isolation
Mini vortexer /centrifuge (Grant, PCV 2400)	Grant, Cambridge, UK	Vortexing, centrifuging samples, reagents
Vortex mixer (SA8)	Bibby Scientific Limited, Staffordshire, UK	All experiments
Test tubes with dual position snap caps	EuroClone, Milano, Italy	Cell treatment (5, 10 mL)
Centrifuge tubes	EuroClone, Milano, Italy	All experiments (15, 50 mL)
Single channel pipettes	Gilson, Middleton, WI, USA; Eppendorf, Hamburg, Germany	All experiments (2.5, 10, 20, 200, 1,000 µL)
Sterile pipette tips	Eppendorf, Hamburg, Germany	All experiments except RNA protocols
Aerosol resistant pipette tips **(**Neptune Brand Barrier)	Gentaur, Kampenhout, Belgium	RNA protocols
Single channel serological	EuroClone, Milano, Italy	Cell cultivation,

pipette controller (Primo buddy)		treatments, Western blotting
Serological pipettes (Corning Costar Stripette)	Sigma-Aldrich, St. Louis, MO, USA	Cell cultivation, treatments, Western blotting (1, 2, 5, 10, 25 mL)
Sterile Eppendorf tubes	Eppendorf, Hamburg, Germany	All experiments (0.5, 1.5, 2, 2.5 mL)
Tube racks	EuroClone, Milano, Italy	All experiments
Vacuum trap	n/a	Cell cultivation and treatments
Counting chamber and cover slip	n/a	Cell counting
Manual tally counter	n/a	Cell counting
Light microscope	Carl Zeiss AG, Jena, Germany	Cell counting
Insulin syringes	BD, New Jersey, USA	RNA samples preparation
Hot plate magnetic stirrer	n/a	WB samples preparation
Hot-plate hold foams for samples	n/a	WB samples preparation
Parafilm	n/a	RNA isolation
Needle	n/a	RNA isolation
Vacuum pot	n/a	RNA isolation
Thermostat	Eppendorf, Hamburg, Germany	RNA isolation
8-tube strips (MicroAmp™ 8-tube strip)	Thermo Fisher Scientific, Waltham, MA, USA	Real-time PCR
8-cap strip (MicroAMp™ 8-cap strip)	Thermo Fisher Scientific, Waltham, MA, USA	Real-time PCR
Cap installing tool (Handle MicroAmp™ Cap Installing tool)	Thermo Fisher Scientific, Waltham, MA, USA	Real-time PCR
Flat-bottom 96-well plates (Corning Costar 9018)	Sigma-Aldrich, St. Louis, MO, USA	LDH test, Protein Assay, ELISA protocols
Optical adhesive cover	n/a	LDH test, Protein Assay
Disposable glass test tubes, 13 x 100 mm	Thermo Fisher Scientific, Waltham, MA, USA	Protein Assay
Repetitive dispensing pipette	Eppendorf, Hamburg, Germany	Protein Assay, ELISA
Combipits (Eppendorf Combitips Advanced®)	Eppendorf, Hamburg, Germany	Protein Assay, ELISA; 5 mL
Multiwell plate washer	n/a	ELISA

Glass plates, casting frame, casting stand, plastic combs, clamping frame with electrode assembly, buffer tank, buffer tank lid, electrode assembly for transfer, gel holder cassette, cooling unit	Bio-Rad, Richmond, CA, USA	SDS-PAGE, transfer (WB)
Power supplies	Bio-Rad, Richmond, CA, USA	SDS-PAGE, transfer (WB)
Foam pads, filter paper	n/a	Transfer of proteins (WB)
Glass suction pipette	n/a	SDS-PAGE (WB)
Gel-loading pipette tips (Corning)	Thermo Fisher Scientific, Waltham, MA, USA	SDS-PAGE (WB)
PVDF membrane	Amersham, Little Chalfont, UK	Transfer of proteins (WB)
pH test strips	n/a	Solutions preparation, WB
Tweezers	n/a	Western Blot
Razor blade	n/a	Transfer of proteins (WB)
Agitator/plate shaker (Heidolph Unimax 1010)	Heidolph, Schwabach, Germany	Western blotting
Powdered milk	n/a	Western blotting
Transparent pallet	n/a	Analysis of WB data
Plastic sheet protector	n/a	Analysis of WB data

3.1.4 Chemicals

All reagents were purchased at the highest purity available.

Cell culture protocols

- RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA)
- RPMI 1640 without Red Phenol (Sigma-Aldrich, St. Louis, MO, USA)
- FBS (Sigma-Aldrich, St. Louis, MO, USA)
- L-glutamine (Sigma-Aldrich, St. Louis, MO, USA)
- Penicillin (Sigma-Aldrich, St. Louis, MO, USA)
- Streptomycin (Sigma-Aldrich, St. Louis, MO, USA)
- 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA)

Treatment protocols

- Avobenzone (Sigma-Aldrich, St. Louis, MO, USA)
- DMSO (Sigma-Aldrich, St. Louis, MO, USA)

- LPS (Sigma-Aldrich, St Louis, MO, USA)
- Cortisol (Sigma-Aldrich, St. Louis, MO, USA)
- PBS (Sigma-Aldrich, St. Louis, MO, USA)
- Trypan Blue solution, 0.4% (Sigma-Aldrich, St. Louis, MO, USA)

RT-qPCR protocols

- Chloroform (Sigma-Aldrich, St. Louis, MO, USA)
- 2-Propranol (Sigma-Aldrich, St. Louis, MO, USA)
- Absolute ethanol (Sigma-Aldrich, St. Louis, MO, USA)
- Diethylpyrocarbonate-treated water (DEPC H_2O) (Sigma-Aldrich, St. Louis, MO, USA)

SDS-PAGE, Western blotting protocols

- Distilled water (Department of Pharmacological and Biomolecular Sciences, Milano, Italy)
- Tris (Bio-Rad, Richmond, CA, USA)
- HCl (Sigma-Aldrich, St. Louis, MO, USA)
- NaCl (Sigma-Aldrich, St. Louis, MO, USA)
- EDTA (pH 7.5) (Sigma-Aldrich, St. Louis, MO, USA)
- 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA)
- Prestained Protein SHARPMASS VI (EuroClone S.p.A., Milano, Italy)
- Methanol (Sigma-Aldrich, St. Louis, MO, USA)
- SDS (Bio-Rad, Richmond, CA, USA)
- Glycerol (Sigma-Aldrich, St. Louis, MO, USA)
- Bromophenol (Bio-Rad, Richmond, CA, USA)
- Acrylamide (EuroClone S.p.A., Milano, Italy)
- Ammonium persulfate (Bio-Rad, Richmond, CA, USA)
- TEMED (Bio-Rad, Richmond, CA, USA)
- Glycine (Bio-Rad, Richmond, CA, USA)
- Tween 20 (Bio-Rad, Richmond, CA, USA)
- Ponceau S (Sigma-Aldrich, St. Louis, MO, USA)
- Acetic Acid (Sigma-Aldrich, St. Louis, MO, USA)

ELISA protocols

- PBS (Sigma-Aldrich, St. Louis, MO, USA)
- Distilled water (Department of Pharmacological and Biomolecular Sciences, Milano, Italy)

3.1.5 Kits

LDH Cytotoxicity Detection Kit User Manual

(Clontech Laboratories, Inc., A Takara Bio Company, USA)

- Material: Catalyst (lyophilized), Dye Solution

TRI Reagent, For procession tissues, cell cultured in monolayer or cell pellets (Sigma-Aldrich, St. Louis, MO, USA)

- Material: TRI Reagent (a mixture of guanidine thiocyanate and phenol in a monophase solution)

High capacity cDNA Reverse Transcription Kit

(Applied Biosystems, Foster City, CA, USA)

- Material: 10X RT Buffer, 10X Random Primers, 25X dNTP Mix (100 mM), MultiScribe^{TM} Reverse Transcriptase (50 U/µL)

TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA)

- Material: TaqMan Universal PCR Master Mix, dye-labelled TaqMan MGB probes (for genes GNB2L1 and 18S)

Bio-Rad Protein Assay Kit II (Bio-Rad, Segrate, Italy)

- Material: Protein Assay Dye Reagent Concentrate (solution contains dye, phosphoric acid and methanol), bovine serum albumin (BSA) standard

Western blotting antibodies:

- Mouse anti-human RACK1 monoclonal antibody (Transduction Laboratories, Affinity, Nottingham, UK)
- Mouse anti-human ß-actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA)
- Anti-mouse secondary antibody (Transduction Laboratories, Affinity, Nottingham, UK)

Clarity^{TM} Western ECL blotting Substrate Kit (Bio-Rad, Segrate, Italy)

- Material: Clarity western peroxide reagent, Clarity western luminol/enhancer reagent

Human TNF alpha ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA)

- Material: anti-human TNF alpha Capture Antibody, recombinant human TNF alpha Standard, anti-human TNF alpha Biotin Detection Antibody, enzyme Avidin-HRP, 5X ELISA/ELISPOT diluent, substrate 1X TMB solution, 10X Coating Buffer

Human Interleukin-8 ELISA, Immuno Tools (ImmunoTools, Friesoythe, Germany)

- Material: anti-human IL-8 Capture-Antibody, anti-human IL-8 Detector-Antibody, recombinant human IL-8 standard, Poly-HRP-Streptavidin

3.1.6 Buffers and solutions

75% Ethanol solution (2 mL): 500 µL of absolute ethanol is mixed with 1.5 mL of DEPC water.

LDH Reaction Mixture: shortly before use, Catalyst and Dye Solution are mixed in ratio 1 + 46 (i.e. 8.5 µL of Catalyst and 391.5 µL of Dye Solution).

0.5 M Tris-HCl Buffer Stock solution, pH 7.5 (1 L): 60.57 g Tris is dissolved in 800 mL of distilled water. pH is adjusted to 7.0 with the appropriate volume of concentrated HCl. The final volume is brought to the final volume of 1 L adding distilled water and then stored at room temperature.

0.5 M Tris-HCl Buffer Stock solution, pH 6/6.6/8.8 (1 L): the protocol is the same as above, for 0.5 M Tris-HCl with pH 7.5, just pH for each solution is adjusted with HCl to the appropriate value.

Homogenization Buffer (100 mL): 0.88 g of NaCl, 1 mL of 0.5 M EDTA (5 mM), 5 mL of 1 M Tris-HCl (pH 7.4) and 1 mL of Triton X-100 (1 % (v/v)) are added to 50 mL of distilled water. All compounds are vigorously mixed and the volume is made up to 100 mL adding distilled water.

10% (w/v) SDS Solution (500 mL): 50 g SDS is dissolved in 350 mL of distilled water and the volume is made up to 500 mL adding distilled water.

10% (w/v) Ammonium Persulfate (10 mL): 100 mg of APS is dissolved in 1 mL of distilled water and then stored in a refrigerator at 4 °C.

Laemmli Sample Buffer 2X (100 mL): 0.1 g of bromophenol and 8.4 g of SDS are dissolved in 30 mL of distilled water. 25 mL of 0.5 M Tris-HCl pH 6, 20 mL of glycerol, 6 mL of ß-mercaptoethanol are added and the volume is made up to 100 mL adding distilled water.

Laemmli Sample Buffer 1X (100 mL): 50 mL of Laemmli Sample Buffer 2X is mixed with 50 mL of distilled water.

5% Stacking Gel (5 mL): 3.0 mL of distilled water, 1.25 mL of 0.5 M Tris/HCl with pH 6.8, 0.75 mL of 30% acrylamide and 0.05 mL of 10% SDS are mixed together to reach the desired gel percentage in final volume 5 mL. 0.05 mL of 10% ammonium persulfate and 0.005 mL of TEMED are added to the solution just before use.

10% Loading Gel (10 mL): 4.05 mL of distilled water, 2.5 mL of 0.5 M Tris/HCl with pH 8.8, 3.3 mL of 30 % acrylamide and 0.1 mL of 10% SDS are mixed together to reach

the desired gel percentage in the final volume of approximately 10 mL. 0.05 mL of 10% ammonium persulfate and 0.005 mL of TEMED are added to solution just before use.

Running Buffer 10X (1 L): 144 g of glycine, 10 g of SDS and 30 g of Tris are dissolved in 1 L of distilled water.

Running Buffer 1X (1 L): 100 mL of Running Buffer 10X is diluted to 1 L with distilled water.

Transfer Buffer 10X (1 L): 144 g of glycine and 30 g of Tris are dissolved in 1 L of distilled water.

Transfer Buffer 1X (1 L): 200 mL of methanol and 100 mL of Transfer Buffer 10X are mixed together, 2 mL of 10% SDS is added. The solution is diluted to 1 L using distilled water and mixed.

TBS 10X (1 L): 24.9 g Tris and 292 g NaCl are dissolved in 1 L of distilled water and adjusted to pH 7.4.

TBST (1 L): 100 mL of TBS 10X is diluted to 1 L with distilled water. 1 mL Tween 20 is added and the solution vigorously mixed.

0.1% (w/v) Ponceau S Staining Buffer: 50 mg of Ponceau S is dissolved in 20 mL of distilled water. 2.5 mL of glacial acetic acid is added (final conc. 5% v/v) and made up to 50 mL with distilled water.

ELISA Wash Buffer: 100 mL of 10X PBS is diluted to 1 L with distilled water; 0.5 mL of Tween 20 (0.05%) is added and the solution vigorously mixed.

3.1.7 Cell culture growth media

Growth medium for the THP-1 cell cultivation

95% RPMI with Red Phenol and 5 % of FCS are aseptically mixed together. Compounds in final concentrations 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin and 50 μM mercaptoethanol are added.

Growth medium during cell treatments

95% RPMI without Red Phenol and 5% of FCS are aseptically mixed together.

3.2 METHODS

3.2.1 Cell culture

Cell protocols are carried out in aseptic environment keeping cells free from contamination. All cell procedures are performed in a laminar flow hood, work surfaces and tools inside are wiped with 70% ethanol before and after procedure. Between uses, ultraviolet light is turned on to sterilise the air and surfaces under the hood. Only sterile glassware, tips, serological pipettes, Petri dishes and tubes are used. Outside containers, pipettes and other tools are disinfected with 70% ethanol before they are placed under the hood.

3.2.1.1 Cultivation of the cells

THP-1 cells (Chapter 3.1.2) are cultured in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50 μM mercaptoethanol, supplemented with 5% heated-inactivated fetal bovine serum. During the cultivation cells are placed in the incubator, set to 37 °C and humidified 5% CO_2 atmosphere.

In all experiments involving treatment, cells (diluted to density 10^6/mL) are cultured in RPMI 1640 medium without phenol red supplemented with 5% charcoal stripped fetal bovine serum (FBS).

3.2.1.2 Cell Subculturing

The growth of cells in culture typically exhibits sigmoid-shaped pattern, starting with the **lag phase** followed by seeding to the **log phase**, where the cells proliferate exponentially. When the cells attend confluency (occupy all of available medium) and/or use up essential nutrients, cell proliferation is greatly reduced or ceases entirely. To keep them at optimal density for continued growth and to stimulate further proliferation, the culture has to be divided and fresh medium supplied. This procedure is called **subculturing** or **passaging**, and enables the further propagation of the cell strain. (62) (63)

Mammalian suspension cells should be passaged when they are in log phase growth, before they reach confluency. After that, cells in the suspension clump together and the medium appears turbid when the culture flask is swirled. (62)

Protocol

1) When the cells are ready for passaging, Petri dishes with THP-1 cells are removed from the incubator and examined under the microscope. Other procedures take place under the laminar flow hood.

2) Using a sterile serological pipette, the suspension is slowly pipetted up and down to evenly distribute the cells. The whole culture from one Petri dish is gathered and equally divided into three Petri dishes (number of the divisions can vary regarding the culture cell density).
3) To dilute cells, the sterile growth media is added to reach the recommended final volume 18 mL in each Petri dish.
4) All the Petri dishes are returned in the incubator.

3.2.1.3 Manual cell counting using a haemocytometer

Many cell culture protocols require an estimate of the number of cells when they are treated, at plating or at harvest. Cells in suspension can either be counted manually, using a haemocytometer and a light microscope, or using any one of numerous electronic, semi-automatic counting devices.

Manual cell counting with a haemocytometer is the simplest, most direct and cheapest method of counting cells in suspension. Using the dye-exclusion method, it also allows determining the percentage of viable (intact) cells.

The haemocytometer is a modified microscope slide that bears two polished surfaces each of which displays a precisely ruled grid subdivided into nine primary squares, each measuring area 1 mm^2, and limited by three closely spaced lines (2.5 μm apart), which are used to determine if cells lie within or outside the grid. The plane of the grids rests 0.1 mm below two ridges that support a sturdy coverslip. There is a bevelled depression at the outer edge of each polished surface, where cell suspension is added to be drawn across the grid by capillary action. When the haemocytometer is properly loaded, the volume of cell suspension that will occupy one primary square is 0.1 mm^3 or 1.0 x 10^{-4} mL. For accuracy and reproducibility, counts must be performed in the same way every time.

Dye exclusion involves mixing of the cell suspension with a volume of buffer or balanced isotonic saline containing a water-soluble (i.e. membrane lipid-insoluble) dye – for example trypan blue – which is visible when it leaks into cells with damaged plasma membranes. By counting the number of unstained (undamaged) and stained (damaged) cells, the viability percentage can be calculated. Caution is needed when interpreting cell 'viability' data obtained by the dye exclusion method. Dye uptake marks cells with grossly disrupted membranes, but may not detect other forms of injury that lead to cell death. In addition, it has greater binding affinity for proteins in solution than for injured cells. (63)

Protocol

1) Cells in the complete medium are mixed well to assure complete homogeneity.
2) The mixture consisting accordingly to the data in Table 4 is aseptically prepared in an Eppendorf.

Table 4: Mixture for cell counting

Mixture	Volume
Sterile PBS	80 µL
Trypan blue	10 µL
Cell suspension	10 µL

3) A clean cover slip is seated on top of the haemocytometer.
4) The haemocytometer is loaded by expelling 10 µL of the suspension at the edge, which is drawn into by capillary action.
5) Examining under a light microscope, unstained (viable) cells in four corner primary squares of the grid using a manual tally counter are counted (Figure 8).

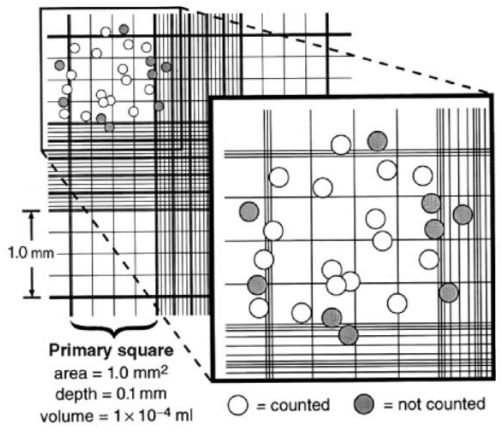

Figure 8: The middle of the triple lines separating the primary square, is the boundary line. Cells that touch the upper or left boundaries are included; those that touch the lower or right boundaries are excluded. (63)

6) The total cell concentration in the original suspension (cells/mL) is then:

$$\frac{A+B+C+D}{4} \times DF \times CF$$

$\frac{A+B+C+D}{4}$	The average number of cells in one primary square
DF	The dilution factor (in the mixture, the dilution factor is 10)
CF	The correction factor (one primary square tells us the number of the cells in 1 x 10^{-4} mL, our measurements are expressed in unit number of the cells in 1 mL, therefore the correction factor is 1.0 x 10^{4})

3.2.2 Cell treatments for investigation of RACK1 production snd LPS-induced cytokine release

THP-1 cells are treated with avobenzone in medium final concentration range from 2 pM to 2,000 pM to investigate RACK1 production and LPS-induced cytokine release.

3.2.2.1 Preparation of cell suspension used in treatments

Protocol

1) Cells are removed from the incubator and examined under the light microscope to check the density and the appearance of the culture.
2) The cell suspension is aseptically transferred to a sterile tube and centrifuged for 5 min at 25 °C and 1,500 rcf.
3) 5 mL of sterile, to room temperature pre-heated PBS is added in each tube and gently pipetted up and down 2 to 3 times to resuspend the cell pellet.
4) Tubes are centrifuged under the same conditions as above (25 °C, 5 min, and 1,500 rcf).
5) The whole amount of supernatant is carefully removed without disturbing the cell pellet, using vacuum and a pipette.
6) 5 mL of previously prepared RPMI 1640 medium without red phenol containing 5% FCS (Materials, Chapter 3.1.7) is added.
7) 10 µL aliquot is removed to determine its viability and the total number of cells, using trypan blue exclusion as described in Chapter 3.2.1.3 of Methods section.
8) After cell counting, the suspension with a concentration 1.0 x 10^6 cells/mL is prepared following the equation:

$$c_1 \times V_1 = c_2 \times V_2$$

c_1 Concentration of the suspension after cell count (cells/mL)

c_2 Required final cell concentration (1.0 x 10^6 cells/mL)

V_1 Volume of the start cell suspension (mL)

V_2 Volume of the suspension with the required concentration (mL)

Determining the final volume of the treatment suspension, the number of samples and one in addition is considered: $V_2 = V_{sample} \times (Number\ of\ samples + 1)$.

To dilute cells to a concentration 1.0 x 10^6 cells/mL, growth medium for cell treatments is used (for the preparation see Chapter 3.1.7).

3.2.2.2 Cell treatment for the investigation of RACK1 mRNA and protein expression

The first steps of cell treatment to investigate the effects of AVB on RACK1 gene expression and protein production are prepared together and then divided into two parts to continue each experiment independently.

Protocol

1) The diluted cell suspension (for the preparation see Chapter 3.2.2.1) is aseptically divided into 5 tubes, each containing 6 mL of the culture.
2) The previously prepared AVB solutions and DMSO as the control (Chapter 3.1.1) are aseptically added to the tubes following the instructions in Table 5 to reach the desired AVB concentrations in the treated samples.

   Table 5: Cell treatment for 5 samples contains 6 mL cell suspension each

Sample	Concentration of added AVB solution (nM)	Volume of added solution (µL)	Final AVB concentration in treatment (nM)
Control	/ (added DMSO)	8	0
2 pM AVB	1.5	8	0.002
20 pM AVB	15	8	0.02
200 pM AVB	150	8	0.2
2 nM AVB	1500	8	2

3) Using serological pipettes, the treated samples are gently pipetted up and down to get the complete suspension homogeneity. Then the culture from each tube is equally divided in 2 tubes with dual position snap caps; 3 mL of treated cells are intended for mRNA investigation and 3 mL for protein examination.
4) To allow the air exchange during incubation, the dual position snap caps should be sealed to the first position. The tubes are incubated at 37 °C and 5% CO_2 for an appropriate period depending on the type of examination.

3.2.2.3 RNA samples preparation

Kit used: TRI Reagent; Sigma-Aldrich, Inc., USA (Principle of action in Chapter 3.2.4.1)

1) RNA is isolated after 16 hours of cell treatment (Chapter 3.2.2.2).
2) Tubes with RNA samples are removed out of the incubator, the snap caps are sealed to the second position to tightly close the tubes and centrifuged at 25 °C for 5 min at 1,500 rpm.

3) 1 mL of the supernatant is transferred to the new Eppendorf to test cell viability during the treatment by using LDH test.
4) The residual supernatant is carefully removed and the pellet is washed with 5 mL of sterile PBS.
5) The tubes are centrifuged under the same conditions as written above (5 min, 1,500 rpm, 25 °C).
6) All the supernatant is removed carefully, without shaking, and the pellet is lysed with 1 mL of TRI Reagent.
7) To completely mix and destruct the cell membrane fragments, the samples are drawn up and down by using insulin syringes. Afterwards, the samples are transferred to sterile Eppendorfs and they can be stored at -70 °C for up to 1 month.

3.2.2.4 Protein samples preparation

1) Proteins are isolated after 24 hours of cell treatment (Chapter 3.2.2.2).
2) The tubes with samples are removed out of the incubator, the snap caps are sealed to the second position to close the tubes and centrifuged at 25 °C for 5 min at 1,500 rpm.
3) 1 mL of the supernatant is transferred to a new Eppendorf tube to test if cells were viable during the treatment by using LDH test.
4) The residual supernatant is carefully aspirated and the pellet is washed with 5 mL of sterile PBS.
5) The tubes are centrifuged under the same conditions as written above.
6) All the supernatant is removed carefully, without shaking, and the pellet is lysed in 100 µL of homogenization buffer (for the preparation see Chapter 3.1.6).
7) After adding the homogenization buffer, all further steps have to be performed on ice.
8) **Preparation of the samples for WB analysis**

   Using a pipette, the sample is gently mixed and 80 µL of the suspension is transferred to a sterile Eppendorf, then 80 µL of Laemmli Sample Buffer 2x (for preparation see Chapter 3.1.6) is added to the same Eppendorf. After that the samples can be removed from ice and boiled for 10 minutes to denaturize proteins.
9) **Preparation of the samples for Protein Assay**

   The remaining volume of the cell lysate (approximately 20 µL) is transferred to a sterile Eppendorf and stored in the freezer until the protein quantization is carried out.

3.2.2.5 Cell treatment for investigation of cytokine release

This cell treatment is performed to investigate dose and time related LPS-induced TNF-α and IL-8 release in THP-1 cells, being treated with AVB in comparison to the control vehicle.

Protocol

Cells are treated for 3 h and 24 h with 2, 20, 200 and 2,000 pM AVB solutions, 10 ng/mL LPS and 1μM cortisol. The preparation of AVB work solutions is described at Materials (Chapter 3.1.1). The culture with an addition of DMSO is used as a control.

1) The diluted THP-1 cell suspension (Chapter 3.2.2.1) is divided into the tubes; each containing 6 mL. Work solutions are aseptically added in the tubes according to the Table 6.
2) The procedure is made in dual, one for incubation time of 3 hours and the other for incubation time of 24 hours.

Table 6: Cytokine release cell treatment, supplements for 6 mL of cell culture

Tubes (samples)	Treatment	DMSO	AVB solutions		10 μg/mL LPS	2 mM Cortisol
1 (1-3)	DMSO	8 μL				
2 (4-6)	2 nM AVB		1,500 nM	8 μL		
3 (7-9)	LPS				6 μL	
4 (10-12)	2 pM AVB + LPS		1.5 nM	8 μL	6 μL	
5 (13-15)	20 pM AVB + LPS		15 nM	8 μL	6 μL	
6 (16-18)	200 pM AVB + LPS		150 nM	8 μL	6 μL	
7 (19-21)	2 nM AVB + LPS		1,500 nM	8 μL	6 μL	
8 (22-24)	1μM cortisol + LPS				6 μL	3 μL

3) After adding the solutions, each tube is gently mixed and equally divided into 3 tubes, containing 2 mL each. The tubes are closed with dual position snap caps to the first position and returned into the incubator (37 °C, 5% CO_2) for an appropriate period, depending on the time of treatment (3 or 24 hours).

3.2.2.6 Cytokine samples preparation

1) After 3 and 24-hour incubation, the tubes are removed from the incubator and centrifuged at 25 °C for 5 minutes at 1,500 rpm.
2) The supernatant is transferred into a new Eppendorf. The pellet is discarded.
3) The samples are stored in the fridge until the implementation of specific ELISA test.

3.2.3 LDH cytotoxicity test

Cell-free supernatants, relocated from cell treatments after 16 and 24 h, are examined to determine the cell death rate during treatments with avobenzone.

Necrotic cell death is evaluated by determing the damage of the plasma membrane. The cytotoxicity assays measuring necrosis are divided into two categories: those based on DNA binding dyes uptake through damaged plasma membranes (i.e. propidium iodide); and the ones based on the leakage of intracellular molecules through the impaired cell membrane. Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme present in almost every cell and is released into the culture supernatant when the plasma membrane is damaged. LDH leakage is determined by a colorimetric assay with a tetrazolium salt. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a catalyst in the reaction mixture (diaphorase) uses NADH to reduce tetrazolium salt INT to a coloured formazan product. The amount of formazan product can be colorimetrically quantified using standard spectroscopy. Because of the linearity of the assay, it can be used to assess the percentage of necrotic cells in a sample. (64) (65)

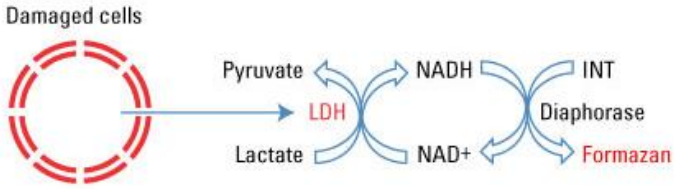

Figure 9: A two-step enzymatic reaction of LDH cytotoxicity assay (66)

Protocol

Kit used: LDH Cytotoxicity Detection Kit User Manual (Clontech Laboratories, Inc.)

1) Cell-free culture supernatants are examined at room temperature right after RNA and protein samples preparation. The supernatants are transferred in 50 µL per well to an optically clear, flat-bottom 96-well plate.
2) 50 µg per well of freshly prepared Reaction Mixture (see Chapter 3.1.6) is added and incubated for 10 - 15 min, protected from light.
3) The absorbance is measured at wavelength 492 nm, using a multiwell plate reader.
4) To calculate the toxicity and cell death during AVB treatments, the resulting values are substituted into the following equation:

$$\text{Viability (\%)} = \frac{\text{Control (DMSO) absorbance}}{\text{Sample absorbance}} \times 100$$

3.2.4 Methods for mRNA investigation

3.2.4.1 Isolation, purification and concentration measurement of RNA

Tri Reagent is a quick and convenient reagent to isolate RNA, DNA and proteins. It is a mixture of quanidine thiocyanate and phenol in a monophase solution, which homogenize samples. After adding chloroform, the mixture separates into three phases:

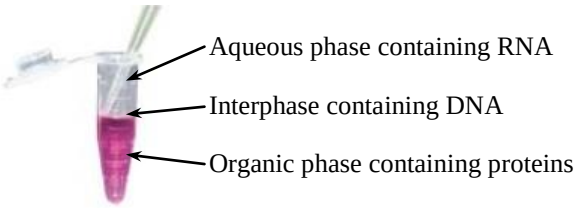

Figure 10: Separation of the lysed sample into three phases (67) (68)

The resulting RNA is intact with little or no contaminating DNA and protein. (67)

Protocol

Additional instructions for RNA examination: you are required to use sterile tubes, tips with a filter and wear gloves.

Phase separation

1) Frozen RNA samples from cell treatment (Chapter 3.2.2.3) are heated to the room temperature and 200 µL of chloroform per Eppendorf is added.
2) The samples are vortexed for approximately 1 minute to mix it thoroughly and incubated for 2 minutes at room temperature.
3) The resulting mixure is centrifuged at 12,000 rpm for 15 minutes at 4 °C.
4) Samples are removed out of the centrifuge. The centrifugation separates the mixure into 3 phases; a red organic phase, an interphase and a transparent aqueous phase.

RNA isolation

1) The upper aqueous phase is completely transferred in a new Eppendorf.
2) 500 µL of isopropranol is added to the RNA solution and mixed.
3) Eppendorfs are placed in the fridge and incubated at 4 °C at least over the night. Samples are proceeded the following day or later.
4) After the incubation, samples are heated to the room temperature and vigorously shaken for 10 – 20 s to be thoroughly mixed.
5) Eppendorfs are centrifuged at 12,000 rpm for 10 minutes at 4 °C. The RNA precipitate forms a pellet on the side and the bottom of the Eppendorf.
6) The supernatant is discarded using the tip with filter.

7) The RNA pellet is washed by adding 1 mL of 75% ethanol solution. (The preparation is described in Chapter 3.1.6.)
8) The samples are vortexed to be mixed thoroughly and centrifuged at 7,500 rpm for 5 min at 4 °C.
9) After the centrifugation, there is a RNA pellet on the bottom, and a supernatant, which should be discarded using a pipette. To ensure complete liquid removal, Eppendorfs' openings are covered with a porous parafilm perforated using a needle. The samples are then placed in vacuum for 30 minutes.
10) 20 μL of DEPC H_2O is added to the RNA pellet.
11) The samples are placed in the thermostat at 60 °C for 10 minutes. Higher temperature ensures the samples dissolving completely.

Assessment of RNA quality

Nucleic acids can be quantified at 260 nm because they possess a clearly defined peak maximum at this wavelength. 40 μg/mL RNA solution has an optical density of 1.0 A in a 10 mm pathlength cell. To calculate the RNA concentration of the sample, factor 40 can be inserted into the formula:

$$Concentration = Abs(260) \times Factor$$

RNA extracted from the cells requires extensive purification to remove protein impurity. The indication of purity is given by the 260/280 nm absorbance ratio. Pure DNA and RNA preparations have ratios of ~ 1.8 and ~ 2.0, respectively, deviations from this indicate the presence of impurities which must be taken into consideration when interpreting the results. An elevated absorbance at 230 nm can also exhibit the presence of impurities. 230 nm is near the absorbance maximum of peptide bonds and may also indicate interference from common buffers such as Tris and EDTA. When measuring RNA samples, the 260/230 ratio should be > 2.0. A ratio lower than this, is generally an indicative of contamination with guanidinium thiocyanate, a reagent commonly used in RNA purification, which absorbs over the 230 - 260 nm range.

Background Correction at a wavelength well apart from the nucleic acid or protein peaks is often used to compensate the effects of background absorbance. The procedure can adjust to the effects of turbidity, stray particulates and high-absorbance buffer solutions. (69)

Procedure

Purity and RNA concentration of the samples is measured using NanoVue^{TM} UV/Visible Spectrophotometer (GE Healthcare) according to the manufacturer's instructions.

DEPC H_2O absorbance at wavelength 320 nm is used as a background correction. RNA concentration, 260/280 and 260/230 ratios are determined.

3.2.4.2 Two-step quantitative reverse transcription PCR

When the starting material is RNA, the quantitative reverse transcription PCR (RT-qPCR) is used. The RNA is first transcribed into a complementary DNA (cDNA) by reverse transcriptase from the total RNA or mRNA. The cDNA is used as a template for the qPCR reaction. RT-qPCR is used in a variety of applications, including gene expression analysis, disease research, genetic testing, microarray validation. (70) In the two-step RT-qPCR, reverse transcription and PCR are performed as two separate reactions. It is usually used when you have a very limited amount of sample and/or you need to examine multiple targets from a single RNA source. You can also optimize the reaction conditions for each individual step.

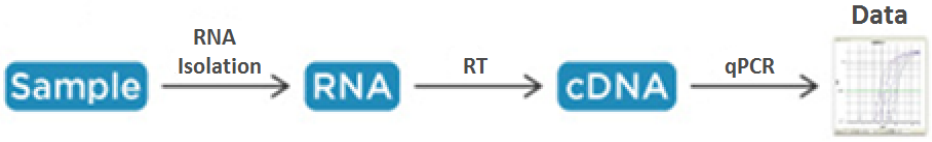

Figure 11: Workflow of the two-step RT-qPCR procedure (71)

At first, the RNA is converted to a cDNA, using random primers, oligo(dT) primers, or even sequence specific primers. The primers anneal to the RNA template and enable the reverse transcriptase enzymes to start the synthesis. (70) (71) cDNA strands are built using deoxynucleotide triphosphates (dNTPs), which are single units of the bases A, T, G and C. (72) At reverse transcription, a mixture of oligo (dT)s and random primers is often used, because they convert all mRNA species in the sample into cDNA. (71)

qPCR is in our case performed with TaqMan Gene Expression Assay, which is based on 5' nuclease chemistry, using a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. A method is based on a pair of unlabeled primers and probe with dye label and nonfluorescent quencher. They anneal to the specific sequences of a single stranded cDNA. Using the unlabeled primers and a template, the DNA polymerase synthesizes a new strand. When it comes to the probe, it cleaves the probe and also separates the dye and the quencher, which results in a fluorescence increase. (73) The reverse transcription and qPCR protocols consist of one (for RT) or more (for qPCR) cycles, where thermal conditions change in order to provide the optimal temperature for every step (e.g. denaturation, annealing and elongation).

3.2.4.2.1 Retro transcription

Protocol

Kit used: High capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA)
The kit is used for quantitatively convert 2 µg of the total RNA in a 20 µL reaction to a single-stranded cDNA, which is suitable for quantitative PCR applications. (74)
The RT samples consist of 10 µL RT RNA sample and a 10 µL 2x RT Master Mix.

1) The 10-µL RT RNA sample preparation: $B = \frac{2\ \mu g}{A}$

$$10\ \mu L - B = C$$

A measured RNA sample concentration [µg/µL]

B sample volume which contains 2 µg of the RNA [µg]

C volume of DEPC H_2O required addition to reach the final volume 10 µg [µL]

Table 7: The 2x RT master mix preparation

Component	**Volume/Reaction (µL)**
10x RT Buffer	2.0
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
Nuclease-free H_2O	4.2
Total per Reaction	10.0

2) The 2x RT master mix is prepared in a way to include additional reaction in volume calculation to provide excess volume for the loss that occurs during the reagent transfer.
3) The mandatory volume of the 2x RT Master Mix is added to the RT RNA samples.
4) Eppendorfs are briefly vortexed and centrifuged to spin down the content and to eliminate air bubbles.
5) A thermal cycler GeneAmp PCR System 2700 (Applied Systems) is set on the program optimized for use with the High Capacity cDNA Reverse Transcription Kits.

Table 8: The thermal Cycler Program for the High Capacity cDNA Reverse Transcription

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

6) At the beginning of step 4 (Table 8) the samples are removed from the thermal cycler and 30 µL of DEPC H_2O is added to each Eppendorf. The samples are stored in the freezer (-15 to -25 °C).

3.2.4.2.2 Real-Time PCR

Protocol

Kit used: TaqMan Gene Expression Assay (Applied Biosystems, CA, USA)

For the gene which encodes RACK1 protein, the dye-labeled GNB2L1 probe is used. Probe for 18S ribosomal RNA is used as an endogenous control. Endogenous controls are genes, used to normalize differences in genes under investigation.

Table 9: PCR reaction mix components for GNB2L1 gene and 18S housekeeping gene

PCR reaction mix component	**Volume per 20-µL single reaction (µL)**	
	GNB2L1 gene sample	**18S sample**
20X TaqMan Gene Expression Assay	1	1
2X TaqMan Gene Expression Master Mix	10	10
cDNA template (1 to 100 ng)	2	1
RNase-free water	7	8

1) Replicates are made for each reaction (duplicates or triplicates).
2) Sample components are pipetted in striped tubes; the protocol is performed on ice, avoiding strong light sources.
3) The tubes are closed with cap-stripes and sealed by using a cap installing tool, and briefly mixed. They are centrifuged to eliminate bubbles and put droplets down the tube wall into the solution.
4) The tube-stripes are loaded into the real-time instrument (ABI PRISM® 7000 Sequence Detection System; Applied Biosystems).

   Table 10: Thermal Cycling Conditions (75)

Initial steps		**PCR (each of 40 cycles)**	
UNG activation	DNA polimerase activation	Melt	Anneal/Extend
2 min at 50 °C	10 min at 95 °C	15 s at 95 °C	1 min at 60 °C

5) After cycling, the baseline and threshold values are set to the amplification plot. Ct (threshold cycle) values are determined.
6) To calculate the relative expression of GNB2L1 gene in each sample in comparison with the control, the comparative method (2^{-DDCt}) of relative quantification is applied:

$$DCt = Target\ Ct - Housekeeping\ Ct$$

$$DDCt = Sample\ DCt - Control\ DCt$$

$$Target\ gene\ expression = 2^{-DDCt}$$

3.2.5 Methods for protein investigation

Western blot (WB) is a common research method to separate, identify and analyze proteins. In this technique a mixture of proteins is separated through gel electrophoresis based on their molecular weight. Protein lines are transferred from the gel to a membrane, which is incubated with labelled antibodies specific to the protein of interest. (76) Labelled antibodies are detected using chemiluminiscent substrate. An image of the membrane with detected signals is taken and the result is densytometric analyzed. (77)

Figure 12: The entire process of producing a Western blot (77)

3.2.5.1 Bradford Protein assay

Bradford assay is a simple and accurate colorimetric method to determine concentration of the solubilised protein. The Coomassie blue dye binds to primary basic and aromatic amino acids residues, especially arginine. When binding to protein occurs, the absorbance maximum for an acidic solution dye shifts from 465 nm to 595 nm. (78) This alternation is visible as a colour change from brown to blue in proportion with the amount of protein present in the sample. Protein determinations are made by comparison to the colour response of protein assay standards, usually prepared as a series of known dilutions of bovine serum albumin (BSA) or bovine gamma globulin (BGG). (79) Colour change in response to protein quantity is measured with a spectrophotometer or plate reader at 595 nm. Interference may be caused by chemical-protein and/or chemical-dye interactions (it is recommended to check in the protocol data if any of buffers, detergents, or other reagents used in the samples, may interfere with the dye reagent). (78)

Protocol

Kit used: Bio-Rad Protein Assay Kit II (Bio-Rad, Segrate, Italy)

For protein samples with low concentrations (< 25 µg/mL, 1 – 20 µg total) the microassay procedure is used. BSA has a role of standard to create a protein standard curve. (80)

1) Protein samples are thawed. Dilutions of a protein standard BSA and sample solutions are prepared according to the Table 11. BSA dilutions with a defined concentration are prepared in duplicates and protein samples are diluted to four different concentrations.

Table 11: Standard and sample dilutions for Bradford Assay

	Distilled water (µL)	BSA (µL)	Sample (µL)	Dye reagent (µL)
Protein standard curve (µg/mL)				
Blank	800			200
1	790	10		200
2	780	20		200
5	750	50		200
10	700	100		200
15	650	150		200
Samples preparation (µL of sample/mL)				
1.5	798.5		1.5	200
2.0	798.0		2.0	200
2.5	797.5		2.5	200
3.0	797.0		3.0	200

2) 800 µL of each standard and sample solution is pipetted into a test tube. Then 200 µL of dye reagent concentrate is added per tube and the mixture is vortexed.
3) 200 µL of each solution is transferred to a 96-well optical plate.
4) The samples are incubated at a room temperature for at least 5 minutes.
5) Absorbance of a standard curve and samples is measured at the wavelength 595 nm using a multiwell plate reader.

3.2.5.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

For protein separation, polyacrylamide gels are formed by the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with TEMED. The separation of molecules within a gel is determined by the relative size of the pores formed inside the gel. (81) For denaturing conditions, sodium dodecyl sulphate (SDS) is added to the system. It forms a negatively charged micelle around the protein, dissolves his 3D structure and the charge of the protein becomes relative to its size resulting in separation of proteins only by size. (77)

The gel typically consists of two parts with different densities: the stacking gel and the separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making it porous, which separates protein poorly but allows them to form thin, sharply defined bands. The lower, separating gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Proteins are separated by their size, as smaller proteins travel more easily and rapidly than larger proteins. The

samples and a molecular weight marker are loaded into the wells, and the empty wells are loaded with a sample buffer. The gel is then connected to the power supply. (76) During the electrophoresis the negatively charged proteins move toward the positively charged anode. (77) The voltage is very important, as a high voltage can overheat and distort the bands. (76)

Protocol

- **Note:** Acrylamide is neurotoxic, therefore wearing gloves is required during the whole procedure.

1) The glass plates are cleaned with distilled water and alcohol, put inside a green casting frame and placed in a casting stand.
2) The ability to keep hold of liquid between two glass plates is checked by using water. After the test, water is discarded and replaced with Running gel (for the preparation see Materials, Chapter 3.1.6).
3) Using a suction pipette, Running gel is carefully added until the level reaches about 1 cm below the glass plates' rim. Water is added to the top of the frame.
4) After approximately 30 minutes the gel solidifies and the residual water is removed completely.
5) The Running gel is overlaid with Stacking gel (for the preparation see Materials, Chapter 3.1.6). Plastic combs are inserted, ensuring there are no air bubbles.
6) After 15 - 30 minutes the second gel solidifies, too, and the combs are carefully removed. The adequacy of wells' formation should be examined.
7) The glass plates are removed from the green casting frame and transferred into the electrophoresis tank. Running Buffer 1x (for the preparation see Materials, Chapter 3.1.6) is poured into the electrophorator.
8) Using gel loading tips, the samples with exact mass of proteins are loaded into the wells (volume calculated on the basis of Bradford Protein assay results), preceded and followed by the marker (2.5 μL per well). Wells without samples or marker are filled with Laemmli Sample Buffer 1x (10 μL per well, for the preparation see Materials, Chapter 3.1.6).
9) The electrophoresis tank is closed with a lid and starts running at 80 - 100 V (constant voltage) for cca 30 minutes and then continues at 100 – 120 V for approximately 1 hour or until the dye front runs off the bottom of the gel.

3.2.5.3 Western blotting

3.2.5.3.1 Electrophoretic transfer

After the separation of proteins, they are transferred to a solid support by using an electric field. As a protein-binding support, two types of membrane are used, nitrocellulose or PVDF. The membrane and the gel are placed together in close contact, filter paper and fiber pads are used on both sides as protection. They have to be situated in the right order, so that the negatively charged proteins, migrating from the gel towards the positively-charged electrode, are stopped and bound by the membrane. The transfer can be performed in wet or semi-dry conditions. (76) Wet conditions are usually preferred for larger proteins and more efficient regarding the quality and the sharpness of bands. (77)

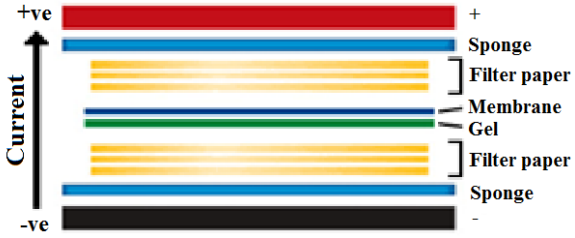

Figure 13: The assembly of the sandwich in an electrophoretic transfer (82)

Protocol

1) The polyvinylidene difluoride (PVDF) membrane is cut to the dimensions of the gel.
2) Before the beginning of the transfer the filter paper and the foam pads are soaked in cold Transfer Buffer 1x (for the preparation see Materials, Chapter 3.1.6) for at least 5 min.
3) The glass plates are removed from the electrophoresis apparatus and placed in cold Transfer Buffer 1x where the gel is retrieved from the plates. To equilibrate gel and prevent shrinking while transferring, it is incubated in Transfer buffer for 3 - 5 min.
4) The PVDF membrane is soaked in methanol for 2 minutes for its activation and then rinsed in distilled water.
5) Transfer sandwiches are created as follows:
   a. The cassette is placed with the black side down and a sandwich is made in the order as follows - foam pad/filter paper/equilibrated gel/membrane/filter paper/foam pad.
      ✓ The side where the sample number 1 is placed should be marked.
      ✓ After sandwiching the gel and the membrane between papers, air bubbles between the gel and membrane can be removed by gently rolling them out with a pipette.
   b. The cassette is clamped firmly together to maintain tight contact between the gel and the membrane.

6) The sandwich cassettes are relocated into the red and black module with the black part of the cassette facing the black support of the module. When the electric field is applied, the negatively-charged proteins travel towards the positively-charged electrode (the red part of the module).
7) The module is placed into the electrophoresis tank, the cooling unit is added next to it and the module is completely filled with cold Transfer Buffer 1x.
8) The lid is closed and the whole running module is dipped in a polystyrene box filled with ice to maintain 4 °C.
9) The transfer runs at constant amperage of 250 A for approximately 80 min.
10) The membrane is removed from the sandwich and any excess margins are cut off.
11) The membrane is relocated in a smooth Petri dish and washed in Tris Buffered Saline Tween (TBST; for preparation see Materials, Chapter 3.1.6). It is also stored in the same solution before further procedures are continued.

- **Note:** During washings and incubations the membranes should always be positioned with the protein side up (the side which was in direct contact with the gel during the transfer).

3.2.5.3.2 Visualization of proteins in membranes

To check the transfer efficiency before the proceeding with WB, the entire protein bands on the membrane are stained with a dye, such as Ponceau Red solution or amido black 10B. (83)

Protocol

1) The stock Ponceau Red (for preparation see Materials, Chapter 3.1.6) is diluted 1:10 in distilled water. The membrane is incubated in a solution for 5 minutes while agitating.
2) The membrane is washed extensively in water until the protein bands are well defined.
3) The membrane is destained completely by repeated washings in TBST or water.

3.2.5.3.3 Blocking the membrane nonspecific sites

Blocking the remaining surface of the membrane is a very important step of Western blotting, as it prevents antibodies during subsequent procedure from binding to the membrane nonspecifically. Common substances for blocking are 5% BSA or non-fat dried milk diluted in TBST to reduce the background. Milk is preferred as an inexpensive and widely available choice, but milk proteins (i.e. casein) are not compatible with all detection labels, so appropriate blocking solution must be chosen carefully. (76) (83)

Protocol

1) A 7-10% milk solution in TBST is prepared.
2) The membrane is soaked in the 7-10% milk solution and incubated for at least 1 hour under agitation at room temperature.

3.2.5.3.4 Antibody incubation

This is an immunoassay procedure, which consists of incubations with different immunochemical reagents separated by wash steps. Washings are necessary to remove unbound reagents and reduce background. (83) At first, the blocked membrane is incubated with the primary antibody which recognizes the target protein. After washing, the membrane is incubated with the secondary antibody that binds to the primary antibody. The secondary antibody is labelled with a reporter. When using a polyclonal antibody as secondary antibody, it may give rise to some background. (77)

Protocol

In the antibody incubation procedure, our target proteins are RACK1 as a protein under investigation and ß-actin as internal reference to normalize results.

1) The membrane is washed several times in TBST while agitating, 5 min per wash.
2) The primary antibodies are diluted in TBST. ß-actin antibody has dilution 1:2,500 and RACK1 antibody has dilution 1:1,000. (Usually 25 mL solution per membrane is prepared.)
3) The membrane is incubated overnight at 4 °C on a plate shaker.

- **Note:** Milk blocking phase can be performed overnight and the antibody can be successfully placed the following morning. In this case the antibody is incubated for 1 hour at 4 °C under agitation.

4) After an overnight incubation in the primary antibody solution, at least 5 washes of 5 min or more in TBST are performed to remove any residual primary antibody.
5) The secondary antibodies are diluted in 5% milk TBST solution (5 g milk powder per 100 mL TBST) at the suggested dilution. After an incubation in primary antibodies for β-actin and RACK1 detection, the anti-mouse antibody is recommended as a secondary antibody and it is diluted in the ratio 1:15,000.
6) The membranes are incubated in the secondary antibody for 1 hour under agitation at room temperature. After incubation, at least five 5-minutes washes in TBST and under agitation are performed.

3.2.5.4 Western Blot detection and imaging

Enzymatic labels are most commonly used for Western blotting detection and they are extremely sensitive when optimized with an appropriate substrate. Horseradish peroxidise (HRP) is the most extensively used enzyme for protein detection and many chromogenic, fluorogenic and chemiluminiscent substrates are available for use. (83)

Chemiluminescence is a chemical reaction that produces light and has become a common detection method for Western blotting because of its high sensitivity. The WB membrane is incubated with a solution containing the chemiluminescent substrate. In the presence of peroxide, the HRP enzyme conjugated secondary antibodies catalyze the oxidation of luminol, which generates light. An enhancer is included in the substrate solution to increase the longevity and intensity of the emitted light. (84) The signal is a transient product of the enzyme-substrate reaction and persists only during the reaction. At the start, when the reaction produces a stable output of light, the sensitive detection of emitted signals can be documented with an X-ray film or a digital imaging equipment. (83)

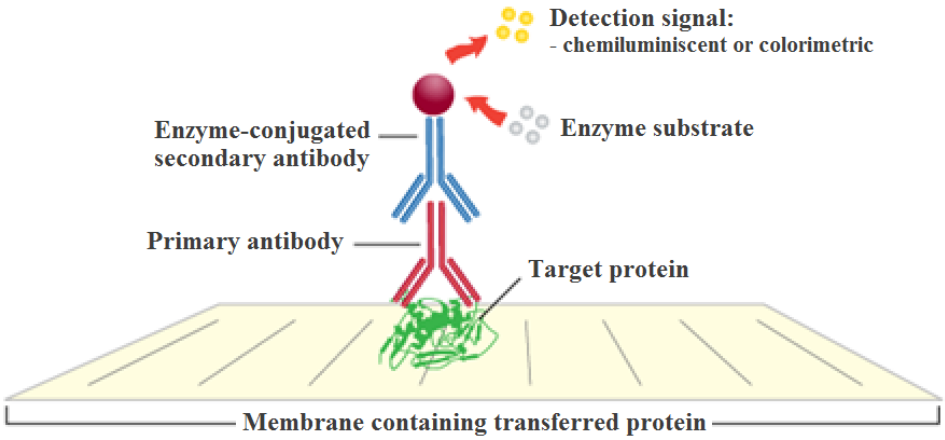

Figure 14: Detection in Western blotting procedure (85)

Protocol

Kit used: Clarity Western ECL blotting Substrate Kit (Bio-Rad)

1) The membrane should be moistened in wash buffer to prevent drying out during the subsequent steps.
2) The ECL substrate is prepared in a tube following the proportion of solution A and B provided by the manufacturer. For Bio-Rad Clarity ECL, the components are mixed in the ratio 1:1. Approximately 0.1 mL of solution/cm^2 of the membrane is prepared.
3) Using a pipette, the ECL mixture is transferred on the transparent pallet and the membrane is completely dipped in the solution with no air bubbles. The membrane is incubated in ECL for 2 minutes at room temperature.

4) The membrane is removed from the substrate solution, the excess is drained off and it is placed in a plastic sheet protector to prevent the membrane from drying.
5) Using the ChemiDoc MP digital imager with Image Lab Software (Bio-Rad), chemiluminiscence is measured by running SAM (Signal Accumulation Mode) Protocol. A digital picture of marker bands on the membrane is taken for subsequent analysis and display of data, too.

NOTE: At first Clarity **ECL substrate** is used to measure the chemiluminiscence. If protein lanes are not visible on SAM picture after the treatment with ECL, the membrane is rinsed for 5 seconds in TBST to completely remove Clarity substrate. Then a stronger substrate is used following the protocol above. The protein lines will be more visible, but possible stains on the background will be exposed as well.

3.2.5.5 Densitometric analysis

Chemiluminescent Western blotting is a semi-quantitative method, because it measures the relative expression of the target proteins, but not an absolute measure of quantity. (77) (86) Using imaging data, the presence of a protein of interest is confirmed. It should be normalized to an internal reference to assess its relative amount. The range that allows an even and precise quantitation where the signal intensity is still proportional to the amount of protein is called the linear dynamic range. It is important to avoid signal saturation due to excessive amounts of protein or high concentrations of antibodies. (77)

Protocol

The chemiluminescent blots are imaged with the ChemiDoc MP imager (Bio-Rad). The Band Analysis tools of ImageLab software (Bio-Rad) are used to select and determine the density of the protein bands. RACK1 bands are normalized to the internal reference protein ß-actin and then relative densities of samples values are compared to the control value.

3.2.6 Methods for cytokine investigation

The enzyme-linked immunosorbent assay (ELISA) is an enzyme-based immunoassay method which is useful for measuring antigen concentrations. Cytokine sandwich ELISA is a sensitive immunoassay that can specifically detect and quantify the concentration of cytokines and chemokines. Highly-purified anti-cytokine antibodies (capture antibodies) are absorbed onto plastic microwell plates and serve to specifically capture soluble cytokine proteins present in samples which are applied to the plate. After washing away the unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin or streptavidin. After the addition of a chromogenic substrate, the level of coloured product generated by the bond can be measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density (Figure 15).

A standard curve is incorporated into a sandwich ELISA assay by making serial dilutions of a standard cytokine protein solution of known concentration. The concentrations of the putative cytokine-containing samples can be interpolated from the standard curve. (87)

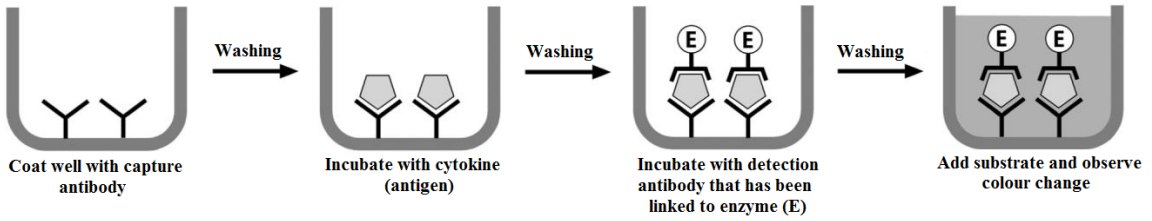

Figure 15: The experimental principle of sandwich ELISA with capture and detection antibodies specific to the cytokine(s) of interest (88)

3.2.6.1 Human TNF alpha ELISA

Kit used: Human TNF alpha ELISA Ready-SET-Go (Affymetrix, eBioscience)

Protocol

1) ELISA plate is coated with 100 µL/well of capture antibody in Coating Buffer. The plate is sealed and incubated overnight at 4 °C.
2) The capture antibody solution is removed and the plate is washed 3 times with > 250 µL/well Wash Buffer (for preparation see Materials, Chapter 3.1.6).
3) 1 part of 5x concentrated Blocking Buffer (5x Assay Diluent) is diluted with 4 parts of distilled water. Wells are blocked with 200 µL/well of 1x Assay Diluent. The plate is incubated at room temperature for 1 hour.
4) Washing is repeated as in step 2).

5) Using 1x Assay Diluent, human TNF alpha standard (1 µg/mL) is diluted to prepare the top standard concentration (1,000 pg/mL). 100 µL/well of top standard concentration and 100 µL/well of 1x Assay Diluent are added to the appropriate wells. 2-fold serial dilutions of the top standards are performed at ELISA plate to make the standard curve for a total of 8 points (the last is blank – just 1x Assay Diluent).
6) Each sample is added in triplets in volume 100 µL/well. The plate is sealed and incubated at room temperature for 2 hours and then washed.
7) Washing is repeated as in step 2).
8) 100 µL/well of biotinylated detector-antibody diluted in 1x Assay Diluent is added. The plate is sealed and incubated at room temperature for 1 hour.
9) Washing is repeated as in step 2).
10) 100 µL/well of Avidin-HRP diluted in 1x Assay Diluent is added. The plate is sealed and incubated at room temperature for 30 minutes.
11) Avidin-HRP is discarded and the plate is washed 3 times, enabled to soak wells 1 - 2 minutes in > 250 µL/well of Washed Buffer prior to aspiration. The plate is blotted on absorbent paper.
12) 100 µL/well of Substrate Solution is added to each well. The plate is incubated at room temperature for 15 minutes or less if the enzymatic colour reaction proceeds faster.
13) Absorbance is measured at 595 nm with a multiwell plate reader.

3.2.6.2 Human IL-8 ELISA

Kit used: Human Interleukin-8 ELISA (Immuno Tools)

Protocol

For IL-8 release analysis the reagents from Immuno Tools Human Interleukin-8 ELISA Kit are used. At the work procedure the same protocol as described above is used, at Human TNF alpha ELISA chapter, with slight changes described below:

a. Prior to the incorporation to the wells, the samples should be diluted by factor 40, so that measurements would remain inside the linearity range.
b. Using 1x Assay Diluent, the human IL-8 standard (50 ng/mL) is diluted to prepare the top standard concentration (500 pg/mL). The preparation of the 8-point standard curve is the same as in the protocol above.

3.2.7 Data analysis

Considering the reliability of data from the measurements, certain rules should be abided by. When spectrophotometric measurements are performed using the standard curve to determine the concentration of samples (Bradford assay, ELISA), the value of the curve ratio (R) has to be greater than 0.98 to continue with the data processing. In qPCR assay, the samples are tested in duplicates, and the difference between values in duplicate should be less than 0.5, otherwise the data are dismissed. ELISA tests are carried out in triplicates. If one out of three values in the triplicate is significantly different, it is dropped and the average is calculated only with regard to the remaining two. In the RT-qPCR and WB, the target gene and the protein respectively are normalized by the measurements of internal standards.

The measurements are statistically evaluated using the SPSS Statistics programme. In every test, at least three independent biological replicates are performed, in which the samples are compared to a control and presented as relative values in comparison to the control value 1.0 or 100%. The results of repetitions are presented as mean values with the standard deviation (mean ± SD). To define the statistical differences between the test samples and the control, Dunnett's test is used. This is a procedure for multiple comparisons, where each treatment is compared to the control, common to all of them (many-to-one comparison). The difference between the two parameters being compared is significant when the probability that the sample and the control belong to the same group is less than 5% ($p < 0.05$). Moreover, the level of significance is also determined for values lower than 1% and 0.1% ($p < 0.01$, $p < 0.001$). The results are displayed graphically as relative values of treatments in comparison to a control (mean ± SD). If some degree of significance is defined there, it is labelled beside the results. Graphs are made using the GraphPad Prism 7 programme.

4 RESULTS AND DISCUSSION

To study the influence of AVB on the immune system, the laboratory testing and the obtained results were divided into three parts: 1. assessment of AVB on cell viability; 2. assessment of the effects of AVB on RACK1 expression at both the mRNA and the protein levels; 3. assessment of the immunomodulatory effects. The same concentration range of AVB (from 2 pM to 2,000 pM) was used for all experiments. DMSO was used as a solvent and in order to avoid possible interferences in measurements, the maximum concentration of 0.2% DMSO was used. The results obtained are presented below.

4.1 DETERMINATION OF CELL VIABILITY

First, we investigated the cytotoxicity of AVB following 16 and 24-hour of incubation. To determine cell viability, LDH leakage was used. The assay is based on a colorimetric change and is described in detail in Chapter 3.2.3 of Materials and Methods section. The purpose of this experiment was to determine if AVB at the tested concentrations possibly has an impact on THP-1 cell viability.

AVB was tested at the final concentrations of 2, 20, 200 and 2,000 pM. We used DMSO (0.13%) in the culture medium as a negative control, to exclude the effect of the solvent alone on cell viability. Cell viability was determined by spectrophotometric measurements of the emerging red-coloured formazan dye product. Its absorbance at 492 nm correlates with the amount of lactate dehydrogenase released in the supernatant from the plasma membrane–damaged cells.

Results were statistically analysed and graphically displayed (Figure 16) as percentage of viable cells after AVB treatment in comparison to the control ± SD (standard deviation). If the measurements with respect to the control would be lower than 80%, i.e. the minimum acceptable metabolic activity of cells, we would conclude that AVB does affect cell viability.

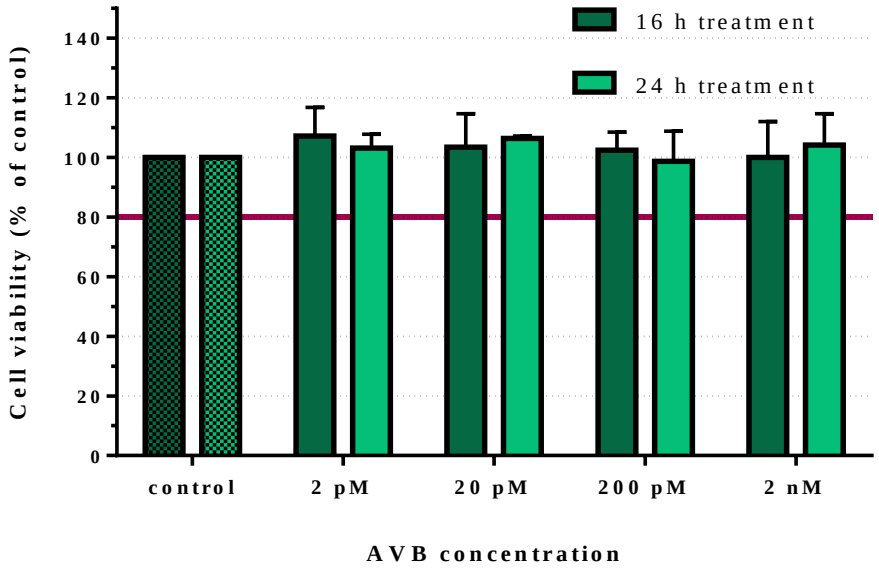

Figure 16: The effect of avobenzone (AVB) on THP-1 cell viability. THP-1 cells were exposed to different concentrations of AVB (2, 20, 200 and 2,000 pM) or DMSO control vehicle (0.13% of the final concentration) for 16 and 24 h. Cell viability was analysed by LDH leakage. The results are expressed as percentage of the control value. The values represent means ± SD (n=3 independent experiments).

As it can be seen from Figure 16, AVB was not cytotoxic at the concentrations tested. After a 16-hour incubation, the highest percentage of cell viability was 107% (2 pM of AVB) and the lowest was 100% (2 nM of AVB). Following a 24-hour incubation, the maximum value was 106% (20 pM of AVB) and the lowest was 99% (200 pM of AVB). No statistically significant deviation from the control was detected at any AVB concentration. Based on these results, we can conclude that AVB in the concentration range from 2 pM to 2 nM does not show cytotoxic effects in THP-1 cells.

4.2 DETERMINATION OF RACK1 EXPRESSION

The impact of AVB on the immune system was first examined through effects on the expression of RACK1, an anchoring protein essential for PKCß activation. Protein kinase C beta is a protein, involved in a number of cascade processes, including the signalling pathways that regulate the production of cytokines. Any possible changes in the expression of RACK1 were investigated by analysing the relative concentrations of mRNA and the protein production in THP-1 cells following the treatment with AVB.

4.2.1 GNB2L1 gene expression

GNB2L1 is the gene whose nucleotide sequence codes for RACK1 protein. A stimulus initiates the transcription of a given DNA sequence to the mRNA molecule whose presence was assessed via RT-qPCR technique. THP-1 cells were treated for 16 h with AVB, followed by RNA isolation, the evaluation of its purity and the concentration determination (Chapter 3.2.4.1). For a good quality of RNA samples, the absorbance ratio of 260 nm and 280 nm has to be $\leq$ 2.0 to generally accept RNA as pure. The secondary measure used to assess nucleic acid purity is at 260/230 ratio and the expected values are commonly present in the range $\geq$ 2.0; otherwise they indicate presence of contaminants. In our experiments, the majority of samples had a 260/280 ratio between 1.9 and 2.0. At the ratio 260/230, samples showed minor deviations from the value 2.0 except for some, which were 0.2 or 0.3 under this margin. We concluded that in these samples the residual phenolic solvent TRIazol, which absorbs in the range near 230 nm, was probably still present. The measured RNA concentrations mostly ranged between 1.0 and 1.2 mg/mL.

As mRNA expression was evaluated by qPCR, mRNA was retrotranscribed to cDNA (Chapter 3.2.4.2). To normalize the results, 18S ribosomal RNA was used. A threshold line in the amplification plot was set to obtain the Ct values, which represented the relative concentration of the target gene. Duplicate samples were discarded if the mutual difference was more than 0.5. The relative changes in gene expression were evaluated by using the 2^{-DDCt} method (Figure 17).

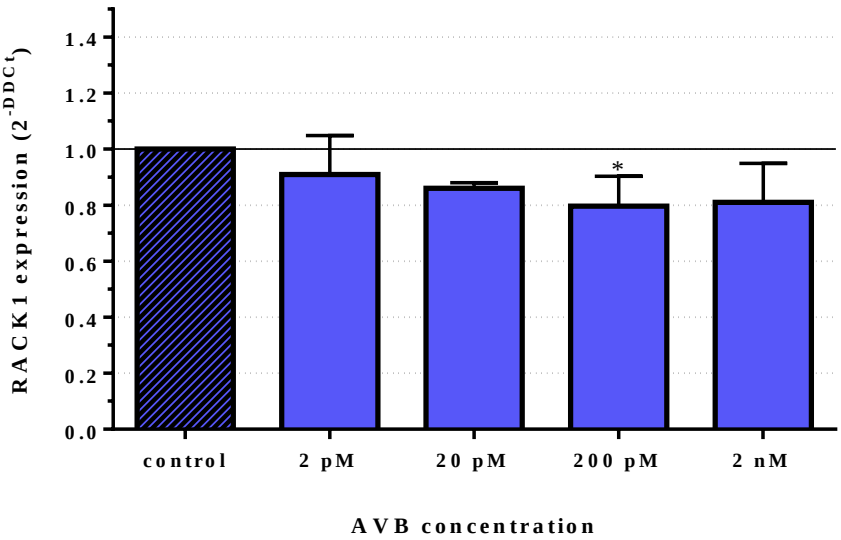

Figure 17: The effect of AVB on RACK1 mRNA expression in THP-1 cell line. THP-1 cells were treated with different AVB concentrations (2, 20, 200, 2,000 pM) or DMSO vehicle control (0.13%

of the final concentration) for 16 hours. The analysis was performed by real-time PCR, using 2^{-DDCt} method and 18S ribosomal RNA as an endogenous reference. The graphic results are presented as means ± SD (n = 3 independent experiments). The statistical analysis was performed by using Dunnett's multiple comparison test, with P values, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, versus control.

After the statistical analysis, we observed a reduction in expression of GNB2L1 gene in THP-1 cells treated with AVB, which reached a statistical significance at 200 pM ($p < 0.05$). The modest reduction in RACK1 mRNA expression observed may be due to the time point of 16 h investigated, while shorter time points (i.e. 6 h) maight give better results. In support of this, we have obtained data with the weak androgen receptor antagonist pp'DDT, which has shown a dose-related decrease in RACK1 expression at a time point of 6 h but not at 16 h.

4.2.2 RACK1 protein expression

The quantitative expression of RACK1 protein in THP-1 cells following 24 h AVB exposure was investigated using Western blot and densitometric analysis.

The Bradford method (Chapter 3.2.5.1) was used to determine the total amount of protein in cell lysates.. We compared the absorbance of samples to standard curve absorbance at defined concentrations to calculate sample protein concentrations.

The samples were examined in four different dilutions and mutually compared to specify the accurate volume of the sample for loading. As an internal control, ß-actin expression was evaluated.

Figure 18: Bradford assay with a standard curve and samples in four different dilutions

10% acrylamide gels were used to separate the cellular proteins (RACK1: 38 - 42 kDa, ß-actin: 31 - 35 kDa). At first, we loaded 10 µg and 20 µg of proteins per well, later we abandoned 20 µg, as it gave saturated signals.

During the evaluation of RACK1 protein expression, some improvements were introduced. At the beginning, we prepared two membranes of each experiment, one to examine RACK1 and the other for ß-actin. Later we changed the way of work, cutting the same membrane into two parts separating the lines of both proteins and continued with each section of the membrane independently. In this way we obtained more consistent results, as

the result of both, RACK1 and ß-actin, were obtained from the same loading and transfer. After electrophoresis (Chapter 3.2.5.2) the transfer and incubation with the specific antibodies (Chapter 3.2.5.3), the detection (Chapter 3.2.5.4) and the densitometic analysis (Chapter 3.2.5.5) were performed. RACK1 expression was normalized with ß-actin immunoreactivity, and later the expression levels were compared with the control. The final results are presented in Figure 19. THP-1 cells showed a decrease in RACK1 expression following AVB exposure. All treatments, except for lowest AVB concentration samples, reached a significant decrease. 2 pM AVB treatment showed a 13% decrease in comparison to the control. 20 pM AVB sample reached 86% ($p < 0.05$), 200 pM 82% ($p < 0.05$), and 2 nM 79% ($p < 0:01$) expression of RACK1 protein in relation to the control.

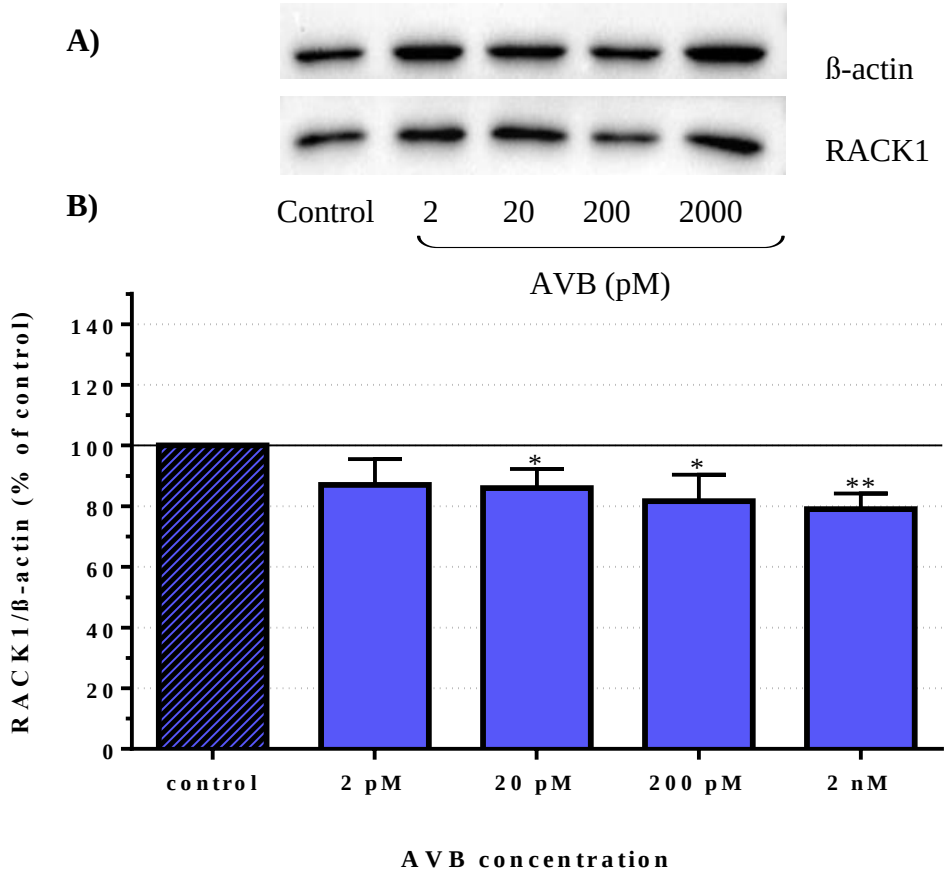

Figure 19: The effects of AVB on RACK1 protein expression in THP-1 cells. THP-1 cells were exposed to AVB (2, 20, 200 and 2000 pM) or DMSO control vehicle (0.13% of the final concentration) for 24 h. Whole cell lysates (10 µg) were separated by SDS-PAGE, transferred and RACK1 protein immunoreactivity in comparison with ß-actin was evaluated. A) Representative Western Blot of RACK1 and ß-actin immunoreactivity following 24 h of treatment. B) The relative densitometric analysis. The results are presented as means ± SD (n = 3 independent experiments). The statistical analysis was performed using Dunnett's multiple comparison test, with P values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, versus control.

Reviewing the results of both investigation methods, RT-qPCR and WB, a modest, but statistically significant, decrease in RACK1 expression at both mRNA and protein levels was observed in THP-1 exposure to AVB, indicating a modest antagonist effect of AVB.

4.3 EFFECT OF AVB ON LPS-INDUCED CYTOKINE RELEASE

Using ELISA tests (Chapter 3.2.6), we examined LPS-induced release of two pro-inflammatory cytokines, namely TNF-alpha and IL-8, in the presence or absence of AVB. THP-1 cells were treated with AVB (2 nM) or DMSO (0.13%) as the vehicle control. To induce the cytokine production, LPS (10 µg/L) was added. As a positive control cortisol in concentration 1 µM was used. Cortisol is known for its inhibitory activity on inflammation. After cell treatments, measurements of each sample were performed in triplicates. The results are calculated as the relative concentration of released cytokines in comparison to the cells treated with LPS only.

4.3.1 TNF-α release

The final results of LPS-induced TNF-α release are presented in Figure 20. As expected, DMSO and 2 nM AVB alone did not induce TNF-α release. The positive control cortisol exhibited a significant inhibition of LPS-induced cytokine release, confirming the proper response of the experimental method. All samples treated with AVB also significantly impaired the LPS-induced cytokine production. After 3 h, the TNF-α release was 76% at 2 pM AVB, 71% at 20 pM AVB, 59% at 200 pM AVB, and 57% at 2 nM AVB cell treatment compared to the LPS-induced sample. After 24 h of incubation, the concentrations of TNF-α were overall similarly reduced.

In view of the effect on TNF-α production, we can conclude that AVB in investigated concentrations displayed antagonistic impact on the production of this pro-inflammatory cytokine.

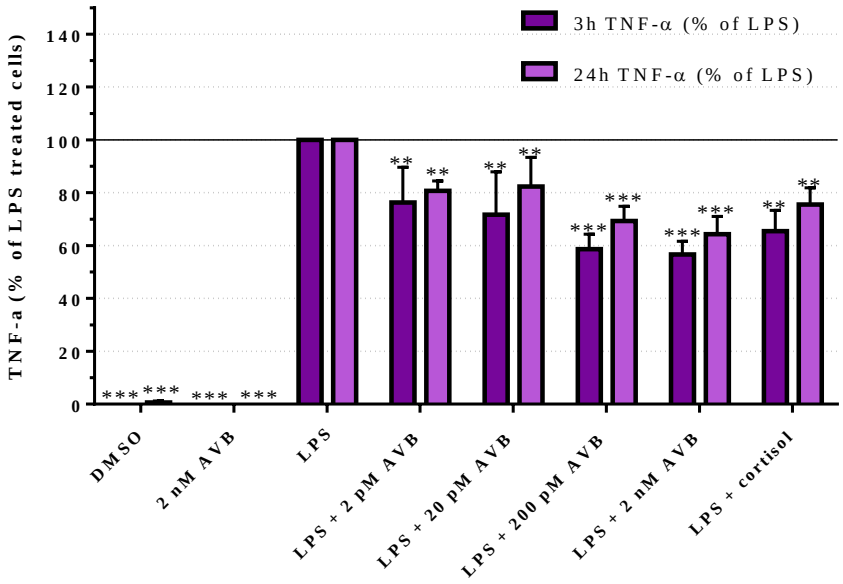

Figure 20: The effect of AVB on LPS-induced TNF-α production in THP-1 cell line following 3 and 24 h of treatment. THP-1 cells were exposed or not to AVB (2, 20, 200 and 2,000 pM), DMSO control vehicle (0.13% of the final concentration), cortisol (1 µM), and stimulated or not with LPS (10 µg/L). Graphic results represent the means ± SD of three independent experiments and are expressed as the percentage of LPS induced TNF-α value. The statistical analysis was performed using Dunnett's multiple comparison test, with P values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, versus LPS treated cells.

4.3.2 IL-8 release

Finally, we evaluated the effect of AVB on LPS-induced IL-8 release. The mean results of the experiment replicates are graphically presented in Figure 21. As one can observe, negative controls did not trigger cytokine IL-8 release and the positive control cortisol significantly inhibited LPS-induced IL-8 production. In line with data observed with TNF-α, a dose-related decline in LPS-induced IL-8 release was observed following the treatment with AVB. The decrease was significant in the range 20 pM - 2 nM. After a 3-hour treatment, 2 pM AVB decreased the production by 19%, 20 pM AVB by 26%, 200 pM AVB by 27%, and 2 nM AVB by 35%. After a 24-hour treatment there is a slight concentration recovery compared to the 3-hour treatment visible. After 24 h, the IL-8 production was decreased by 17% at 2 pM, 30% at 20 pM, 27% at 200 pM and 29% at 2 nM of AVB treatment concentration in comparison to the LPS sample value.

Compared to TNF-α, we observed a lower level of significance. The reason for a greater deviation in results may be the additional step in IL-8 ELISA, where we had to dilute samples prior to ELISA procedure.

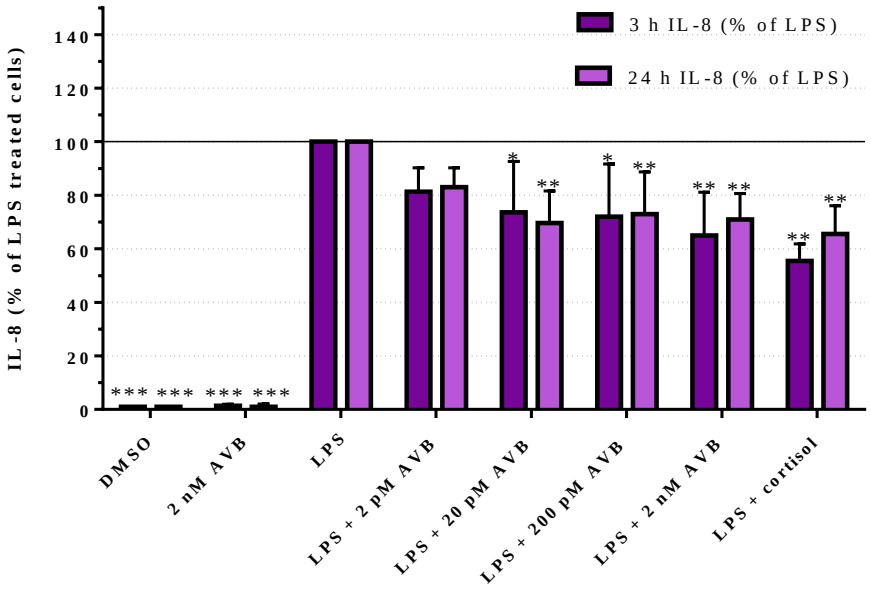

Figure 21: The effect of AVB on LPS-induced IL-8 production in THP-1 cell line following 3 and 24 h of treatment. THP-1 cells were exposed or not to AVB (2, 20, 200 and 2,000 pM), DMSO control vehicle (0.13% of the final concentration), cortisol (1 µM), and stimulated or not with LPS (10 µg/L). The graphic results represent the means ± SD of three independent experiments and are expressed as the percentage of LPS induced IL-8 value. The statistical analysis was performed using Dunnett's multiple comparison test, with P values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, versus LPS treated cells**.**

Based on these results, we can conclude that AVB has antagonistic effects on the release of TNF-α and IL-8 in monocytic cells following exposure to LPS.

There was another research investigating the effect of AVB in the promyeloid cell line THP-1. They tested cell viability and IL-8, IL-1ß release following 24h exposure to AVB in the concentration range from 1 to 250 µg/mL (3.22 - 805.4 µM). AVB did not reach EC_{50} value for cytotoxicity. IL-8 and IL-1ß, measured in monocytes and macrophages, showed an increase in the cytokine production (without additional LPS stimuli), as many other organic filters examined in this research. (39) AVB concentrations in this article were much higher than ours, which were from pM to nM range. Comparing these results, we suspect, that AVB may possess non-monotonic behaviour as many other EDCs. In low concentrations it has antagonistic effect on the immune system, in contrast by high concentrations, where it may act as a sensitizer.

4.4 AVB EXPOSURE LEVELS

It is of high concern, how much humans actually are exposed to avobenzone and what concentration rank we can expect. In the environment or through our skin, we usually contact very low EDC concentrations. To establish avobenzone exposure, published data were investigated. Because of AVB lipophilic nature, we suspected percutaneous absorption after topical application and bio-accumulation in fatty tissues.

To study percutaneous penetration and distribution in skin layers, researchers mainly use four methodologies: very common tape-stripping and diffusion cells, in contrast with less used determination of content in body fluids and tissues. (18) AVB penetration through the skin of humans and animal models were evaluated (Appendix, Table 13). AVB in range from 14 $\mu g/cm^2$ to 3 mg/cm^2 (e.g. 45 $nmol/cm^2$ – 9.66 $\mu mol/cm^2$) was applied on the human skin. People normally apply amounts from the lower part of this range on their skin, i.e. 15 – 25 $\mu g/cm^2$. The results showed that in the human skin the majority of the product penetrates into the *stratum corneum* (approximately 10 – 15% of the applied dose). In the epidermis, the layer under the *stratum corneum*, they measured lower concentrations (0.1 – 2.4% of the applied dose). Furthermore, in the dermis and the receptor fluid (presenting plasma), AVB was undetectable in most cases. Using animal models, the trend was the same, except for higher values detected. AVB was detectable also in the dermis and sometimes in the receptor fluid. Skin of many animal models (mouse, rat) is thinner than human skin, which may affect the penetration level.

Organic UV filters and their metabolites end up in the aquatic environment via bathing, swimming, sewage and landfill. WWTPs are not able to remove or degrade them from the wastewater, so they may also persist in the rivers, which can present 30 - 40% of the total drinking water supply. There is a concern that they possess the ability to pass through the additional treatment from the water source to potable water, and eventually be present in the drinking water. (15) Moreover, organisms in all water environments can be in danger due to the continuous exposure. In publications investigating UV filter occurrence, they usually detect AVB in the ng/L (e.g. 50 pM – 8 nM) range (Appendix, Table 14). According to AVB monitoring in the environment, exposure concentrations are highly variable, dependent on the population density, the geographical area (open or enclosed profile), and the season (peaks at summer seasons, when there is an increase of tourists and water activities). (22) (19)

Because of its lipophilic character, AVB is expected to retain in humans or biota, and consequently it may be stored rather faster than metabolized or excreted. Analysis studies of UV filters residues in aquatic biota were mainly focused on fish, the representative environmental aquatic biota and assumed to be able to bioaccumulate UV-filters because of the lipophilic tissues. (17) In the research, AVB was found in fish tissues (in concentrations ng/g of dry weight) and it preferentially accumulated in body parts which contain fat (Appendix, Table 15). Asit is known, the lipophilic substances bioconcentrate and transfer through the food chain, therefore, people are may also be indirectly exposed to AVB with the consumption of fish and other aquatic organisms.
It can be concluded that concentrations used in our experiments (e.g. 2 pM – 2 nM) are representative for human and animal exposure to AVB.

Based on our results, we can say that we have demonstrated the impact of AVB on the immune system. The modulation of RACK1 expression, together with other factors affected by GR, is a plausible explanation of the decreased response observed. We only have data on the effect of AVB on LPS-induced cytokine production in a human cell line, whereas additional studies using primary cells and different immune parameters are necessary to characterize the immunomodulatory effects of AVB better. The concentrations tested are relevant for the human exposure. Future perspectives are a better determination of AVB mechanisms, effects on the endocrine and the immune system, and the continuation of testing according to the OECD test strategies for EDCs. The mixture effects and the occurrence of AVB metabolites should also be included in the exposure and the risks' assessment of avobenzone.

5 CONCLUSION

The aim of this thesis was to identify the impact of AVB on the immune system. AVB is an ingredient widely used in cosmetic products, which is suspected of disrupting actions on the endocrine regulation and all other systems associated with it.

The results indicate:

- AVB in a concentration range from 2 pM to 2 nM displayed no cytotoxicity effect on the THP-1 cell line. None of the treatments significantly deviates from the control samples.
- AVB partially inhibited the GNB2L1 gene expression, but the change was significant only at the concentration of 200 pM, where GNB2L1 expression was inhibited by 20% in comparison to the control.
- AVB also reduced RACK1 protein. Significance was observed in all treatments from 20 pM to 2 nM, the decrease in protein production was modest, ranging from 12% to 21%.
- In LPS-induced TNF-α production AVB revealed antagonistic action at all experimental concentrations. After a 3-hour treatment, AVB inhibited TNF-α release by 24 to 41%. After 24 hours, the inhibition was still significant, in the range of 18 to 36% compared to the LPS sample.
- AVB in the range from 20 pM to 2 nM significantly inhibited IL-8 release. After 3 hours of treatment, the IL-8 release was by 19 to 35% lower than the control. After 24 hours, the release was 17-30% in comparison to LPS-treated cells.

According to the results:

Hypothesis 1) is confirmed. AVB in concentrations under investigation was not cytotoxic to the cell line THP-1.

Hypothesis 2) is confirmed, even if a minor statistically significant reduction in RACK-1 gene expression and protein production was observed. It is reasonable to speculate that additional mechanisms may be involved in AVB-induced immune modulation.

Hypothesis 3) is confirmed, AVB modified LPS-induced TNF-α and IL-8 release.

Reviewing the literature data, avobenzone as an environmental pollutant in water is usually detected in the nM range, and findings indicate that it can accumulate in aquatic organisms (e.g. fish). In humans, skin penetration investigations resulted in perceivable

concentrations in the upper skin layer, e.g. *stratum corneum*, in contrast with the dermis and the receptor fluid, where AVB was not detectable.

To confirm the AVB antagonistic effect on the immune system and its mechanism of action, further investigations should be applied, using *in vitro* and also *in vivo* testing strategies.

To assess health risk and long-term effects on humans and biota, we have to take the total burden of exposure through the products use, the environment exposure and the transfer through the food chain into account. Furthermore, the potency of drug metabolites and the joint toxicity of different chemical mixtures, we normally come into contact with, should be assessed.

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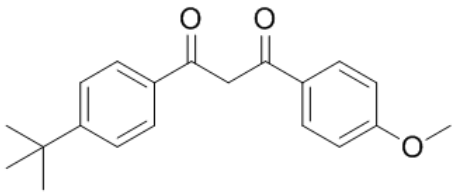
7 APPENDIX

7.1 Avobenzone properties

Table 12: The avobenzone structure and its properties (89) (90) (91)

Avobenzone (AVB, AVO)	
	
IUPAC name	1-(4-Methoxyphenyl)-3-(4-*tert*-butylphenyl)propane-1,3-dione
INCI name	Butyl methoxydibenzoylmethane
Other chemical names, abbreviations	4-tert-butyl-4'-methoxydibenzoylmethane, BM-DBM, BMDM, DBM
Trade names	Eusolex 9020, Parsol 1789, Escalol 517, NeoHeliopan 357, Milestab 1789
PubChem CID	51040
CAS number	70356-09-1
Chemical formula	$C_{20}H_{22}O_3$
Molar weight	310.39 g/mol
Appearance	Off-white to yellowish, crystalline powder with a weak, slightly aromatic smell
Melting point	83.5 °C
Solubility	In water, 25 °C: 2.2 mg/L (insoluble) In glycerol, 20 °C: 0.1% w/w In ethanol and isopropanol, 20 °C: 2% w/w (soluble)
log K_{ow}	4.51
Wavelength range of absorption	Enol-tautomer: 350 – 363 nm (28) Keto-tautomer: 260 – 280 nm (27)
Photostability	Photostable in polar protic solvents (methanol) Unstable in polar aprotic solvent (DMSO) Stabilizers/triplet state quenchers: octocylene (OC), benzylidene camphor, benzylidene malonates, cinnamates, triazines (27) (32)

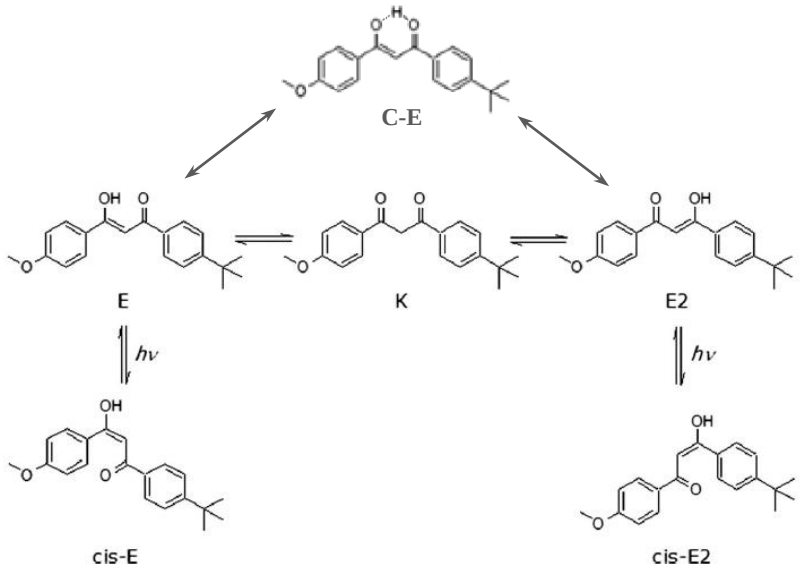


Figure 22: Keto-enol tautomerism and photoisomerism of avobenzone (C-E chelated-enol; E, E2 non-chelated enol-tautomers; K keto-tautomer) (28) (29)

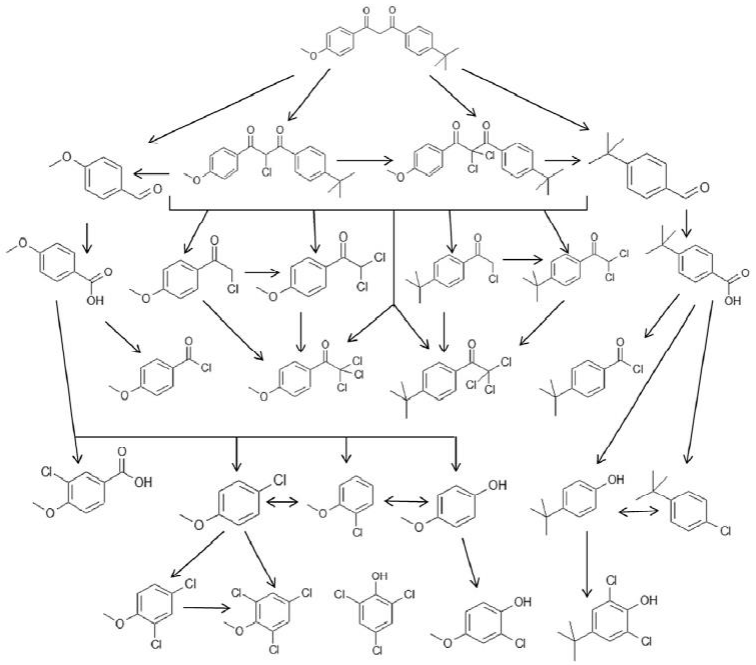


Figure 23: Avobenzone transformation pathways under aquatic chlorination and UV irradiation (30)

7.2 Protein kinases C pathways in monocyte

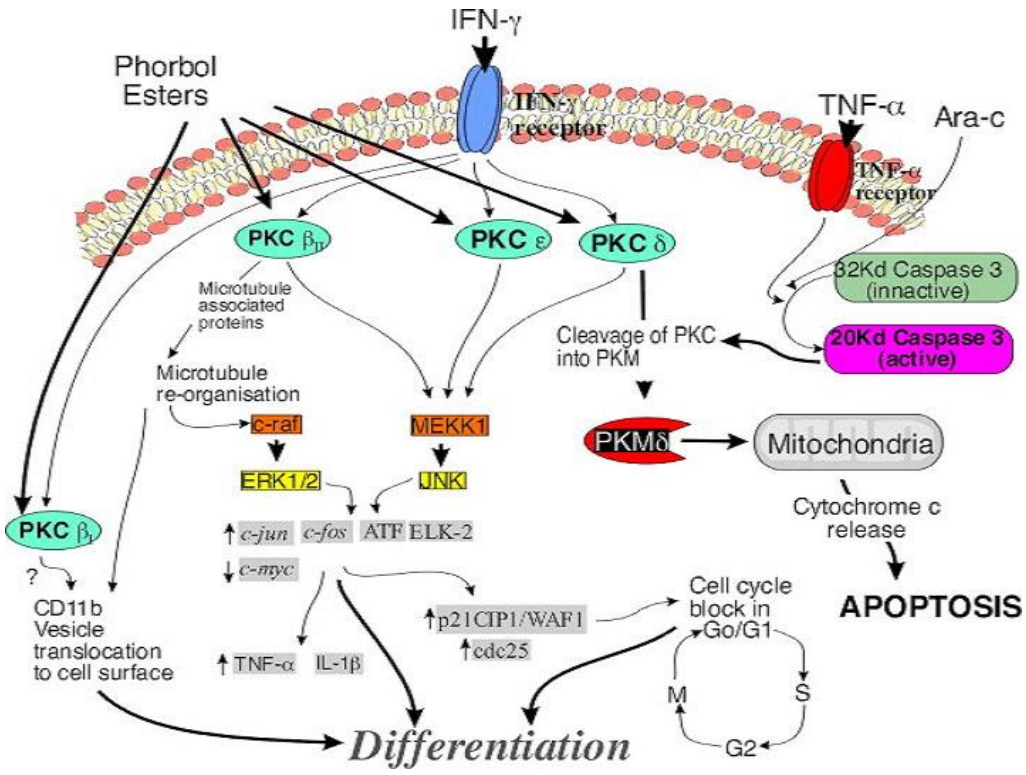


Figure 24: Roles of different PKC isoforms in the monocytic white blood cell.

PKC isoforms have distinct roles: PKC $ß_{II}$ affects the differentiation, PKC ε collaborates in anti-apoptotic signals and PKC δ is implicated in cell apoptosis (programmed cell death). (92)

7.3 Avobenzone percutaneous penetration and its concentrations in the environment and biota

Table 13: An overview of AVB percutaneous absorption in human, animal and artificial skin membranes with methods tape stripping and/or diffusion Franz cells

Skin	Thickness/ Material	Vehicle, applied dose	Time (h)	Analysis method	Recovery (%)	MLOD	% Applied dose absorbed				Year
							Stratum corneum	**Epidermis**	**Dermis**	**Fluid**	
Human, *in vitro*	Full-thickness	15 µg/mL, 1 mL	6	HPLC	/	/	13.01 – 14.49	0.66 – 2.37	n.d.	n.d.	2004 (24)
Human, *in vitro* and *in vitro*	Full-thickness (in vivo – str.c., in vitro - epidermis, dermis)	Emulsion gel 3.0 ± 0.4 mg/cm^2	0.5	HPLC	In vivo: 83 In vitro: /	0.01 µg/L	25.8	0.1 (0.1 µg/cm^2)	n.d.	n.d.	2002 (93)
		in vitro, 2 mg/ cm^2 in vivo	6				Not meas.	0.1 (0.1 µg/cm^2)	n.d.	n.d.	
		Petroleum 3.0 ± 0.4 mg/cm^2	0.5	HPLC	In vivo: 93 In vitro: /	0.01 µg/L	10.3	0.1	n.d.	n.d.	
		in vitro, 2 mg/ cm^2 in vivo	6				Not meas.	0.2	n.d.	n.d.	
Human, *in vivo*	Full-thickness	Ethanol solution 0.57 mg/cm^2	3	UV/VIS	/	/	Blister fluid between epidermis and dermis: 0.15%				2007 (94)
Human, *in vivo*	*Stratum corneum* (tape stripping)	O/W emulsion, free AVB 14 µg/cm^2	1	HPLC	> 94.4	/	9.7 ± 2.5	/	/	/	2010 (95)
		O/W emulsion, AVB lipid microparticles 14 µg/cm^2	1	HPLC	> 94.4	/	15.1 ± 2.7	/	/	/	
Human, *in vitro*	Epidermal membrane	Mineral oil 128 µg/cm^2	24	HPLC	93. 2 - 97.3 (96)	0.1 µg/mL (96)	/	0.45	/	n.d.	2004 (97)

Cellulose membrane	Cellulose acetate	6 different O/W emulsions 2.67 mg/cm^2	22	HPLC	100	0.1 µg/mL	/	/	/	0.027 - 0.521	2008 (98)
Human skin, *in vitro*	*Stratum corneum*, epidermal membrane	6 different O/W emulsions 2.67 mg/cm^2	22	HPLC	100	0.1 µg/mL	/	/	/	0.06 – 0.28	
Mouse, *in vitro*	Full-thickness	Ethanol solution 4.4 mg/cm^2	24	UV/VIS spectrophot.	/	/	Not meas.	Not. meas.	Not. meas.	0.80 ± 0.28	2007 (94)
Hairless mouse, *in vitro*	Full-thickness	Aqueous suspension 37.5 µg/cm^2	24	HPLC	/	/	Not. meas.	Not. meas.	Not. meas.	2.35 (0.88 µg/cm^2)	2008 (99)
Rat, *in vivo*	Full-thickness	Emulsion 11.7 mg/kg BW	12	LC-ESI-MS/MS	93.7 – 106.5	Plasma 0.5 ng/mL, skin 5-10 ng/mL	17.8 ± 2.1	2.0 ± 1.0	0.15 ± 0.10	n.d.	2015 (100)
		Lotion 11.7 mg/kg BW	12	LC-ESI-MS/MS	93.7 – 106.5	Plasma 0.5 ng/mL, skin 5-10 ng/mL	16.3 ± 4.9	3.4 ± 1.5	0.11 ± 0.08	n.d.	
Pig (ear), *in vitro*	Full-thickness	W/O emulsion 25 µg/cm^2	6 24	HPLC	/	* LOQ: 0.350 µg/cm^2 (dermis, epidermis) 0.406 µg/cm^2 (fluid)	/ /	6.4 ± 1.6 12.4 ± 2.8	6.0 ± 3.6 8.8 ± 1.2	<LOQ <LOQ	2015 (101)
		W/O emulsion 100 µg/cm^2	24	HPLC	/	*	/	8.3 ± 1.5	11.2 ± 1.6	1.1 ± 0.3	
		O/W emulsion 25 µg/cm^2	6 24	HPLC	/	*	/ /	7.2 ± 2.0 10.8 ± 4.8	5.2 ± 0.16 13.6 ± 6.4	<LOQ <LOQ	
		O/W emulsion 100 µg/cm^2	24	HPLC	/	*	/	14.8 ± 1.7	10.8 ± 1.5	0.5 ± 0.09	

Table 14: Avobenzone concentrations in environment waters

Location	Matrix	Instrumental method	Rec. (%)	LOD (ng/L)	Conc. (ng/L)
Bangkok (20)	River water	HPLC-ESI-MS/MS	63-106	0.03-1.38	36-38
Germany (20)	River water	TD-GC-MS	101	63	<LOD
Germany (20)	Lake water	LC-APPI-MS/MS	76-94	10	2431
Germany (20)	Lake water	TD-GC-MS	82	63	<LOD
Switzerland (20)	Lake water (midland)	GC-MS	42	20	<LOD
Switzerland (20)	Lake water (small)	GC-MS	42	20	<LOD-24
Honkong (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	24-721
Japan, Tokyo (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	78-104
USA, New York (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	70-87
USA, Los Angeles (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	67-109
China, Shantou (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	53-100
China, Chaozhou (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	<LOD
The arctic (20)	Seawater (surface)	HPLC-ESI-MS/MS	63-106	0.03-1.38	18-70
Greece (20)	Seawater	LC-UV-DAD	96.5-98-0	1270	n.d.
Greece (20)	Seawater	LC-UV-DAD	87	24	n.d.
Greece (20)	Swimming pool water	LC-UV-DAD	88	24	n.d.
Greece (20)	Shower wastes water	LC-UV-DAD	86	24	n.d.
Canary Islands (19)	Seawater, semi-enclosed beaches	GC-MS (16)	50-98 (16)	2	<LOD-737
Canary Islands (19)	Seawater, open profile beaches	GC-MS (16)	50-98 (16)	2	<LOD-1770
USA, South Carolina (22)	Seawater (six beaches)	LC-MS	80-120	1	<LOD-425 (highest 1298)

Legend: LOD – limit of detection; Rec. – recovery

Table 15: AVB concentrations in aquatic biota

Location	Matrix	Instrumental method	Rec. (%)	MLOD ng/kg body weight	Concentration
Meerfelder Maar lake, Germany (102)	Fish; Perch (*Perca fluviatilis*)	GC-EI-MS	89-106	50-90	**(ng/g lipid)** Muscle: / Offal: 210 Rest: / Whole fish: 44
China (103)	Wild species	HPLC-MS/MS	41,1-82.8	1	**(ng/g d.w.)** n.d.
China (103)	Farmed red snapper	HPLC-MS/MS	41,1-82.8	1	**(ng/g d.w.)** Fillet: 33 ± 12 Belly: 52 ± 14

Legend: MLOD – method limit of detection; Rec. – Recovery; n.d. - not detected; d.w. - dry weight