universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Combinatory Effects of Mycoestrogens and Isoflavones“

verfasst von / submitted by

Rebeka Früholz, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2022 / Vienna, 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 659
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Lebensmittelchemie
Betreut von / Supervisor:	Univ.-Prof. Dr. Doris Marko
Mitbetreut von / Co-Supervisor:	Dr. Elisabeth Varga

Acknowledgments:

First and foremost, I would like to thank Univ.-Prof. Dr. Doris Marko for giving me the opportunity to become part of such an insightful project within her research group at the Department of Food Chemistry and Toxicology at the University of Vienna.

Furthermore, I would like to thank Dr. Elisabeth Varga for her great supervision and patience throughout this entire learning process, in addition to allowing me to conduct research towards this topic. Simultaneously, I would like to thank Dino Grgic for his co-supervision, acquainting me with lab skills required for the performed assays, and passing on his knowledge, whilst never ceasing to help. Throughout this journey I have attained various new skills in the field of toxicology, statistics, and many more, which I am forever grateful for.

Furthermore, I would like to thank the Austrian Research Promotion Agency (FFG) and DSM BIOMIN Holding GmbH for funding the “ISOMYCOTOX” project (project number 880656).

I would also like to express my gratitude towards the remaining department for all the help I have received whenever required and for the all-round pleasant work atmosphere.

Lastly, I would like to thank my parents for their financial as well as emotional support, my friends for listening to my struggles and their babysitting skills, my partner for his diverse set of computer know-how, and finally my son for forcing me back into reality.

Table of Contents

Abbreviations

2-OH-E2	2-hydroxyestradiol
4-OH-E2	4-hydroxyestradiol
α-ZAL	α-zearalanol
α-ZEL	α-zearalenol
β-ZAL	β-zearalanol
β-ZEL	β-zearalenol
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
AF	activation function
AhR	aryl hydrocarbon receptor
ALP	alkaline phosphatase
AP-1	activation protein 1
cAMP	cyclic adenosine monophosphate
CD-FBS	charcoal-dextran-treated fetal bovine serum
cDNA	complementary DNA
c-Src	tyrosine kinases
CTB	CellTiter Blue
CYP1A1	cytochrome P450 1A1
CYP1B1	cytochrome P450 1B1
DAI	daidzein
DHZEN	decarboxylated hydrolyzed zearalenone
DNA	deoxyribonucleic acid
DBD	DNA-binding domains
DMEM/F-12	Dulbecco's Modified Eagle Medium/ nutrient mixture F-12
DMSO	dimethyl sulfoxide
E1	estrone
E2	17β-estradiol
E3	estriol
EC_{50}	half maximum effect concentration
EGFR	epidermal growth factor receptor
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EQ	equol
ER	estrogen receptor
ERE	estrogen response element
ERK1/2	extracellular-signal-regulated kinase
ESR1	estrogen receptor 1
ESR2	estrogen receptor 2
EU	European Union
FBS	fetal bovine serum
FFG	Austrian Research Promotion Agency
GEN	genistein
GPER	G-protein coupled estrogen receptor
GLY	glycitein
HAT	histone acetylase

HDAC	histone deacetylase
Hsp	heat shock protein
HZEN	hydrolyzed zearalenone
LBD	ligand binding domain
LD_{50}	lethal dose 50%
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinases
MEM	minimal essential medium
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
NHR	nuclear hormone receptors
NLS	nuclear localisation signals
O-DMA	o-desmethylangolensin
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PI3K-Akt	Akt/protein kinase B
PKA	protein kinase
ProHB-EGF	pro-heparin-binding-epidermal growth factor
P/S	penicillin/streptomycin
qRT-PCR	real-time reverse-transcription polymerase chain reaction
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SRB	sulforhodamine B
T75	cell culture flasks with 75 cm^2 surface
T175	cell culture flasks with 175 cm^2 surface
TBP	TATA-binding protein
TCA	trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	tolerable daily intake
TF	transcription factor
YES	yeast estrogen screen
ZEN	zearalenone
ZEN-14-glc	zearalenone-14-O-β-glucoside
ZEN-14-S	zearalenone-14-sulfate

Introduction

Observing the world from a chemist's perspective, all matter is composed of either anthropogenic or natural compounds, undergoing multitudinous interactions and impacting our daily lives. At the same time, the quintessence of toxicology imparts the idea of every compound owning the capacity to act adversely, indifferent of their origin, yet depended on the inflicted dosage. As various anthropogenic factors continuously influence the dynamics of natural compounds, new toxicological concerns arise, calling for new assessments. Furthermore, interdisciplinary approaches become necessary, as studying the acute toxicity of single compounds on an organism portrays an unrealistic scenario of exposure, hence limiting the predictive powers of such investigations.

The impact of mixture toxicity has recently gained increased awareness, leading to governmental authorities calling for a need to assess combinatory effects of chemical mixtures. The evaluation of the effects of simultaneous exposure to chemical mixtures dates back to the early 1900s, where Loewe and Muischnek formulated a concept concerning the additivity of chemicals (Loewe and Muischnek 1926). This principle of "Loewe Additivity" remains relevant today, yet newer models such as the Bliss Independence model or the Chou and Talalay's method, have contributed to a progression of this topic (Bliss 1939; Chou and Talalay 1984). Nonetheless, the development and standardization of methodologies utilized to assess risk scenarios accommodating simultaneous exposure to stressors, still poses a challenge for different stakeholders across continents.

Climate change as a result of global warming is a common cause of concern impacting a broad spectrum of fields, including toxicology. An alteration in climatic condition has been associated with a change in the toxins present, as well as their respective toxicities. To exemplify this matter, water redistribution occurs as a consequence of changing climatic patterns, leading to global changes in stream acidity. Certain chemical groups, such as pharmaceuticals which are highly sensitive to pH changes, are thought to experience a shift in their toxicity (Lovett 2010).

Another group of toxins benefitting from changing climatic conditions, having recently gained more popularity, partially due to improved analytical detection methods, are secondary metabolites of fungi named mycotoxins. Mycotoxins are contaminants of natural origin which have been associated with adverse effects on human and animal health. They derive from fungi, such as *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp., which infest crops and subsequently produce an expansive class of toxins (Lui and Fels-Klerx 2021). This work will specifically focus on the mycotoxin "zearalenone" (ZEN), produced by *Fusarium graminearum* and *Fusarium culmorum* species, reputable for its mycoestrogenic properties, and most commonly afflicting damage to maize crops (EFSA 2011).

The European Food Safety Authority (EFSA) published an evaluation concerning potential risks to human health related to exposure to ZEN in the year 2011. After a thorough assessment of the present situation, the scientific panel concluded chronic human exposure to ZEN to be below or within the range of the accepted tolerable daily intake (TDI) of the mycotoxin, hence dismissing potential assumptions concerning risks to human health (EFSA 2011). However, human dietary patterns are thought to be conspicuous due to their heterogeneity, whereas certain livestock is exempted from the enjoyment in variation of daily meals. Swine and poultry feed mainly constitutes of corn and soybean meal in order to cover their needs for energy and proteins (Zentek and Goodarzi 2020). Soybeans represent a rich source of another natural group of compounds, the isoflavones. Isoflavones classify as phytoestrogens, which as the name suggests, may act in an antagonistic or agonistic fashion on estrogen receptors (Thompson 2010). Structurally speaking, phytoestrogens are nonsteroidal phenols and subdivided into two groups: flavonoids and non-flavonoids. Due to their structural similarity to 17β-estradiol, they possess the ability to mimic estrogens, hence are able to bind to their respective receptors (Krizova, et al. 2019).

In this thesis, combinatory effects between the mycoestrogen ZEN along with its metabolites, and the phytoestrogens genistein (GEN), daidzein (DAI), glycitein (GLY), and equol (EQ), in regards to their cytotoxicity and potential mechanisms shall represent the focus of this investigation. ZEN is naturally subject to plant, fungal, and animal metabolism. The metabolites considered in this thesis are: α-zearalanol (α-ZAL), zearalenone-14-sulfate (ZEN-14-S). In previous literature, data on these combinations is still limited: Yet the relevance

of this topic is undeniable as factors such as climate change, increased usage of soybeans, in addition to homogenic livestock feed encourage the co-occurrence of ZEN and phytoestrogens, thus posing the question of whether their similar modes of action cause an amplification of the effects observed by each individual compound.

Theoretical Background

Mixture Toxicology

The term "toxicology" originates from the Ancient Greek words *toxikos* and *logos*, translating into "the study of poisons". Poisons, also referred to as toxins, possess the ability to induce potentially harmful effects on organisms and their surrounding environments (Hussain, Kolli and Sethi 2015). Nevertheless, the effect of a toxin cannot be regarded as a singular event, where E. Hodgson offers an illustrative definition of toxicity as:

"Toxicity itself can rarely, if ever, be defined as a single molecular event but is, rather, a cascade of events starting with exposure, proceeding through distribution and metabolism, and ending with interaction with cellular macromolecules (usually DNA or protein) and the expression of a toxic end point. " (Hodgson 2004)

Toxicity is a quantitative concept, imparting the assumption of every substance being able to induce harmful effects at a certain dosage, however being harmless or even advantageous at a lower dosage (Hodgson 2004). The scope of toxicology encompasses parameters such as acute and chronic exposure, dosage, route of exposure, as well as the traits of the affected species or environment (Hussain, Kolli and Sethi 2015).

The classical evaluation of toxicity is generally performed on a single compound basis. Yet in a real-life setting, exposure to multiple chemicals simultaneously is to be the expected scenario. A chemical mixture is generally described as:

"Any set of multiple chemicals regardless of source that may or may not be identifiable that may contribute to joint toxicity in a target population" (EPA 2000)

The potential harm of exposure to chemical mixtures or multiple stressors can also be described as cumulative risks. The form of exposure can be acute or chronic, accumulated over time, following different pathways and differing in their source (US EPA 2003).

Chemical compounds derive of either natural or anthropogenic origin. Worldwide approximately 350 000 chemicals have been registered as innocuous compounds, to be used in a variety of industries (Wang, et al. 2020). Certain regional unions, such as the European Union (EU), implement stricter precautionary measures, where currently around 26 000 chemicals are registered for safe usage (ECHA 2021). However, often these lists do not take into consideration compounds with potentially harmful effects of natural origin such as heavy metals, alkaloids, or mycotoxins. Such substances are considered to be contaminants, which are regulated by authorities and defined in the European Union as:

> *„Any substance not intentionally added to food which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food, or as a result of environmental contamination"* (Council Regulation (EEC) No 315/93)

Other bioactive constituents, generally considered to be harmless or even beneficial to our health, are found in plants. Flavonoids, quercetin, isoflavones, or carotenoids are examples of such compounds, found in a wide variety of fruits, grains, and vegetables (Gökmen 2015).

Hence mixture toxicology can be applied universally, as confrontation to multiple chemical compounds simultaneously represents the prevalent scenario of exposure. Industrial chemicals, contaminants, and bioactive plant constituents are entwined in our modern-day world, affecting us on a daily basis. From a liberal stance, the simultaneous and constant exposure to combinations of chemicals is the price paid for an extended life span in addition to living in a developed world with all its amenities. On the contrary, from a conservative point of view, the toxicological effects of this continuous exposure to mixtures of chemicals remains to be unknown, and hence should be investigated further (Mumtaz 2010).

Until now, the complexity of the assessment of mixture toxicology remains an obstacle to be overcome. Recent amends in establishing standardized methodologies in order to evaluate combinatory effects of multiple compounds are being made by different stakeholders. In 2011 the Scientific Committee on Health and Environmental Risks (SCHER), Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), and Scientific Committee on Consumer Safety (SCCS) of the European Commission published an opinion on "potential adverse effects of the interactions between substances when present simultaneously in a mixture" (SCHER, SCENIHR, and SCCS 2012). Since then, multiple projects have been launched by the European Union in order to improve method development and assessment of combinatory effects of chemicals on human and ecological health. Mixtox and EuroMix are examples of such programs, both aiming at developing test strategies to assess mixtures of multiple chemicals (EFSA Scientific Committee 2019, EuroMix 2017). In North America, similar governmental programs are being encouraged by the Environmental Protection Agency (EPA), US Food and Drug Administration (FDA), and Health Canada (HC), in order to develop appropriate methodologies and generate data for cumulative risk assessment of chemicals (Health Canada 2017, US EPA 2003). These joint efforts across continents allow continuous amelioration in the methodologies available for the assessment of chemical mixtures, with the ultimate goal to protect human health and our surrounding environment.

Important parameters frequently discussed during the assessment of toxicological properties of mixtures include the terms "synergism" and "antagonism". Synergism describes an effect beyond the anticipated additive effects induced by two or more agents applied in combinations. On the other hand, opposing synergism, antagonism is observed once combinations of two or more agents result in an effect less than the anticipated additive affect. As the baseline of synergistic or antagonistic effects, the term "additivity" cannot be neglected. Additivity is seen as the expected effect of two or more compounds in combination, often deemed as inertism or non-interaction (Roell, Reif and Motsinger-Reif 2017). On the basis of these terms, mixtures of compounds are categorized, contributing to an improvement in evaluating toxicological properties.

Mycotoxins: Zearalenone and Metabolites

Brief Introduction to Mycotoxins

Mycotoxins are representatives of one major class of natural contaminants found in food and feed. They are considered to be:

"Secondary metabolites produced by filamentous fungi either pre-or postharvest and which can contaminate agricultural food and feed products and have detrimental effects on human and animal health" (Botana and Sainz 2015).

Although general public awareness of mycotoxins is relatively low, historically speaking living species have been entangled with these toxins since the first settlement of civilizations. For example, ergotism, caused by the fungus *Claviceps purpurea,* is one of the most documented fungal diseases, afflicting damage since antiquity until the 1900s (Lee 2009).

Today, due to improved analytical methods, multiple types of mycotoxins have since been identified. Often these discoveries followed a cluster of diseases which could be traced back to a consumption of a certain type of food. Cardiac beriberi had been reported since the 17th century, where in 1881 the cause could be traced back to moldy rice contaminated with the mycotoxin citreoviridin. Meanwhile the deaths of 100 000s of turkeys could be acknowledged for the discovery of aflatoxins in 1960. On the other hand, zearalenone (ZEN) was isolated after crop infestation with *Fusarium graminearum* and had been associated with estrogenic symptoms in pigs. Its chemical structure was defined in the 1960s (Pitt and Miller 2017).

Introducing Zearalenone

ZEN (Figure 1) is characterized by its resorcyclic acid lactone structure, possessing a double bond between C11 and C12, and a ketone at C7 (Lu, et al. 2022). It is synthesized through the polyketide pathway of *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium equiseti*, and *Fusarium crookwellense* (Bennett and Klich 2003). As a result of its structure, ZEN is lipophilic and has a high melting point of approximately 165 °C. It is a stable compound, hence does not

undergo chemical changes during storage, milling, or processing of food products (EFSA 2004).

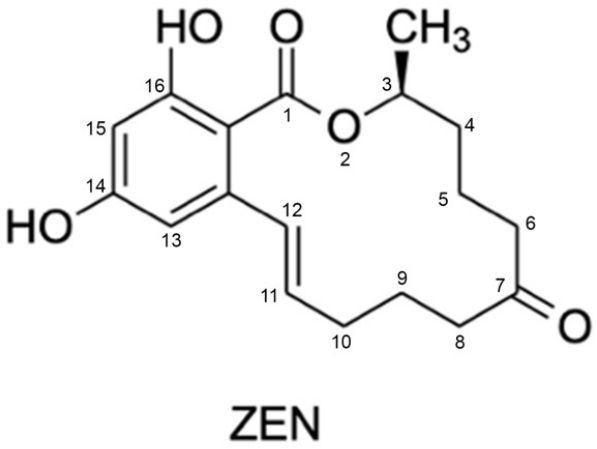


Figure 1: Structure of ZEN

Due to its toxicological properties, ZEN is often considered to be a mycoestrogen, on top of being a mycotoxin with genotoxic, carcinogenic, and immunotoxic potential. The structure of ZEN bears resemblance to the structure of 17β-estradiol (E2), which is illustrated in Figure 2. This structural resemblance, allows ZEN to act on estrogenic receptors. Since the 1920s, observations concerning the correlation between the consumption of infested grain and hyperestrogenism, especially in swine, had been made. Characteristically, the exposed animals would struggle with reproductive problems, such as impaired conception and abortion (Han, et al. 2022).

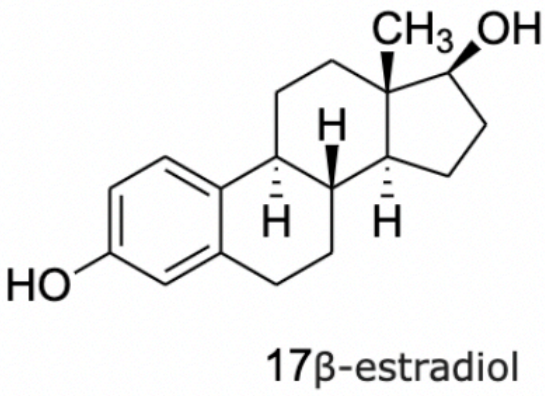


Figure 2: Structure of E2

Metabolization of Zearalenone

Upon ingestion of ZEN, the mycoestrogen is metabolized to a certain degree by a variety of enzymes. This metabolism can occur in animals, plants, and fungi. The process of metabolization allows the detoxification of toxins which can ultimately result in rapid excretion. Nevertheless, at times, the opposite effect is achieved. ZEN can undergo a reduction of the keto group to form the metabolites α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) in phase I metabolism, where, α-ZEL has been reported to be around 60 times more estrogenic than the parent mycoestrogen. Additionally, α-ZEL and β-ZEL can be transformed further into α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) respectively (EFSA 2016). All metabolites can then undergo phase II metabolism, forming different hydrophilic conjugated forms, allowing easy excretion. An overview of all currently recognized metabolites and their reaction pathways is illustrated in Figure 3.

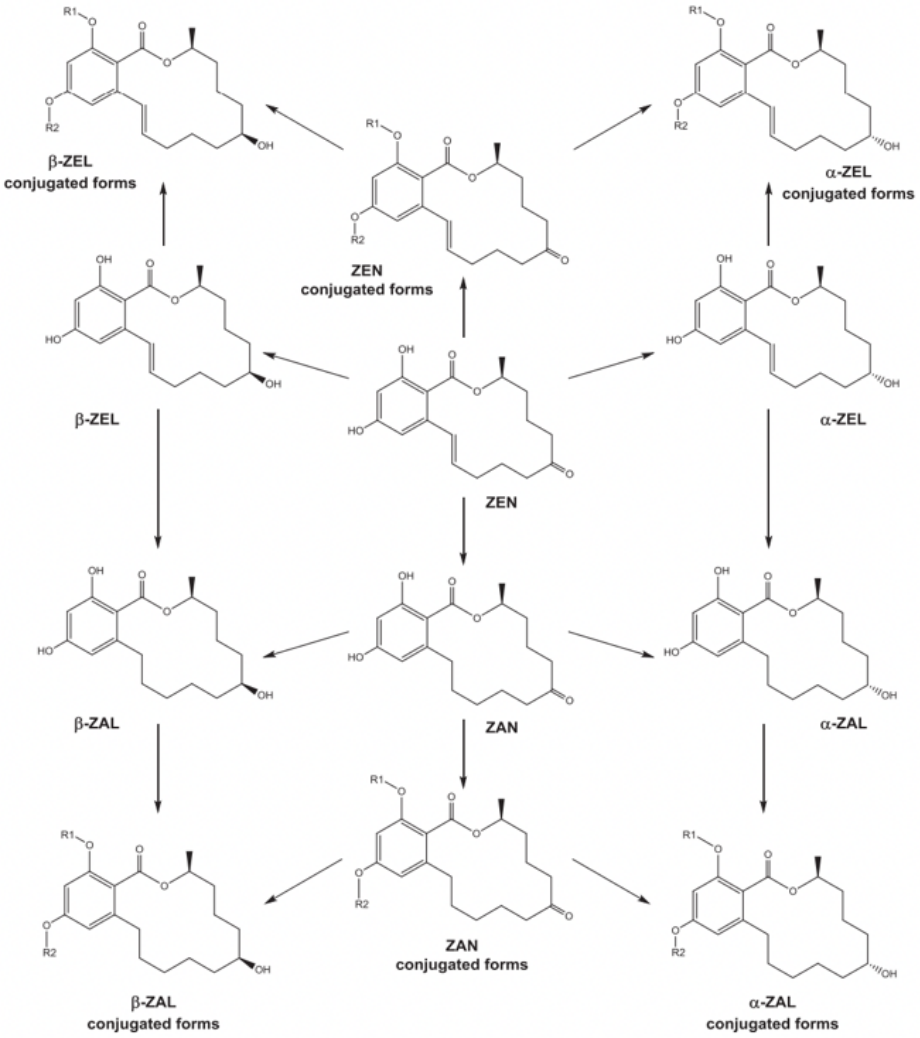


Figure 3: Reaction pathway of ZEN (EFSA 2016)

Different possibilities of reaction pathways of ZEN with R1=R2= glucoside, sulfate, glucuronide

The preferred mode of metabolization is species dependent, resulting in a species dependent sensitivity to ZEN exposure. In pigs, a high amount of α-ZEL compared to β-ZEL was found in the bile, urine, and blood, whereas in cattle or poultry β-ZEL seemed to be the more dominant metabolite (Kovalsky and Gruber-Dorninger 2021). Notably, the intestinal system possesses the capacity to hydrolyze conjugated compounds, which then results in a reuptake of the parent mycotoxin. In pigs, such hydrolyzation of zearalenone-14-O-β-glucoside (ZEN-14-glc) and zearalenone-14-sulfate (ZEN-14-S) had been observed, where these conjugates, or modified mycotoxins, are of plant or fungal origin, contributing to further crop contamination (Binder, et al. 2017). Upon analysis of human urine samples, the main metabolites next to the parent compound identified were glucosides of ZEN and low concentrations of α-ZEL (Carballo, et al. 2021).

Toxicity of Zearalenone

ZEN has been titled a mycoestrogen rather than a mycotoxin, due to its low acute toxicity. Virtually preposterously high dosages are required to achieve lethality, which have been studied in rats and guinea-pigs. On the contrary, ZEN and its metabolites are highly biologically potent compounds, capable of acting on estrogenic receptors (Bennett and Klich 2003). A study conducted on piglets being fed with a ZEN contaminated diet for 28 days demonstrated an increase in the size of the vulva and the level of certain immunoglobulins (Su, et al. 2018). Meanwhile Pang et al. found exposure to low doses of ZEN (20 or 40 µg/kg body weight) over the timespan of 14-42 days impairing the capacity of male reproductivity in male mice (Pang, et al. 2017). Often ZEN is categorized as an endocrine disruptor, owning the capacity to cause hormonal dysregulation. Kowalska et al. reviewed a broad spectrum of hormone-related endpoints observed in different animal models following exposure to low dosages of ZEN. The effects seen varied from decreased plasma testosterone concentrations in mice to glandular hyperplasia in the endometrium of gilts. In humans, studies rely on *in-vitro* models such as the estrogen sensitive MCF-7 cell line undergoing proliferation once exposed to naturally occurring concentrations of ZEN, giving rise to postulations regarding the role of ZEN in hormonal carcinogenesis (Kowalska, et al. 2016). Furthermore, in Ishikawa cells, the potential estrogenic activation induced by ZEN at concentrations above 100 nM (Li, et al. 2012).

Zearalenone Contamination

Contaminations with ZEN predominantly affects particular agricultural crops such as corn, soybean, or wheat. The TDI has been defined by the EFSA as 0.25 µg/kg body weight per day (EFSA 2011). In Europe, biomonitoring studies demonstrated prevalent exposure to ZEN, yet predominantly these exposure levels seem to lie below the established TDI. A systematic review summarizing the ZEN exposure outside of Europe concluded a low prevalence of ZEN. In Africa less than 6% of the samples were found to be contaminated whereas for the remaining continents data was insufficient (Al-Jaal, et al. 2019). However, an increasing global population calls for an expansion of agricultural activity in order to ensure global food security. Changing environmental conditions such as elevated CO_2 levels in combination with water scarcity and fluctuating temperatures favor the growth of certain fungal species, including *Fusarium* species (Bencze, et al. 2017). Consequently, posing the conundrum of achievable food security without potential health hazards due to potentially increased mycoestrogenic contamination. Furthermore, the established TDI threshold solely takes into consideration the impact of a single compound, hence disregarding plausible combinatory effects. In this fashion, combinations of different mycotoxins itself or mycotoxins alongside secondary plant metabolites could potentially act synergistically, circumventing established TDIs for single substances, thus posing impending risks to consumers.

Secondary Plant Metabolites: Isoflavones

Introducing Secondary Plant Metabolites

Secondary plant metabolites are small molecules produced by the plant itself to ensure competitiveness in their surrounding environment. In plants, these metabolites own the capacity to induce certain plant-specific phases such as flowering and fruiting, perennial growth, or abscission. Over 50 000 secondary plant metabolites have been identified, where many have demonstrated bioactivity in humans (Teoh 2015).

One major group of the secondary plant metabolites constitutes of the polyphenols, or also named phenolic compounds. They are characterized by the presence of aromatic ring structures and can be grouped into subdivisions, based on their total number of carbon atoms (Figure 4). Flavonoids represent such a subdivision of polyphenols, consisting of

15 carbon atoms with a C6-C3-C6 framework, deriving from phenylalanine. Flavonoids can then be further separated into isoflavones, flavonols, flavones, flavanones, flavanols, and anthocyanins depending on the configuration of the C-ring (Keller 2009).

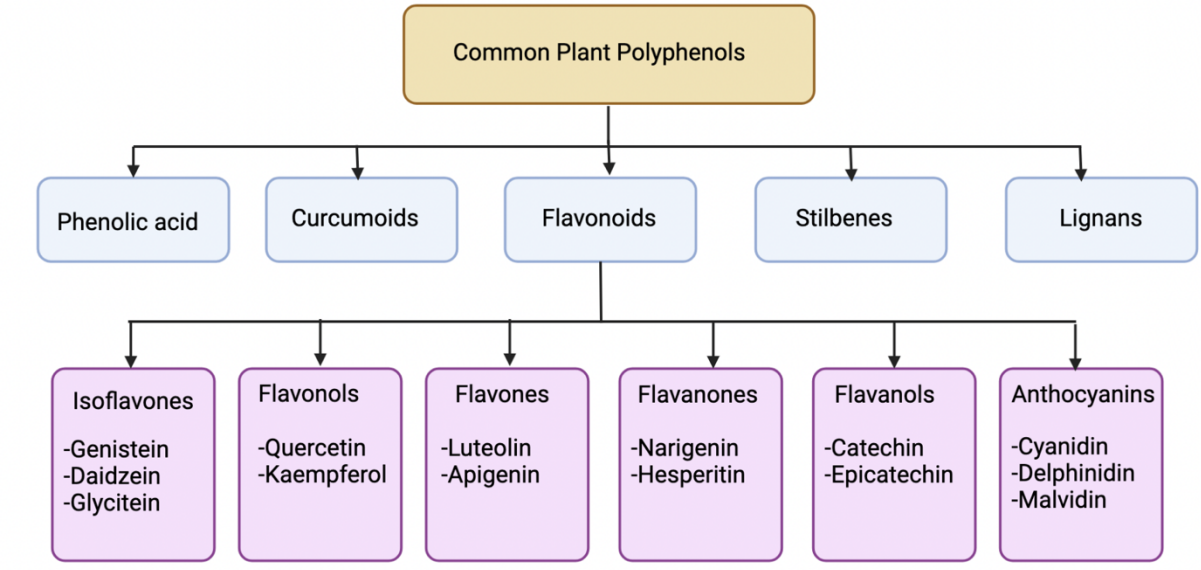


Figure 4: Schematic Diagram: Classification of polyphenols modified from: (Amararathna, Johnston and Rupasinghe 2016)

What are Isoflavones?

Isoflavones are found mainly in legumes of the family *Fabaceae*, most notably in soybeans. The major isoflavones found in plant-based foods are genistein (GEN), glycitein (GLY), biochanin A, formononetin, and daidzein (DAI). Biochanin A and formononetin are derivatives of GEN and DAI, owning an additional methyl group (Committee on Toxicity 2003). Equol (EQ) is often affiliated with the previously mentioned isoflavones, yet it is not of plant origin, as it is a bacterial metabolite of DAI produced in the intestines of certain animals (Krizova, et al. 2019). Isoflavone are predominantly present as glucoside conjugates in foods and plants. The glucose group then can at times be found esterified to an acetyl- or malonyl group (Committee on Toxicity 2003).

In plants, isoflavones function as phytoalexins, being part of the natural defense system, the plant has to offer against microbes or fungi. Meanwhile in animals, isoflavones have demonstrated estrogenic activity, hence acting upon estrogen receptors and resulting in the exertion of estrogenic or anti-estrogenic effects. Again, the estrogenic activity is a result of

structural similarity to E2. This type of bioactivity allows isoflavones to be classified as phytoestrogens (Krizova, et al. 2019).

Metabolization of Isoflavones

Upon hydrolyzation of isoflavone glycosides in the gut, absorption of the aglycones of isoflavones occurs by the means of passive diffusion in the upper small intestine, where then in the liver glucuronidation or sulfation can occur as part of phase II metabolism. As these metabolites are excreted with the bile back into the intestines, the glucuronide or sulfate groups can be cleaved off in the large intestine, resulting in enterohepatic circulation. Excreted metabolites are found to be EQ, O-desmethylangolensin (O-DMA), or 6-hydroxy-O-DMA. In humans the metabolites vary between different geographical regions, whilst EQ is excreted consistently by animals, however here too, classification between EQ and non-EQ producers apply. Cattle are considered to be EQ producers whereas in piglets this cannot be observed (Preedy 2013).

Physiological Effects of Isoflavones

In terms of physiological effects on humans, isoflavones have been associated with a variety of health beneficial effects such as lowering the risks for cardiovascular diseases, reducing risks of certain types of cancers, or improving menopausal symptoms. A metanalysis studied the correlation between cardiovascular disease mortality and isoflavone intake, where a tendency towards a risk reduction was noted, yet the results were overall not significant (Nachvak, et al. 2019). Similar conclusions were drawn regarding all-cancer-mortality and isoflavone consumption, where a 20% lowered cancer mortality risk was found to correlate with the highest intake of isoflavones (Nachvak, et al. 2019). Nevertheless, a diet high in isoflavones could pose as a marker for a plant-based diet, which is generally thought to act cancer preventive. Individual characteristic differences of this sort cannot be neglected, as countless factors contribute to the etiology of cancer. Looking at specific types of cancer, isoflavone consumption and breast cancer are either inversely or not related at all. Particularly noteworthy is the difference between Western and Asian populations, where a high isoflavone intake had a higher protective effect in Asian regions compared to Western

countries (Chen, et al. 2014). This peculiarity might be due to differences in the gut microbial communities, where individuals can be classified as EQ and O-DMA producers or non-producers based on their metabotype. In Asian regions the prevalence of EQ and O-DMA producers is higher compared to Western regions, as a result of historical dietary differences, thus offering a tentative explanation to regional differences in the observed health effects of isoflavones (Frankenfeld 2021). Concerning menopausal symptoms treatment, the evidence is more coherent regarding isoflavone consumption in post-menopausal women. The frequency as well as severity of symptoms seemed to be slightly reduced, although a distinction between the type of isoflavones and the effects observed was not clarified (Bolaños, Castillo and Francia 2010, G. Howes, Howes and Knight 2006). Overall, the results of different systematic reviews focusing on randomized, controlled trials and epidemiological studies were not able to provide substantial evidence for positively associated effects linked to isoflavones consumption.

Dietary Sources of Isoflavones

Currently the most prominent source of endocrine disrupting compounds, apart from pharmaceuticals, are dietary phytoestrogens (Behr, Oehlmann and Wagner 2011). Dietary patterns are not only subject to geographical regions, but also to time, globalization, industrialization, and trends. On average, in Asian populations around 15-20 mg of isoflavones are consumed daily. In comparison, in Western countries the daily consumption of isoflavones amounts to less than 2 mg (Eisenbrand 2007). Lately, as a product of globalization, appropriation of new food groups has increased the global demand for soy-based products such as soymilk (Colletti, et al. 2020). The main source of isoflavones in our diet are soy and soy products. A variety of food products and their respective isoflavone concentration is listed in Table 1 below:

Table 1: Isoflavone content in different food items

Food item	Total isoflavone (mg/100 g)
Soy yoghurt	33.17
Soy protein drink	81.65
Tempeh	60.61
Soy meal, defatted, raw	209.58
Soy beans green	48.95
Soybeans boiled	17.92
Miso	41.45
Chicken nuggets, meatless	14.60
Infant formula	2.21–28.01
Modified from: (Inbaraj and Chen 2013)	

Supplementation of Isoflavones

Another source of isoflavones are supplements, on the basis of red clover or soy extracts, which have gained popularity to treat symptoms during menopause. These supplements are characterized by their high concentrations of isoflavones, although vary greatly in their constitution (Setchell, et al. 2001). Food supplements fall under the regulation of EU General Food Law Regulation (EC) No 178/2002, hence are considered to be foodstuffs and do not undergo the same stringent application procedure as pharmaceutical products. Nonetheless the potential estrogenicity of highly concentrated isoflavones called for a risk assessment. In 2015 the EFSA published a scientific opinion on the potentially adverse effects of food supplements containing isoflavones, alleviating suspicions concerning post-menopausal women (EFSA 2015). In this statement the focus was laid on post-menopausal women, representing the main target audience for such type of supplementation. However, supplementation containing isoflavones can also aim at different target populations, with different marketing strategies such as promising breast augmentations (Setchell, et al. 2001). For these target populations there is still a lack in conclusive risk assessments.

Isoflavone Exposure: Children

Children also represent a potentially underestimated population group affected by phytoestrogens. Exposure to isoflavones in young children can occur prenatally or via breastmilk, soy-based infant formula, or consumption of soy products. Prenatal exposure is due to the mother´s diet, where isoflavones are able to cross the placental barrier and enter

the fetal circulation (Balakrishnan, Thorstensen and Mitchell 2010). Yet it must be noted that the amount of potent endogenous hormones circulating increases significantly upon pregnancy, where then the estrogenicity of dietary isoflavones might essentially be minimal (Fleck, et al. 2016). Analytical investigations of DAI and GEN concentrations in breast milk found respective concentrations of approximately 24 µg/L and 10 µg/L (Franke and Custer 1996). In contrast to this, infants fed with soy-based formula, would be exposed to 14.7–25.6 mg/day of isoflavones. Estimations declare a daily intake of 500 mL of follow-on formula in weaning babies, thus equating to concentrations ranging from 29.4 mg/L–51.2 mg/L (Morandi, et al. 2005). Principally infants are fed exclusively with appropriate breastmilk substitutes in the first few months, consequently leading to a high daily intake of isoflavones. Contrarily, breastfed infants consume endogenous E2, suggesting a generally high estrogenicity of breastmilk, thus challenging the relevance of a high isoflavone content in infant formula (Behr, Oehlmann and Wagner 2011). Young children can easily consume 2-16 mg of isoflavones on a daily basis depending on their diet. Celiac or vegetarian children in addition to children with certain intolerances and allergies might depend more on processed food substitutes which often contain higher amounts of soy. This in turn results in a higher intake of isoflavones (Morandi, et al. 2005). Infants and young children have been associated with higher sensitivity to exogenous estrogens as their endogenous hormonal levels are low, yet certain organs express the appropriate estrogen receptors. Early breast development and sexual maturation has been linked to endogenous hormone imbalances, possibly caused by endocrine disrupting compounds such as isoflavones (Aksglaede, et al. 2006). All in all, albeit the low estrogenicity of isoflavones, the time of exposition in addition to the dosage portray important parameters in assessing the impact of these phytoestrogens. Moreover, the continuous exposition to isoflavones in combination with a multitude of different bioactive compounds, including various endocrine disrupting compounds, could potentially enhance adverse effects observed during the pubertal development of children.

Zearalenone and Isoflavones Co-occurrence

In order to assess the relevance of plausible combinatory effects between the mycoestrogen ZEN and isoflavones, their co-occurrence in the environment, human diet, and animal feed

has to be examined first. Often analytical methods such as gas or liquid chromatography coupled to mass spectrometry are utilized. An investigation of seasonal water samples withdrawn from the Douro River estuary in Portugal found concentrations up to 277.4 ng/L of DAI in the summer and 130.0 ng/L of GEN in the spring. On the other hand, ZEN was found to be below a concentration of 137.5 ng/L in spring and summer. The sources of the isoflavones and ZEN were hypothesized to originate from agricultural effluents, as the fertility of the area around this estuary is utilized to grow agricultural products (Ribeiro, et al. 2016). In this case, the water samples mirror the co-occurrence of these estrogenic compounds in the local agricultural fields. Meanwhile an analysis of pasture field samples from 2019 in Austria determined a large variety of mycotoxins in addition to phytoestrogens and other secondary plant metabolites. Half of the samples were contaminated with ZEN with an average concentration of 29.6 µg/kg whilst 83 % of the samples were found to contain GEN, DAI, and GLY with average concentrations being 2760, 936, and 7470 µg/kg respectively (Penagos-Tabares, et al. 2021). The detected levels of ZEN fall below the EU guidance value of 500 µg/kg, intended for dairy cattle and sheep feed (European Commission 2006). The presence of ZEN and different isoflavones in multiple livestock feed products was reviewed by Grgic et al. in 2021, where the highest percentages of co-occurrence of ZEN with an isoflavone ranged from poultry feed (≈ 55%), pig feed (≈ 30%), and cattle feed (≈ 15 %) (Grgic, et al. 2021). At times, individual samples surpassed the guideline values set by the European Commission for ZEN concentrations. Nevertheless, solely the mean ZEN concentration of pig feed was found to be above the limit defined for piglets and gilts (100 µg/kg) in addition to sows and fattening pigs (250 µg/kg) (European Commission 2006). Another study simultaneously determined the mycotoxins and isoflavones in soy-based burgers. The highest average concentration of 0.125 mg/100 g (1250 µg/kg) was found for DAI which was followed by 0.057 mg/100 g (570 µg/kg) of GLY and 0.017 mg/100 g (170 µg/kg) of GEN. ZEN was not to be detected in any of the analyzed samples (Rodríguez-Carrasco, et al. 2019). Data regarding co-occurrence of ZEN and isoflavones in the human diet is rather scarce, nevertheless investigations of animal feed products confirm the possibility of the simultaneous presence of both estrogenic compounds.

A different approach is to measure the total amount of estrogenic active substances using *in vitro* assays. For instance, Behr et al. analyzed a variety of human dietary staples utilizing a

yeast estrogen screen (YES). They found foodstuff to be more estrogenic once containing soy ingredients such as soy lecithin. Bread products demonstrated minimal estrogenicity, whilst dairy products seem to be a relevant source of dietary estrogens. Besides endogenous E2 and phytoestrogens, milk can also contain mycoestrogens, where ZEN has been described to transfer from cattle feed into the milk (Behr, Oehlmann and Wagner 2011). A determination of the sum of the estrogen activity might offer an insight to which food commodities act as sources of exogenous estrogens, suggesting possible co-occurrence scenarios of isoflavones and ZEN.

Mode of Action

Due to structural similarities to 17β-estradiol, isoflavones and ZEN (including its metabolites) can emulate the actions of 17β-estradiol. In Figure 5 this structural similarity especially upon consideration of the phenol ring is shown.

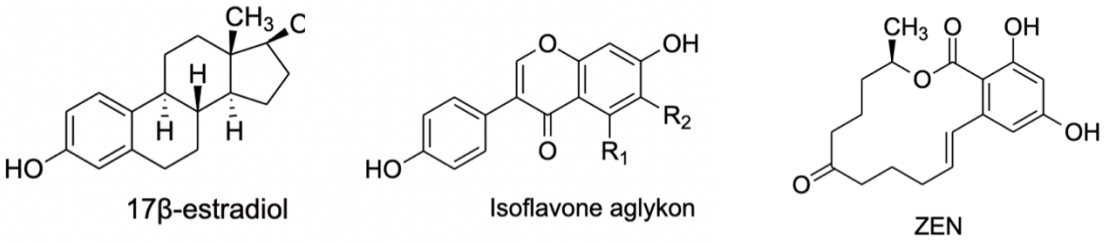


Figure 4: Structural similarities

Estrogen Receptors

17β-estradiol (E2) is an important steroidal hormone responsible for regulating cell growth, differentiation, and a broad spectrum of physiological functions. These actions are exerted through binding of E2 to intracellular receptor proteins named estrogen receptors (ERs). Estrogen receptors belong to the family of nuclear hormone receptors (NHRs), which are considered to be ligand-activated transcription factors. Generally nuclear hormone receptors, including estrogen receptors, comprise of six distinct domains with various functions. These domains are illustrated in Figure 6. Two types of estrogen receptors are presently known, ERα and ERβ, where the highest homology of these receptors exists between the DNA-binding domains (DBD).

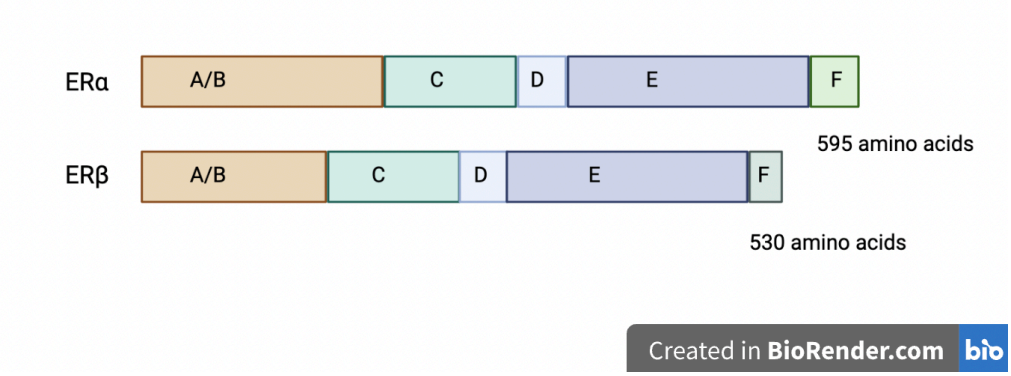


Figure 5: Structural domains of nuclear hormone receptors modified from (Yasar, et al. 2016)

Structural composition of different regions of both estrogen receptors: A/B representing the amino terminal domain, C being the DNA-binding region, D is equal to a flexible hinge, E acting as the ligand-binding domain, and lastly F as the carboxyl-terminal end.

ERα is built by 595 amino acids whilst ERβ is composed of 530 amin acids. A/B represent the amino terminal domain, C is the highly homologous DBD region, and D is called the flexible hinge with nuclear localization signals (NLS) linking the C domain to the following E domain. The E domain is considered to be the ligand-binding domain (LBD) which is the area designated as a hormone binding site as well as acting as the dimerization interface and allowing interactions between ligand-dependent co-regulatory functions such as the activation function AF-2. Lastly, the F domain represents the carboxyl-terminus with an unclear function in ERβ. In ERα it seems to act as a modulator for transcriptional activity and enhance receptor stability (Yasar, et al. 2016). In terms of ERα and ERβ distribution throughout organisms, ERα seemingly predominates in the uterus, mammary glands, pituitary gland, skeletal muscle, bones, and adipose tissue. ERβ is found in the ovary, prostate, lung, cardiovascular, and central nervous system (Yasar, et al. 2016). Co-expression of both receptors has been detected in the thyroid, uterus and brain, where ERβ often counteracts the effects of ERα (Amandusson and Blomqvist 2013).

Binding to ER: ERE-dependent Pathway

Three endogenous ER ligands are known to bind to ERs: estrone (E1), 17β-estradiol (E2), and estriol (E3). E2 is by far the most abundant and potent, as it possesses the strongest affinity to the estrogen receptors. The binding of ligands to estrogen receptors may result in variety of signaling cascades where the main two pathways represent the estrogen response element dependent pathway and the estrogen response element independent pathway. The estrogen response elements (ERE) can be seen as an area on the DNA defined to be an archetypal

5´-GGTCAnnnTGACC-3´ consensus sequence with “nnn” representing three unspecified nucleotides. Yet only a handful of estrogen responsive genes contain this exact sequence. Deviations of this sequence are found in the majority of the genes described to be sensitive to estrogens (Amandusson and Blomqvist 2013). As illustrated in the diagram below (Figure 7), estrogen receptors are bound to heat-shock protein (Hsp`s) complexes. Principally estrogen receptors are found in the nucleus as monomers, but can also be located in the cytoplasm, plasma membrane and mitochondria. Once E2, or another ligand, diffuses through the plasma membrane into the cell, it binds to the ER, consequentially leading to a disassociation of the ER from the Hsp. During the ERE-depended pathway, often referred to as the classical pathway, binding of the ligand to the ER results in a dimerization in the ligand binding site, which then allows the ER to attach to the appropriate EREs on the DNA by means of the DNA-binding domain of the ER. Furthermore, the binding of the ligand mediates conformational changes, exposing new binding surfaces of the ER complex. These surfaces are important for the activation of the transcription process as coactivators are able to bind there. Coactivators are involved in the process simplified as the untangling of chromatin. In eukaryotes, DNA strands are tightly coiled up with the help of histones to form chromatin, which is inaccessible to the transcriptional machinery. Coactivators are responsible for the uncoiling of certain areas of chromatin to liberate DNA strands and allow the commencement of transcription. For instance, histone acetylases (HATs), which are responsible for the acetylation of histones, have been linked to the activation of transcription. The acetylation of histones weakens the negative charge of the histones and hence reduces that electrostatic attraction between DNA and histones (Srinivasan and Nawaz 2009). Contrarily, corepressors can suppress transcriptional activity, due to the recruitment of histone deacetylases (HDACs), acting in a repressive manner on chromatin. ER antagonists are considered to demonstrate such an effect, resulting in a reduction in the translation of certain proteins (Srinivasan and Nawaz 2009).

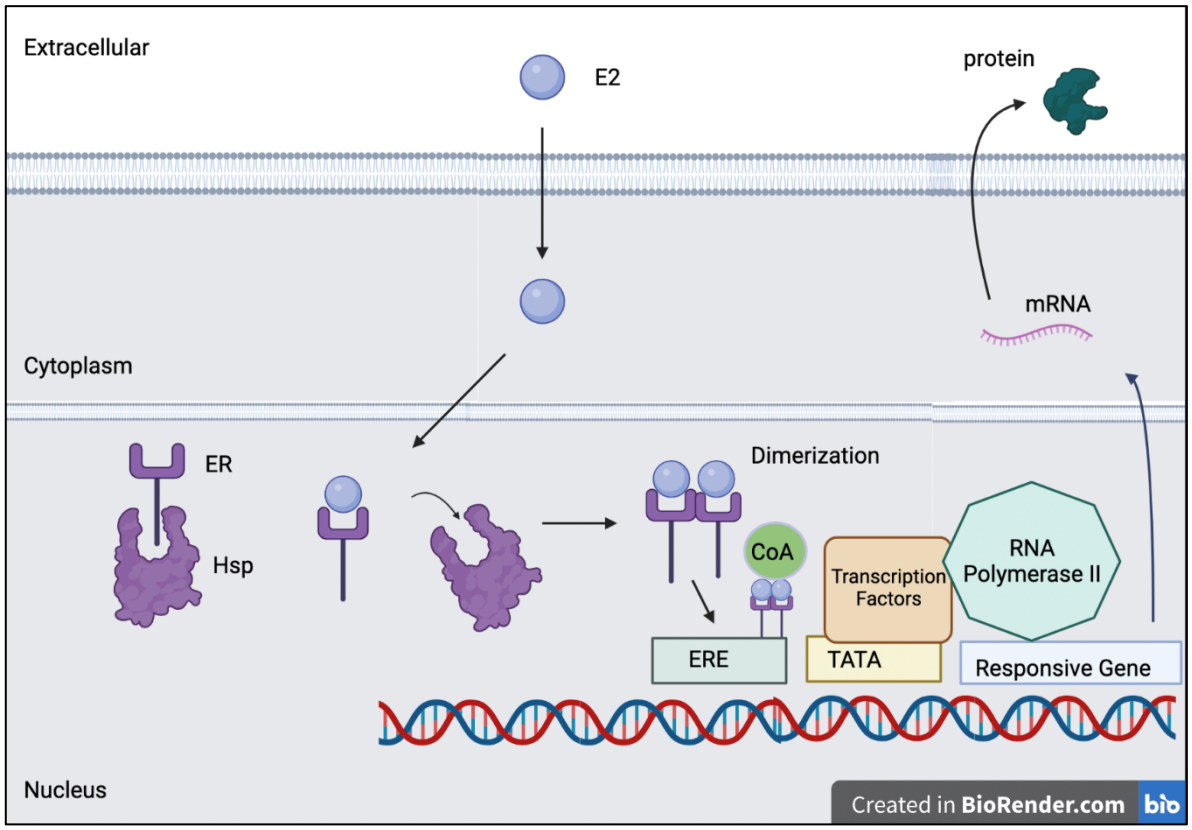


Figure 6: ERE-dependent genomic action modified from: (Srinivasan and Nawaz 2009)

Binding to ER: ERE-independent Pathway

As mentioned previously, different pathways of estrogenic signaling have been elucidated where another approach to the ERE-dependent pathway is illustrated by the ERE-independent pathway. Approximately 30% of the genes undergoing regulation through ERs, do not contain the ERE consensus sequence, calling for an alternative route of regulation (O'Lone, et al. 2004). Protein-protein interactions, sometimes referred to as "transcriptional crosstalk" represent such alternative pathways without the need for an ERE sequence on the transcribed gene. In Figure 8 below two variations of such protein-protein interactions are depicted which have been described for estrogen receptors. In a) E2 is able to interact with Jun and Fos proteins located at the activation protein 1 (AP-1) binding site. Through the involvement of the activation function (AF) domain on the ER, the protein complex is stabilized and the recruitment of general transcription coactivators at the promotor region allows the subsequent transcription of a certain estrogen responsive gene. In b) the transcription factor Sp1 interacts with ERs and seems to activate GC-rich motifs located in promotor regions of estrogen responsive genes (Björnström und Sjöberg 2005). The

activation of gene transcription is depicted, yet these mechanisms possess the ability to work in a reversible manner of fashion, hence acting repressive on transcription.

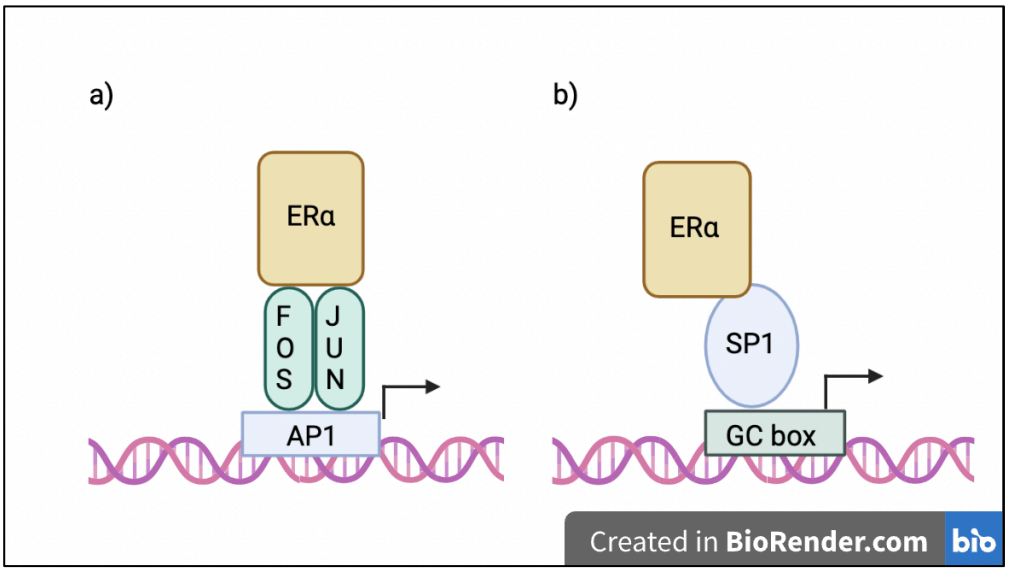


Figure 7: ERE-independent genomic action modified from (Srinivasan and Nawaz 2009)

Recent research suggests non-genomic mechanisms of estrogens, which are induced too rapidly to be accredited to mRNA transcription. In order to achieve such effects an alternative transmembrane ER has been suggested, named G-protein coupled estrogen receptor (GPER or GPER30), often found to be located intracellularly acting alongside the classical ER (Prossnitz and Barton 2011). The influx of calcium cations from extracellular regions to the interior of the cell is one example of such a rapid action and has been linked as a response to the activation of estrogen receptors, provoking regulatory physiological processes (Zhang, et al. 2009). The mobilization of calcium ions leads to potential activation of the extracellular-signal-regulated kinase (ERK1/2) pathway, the mitogen-activated protein kinases (MAPK) pathway, and the phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B (PI3K-Akt) pathway (Prossnitz and Maggiolini 2009). This complex interplay between these pathways ultimately results in the regulation of the cell cycle, apoptosis, cell proliferation and growth, and cell differentiation (Ramos 2008). Yet the activation of the above-mentioned pathways may additionally prompt genomic actions by means of activation of certain transcription factors such as AP-1 (Prossnitz and Barton 2011). Figure 9 below depicts the molecular mechanisms of genomic and non-genomic effects upon activation of the GPER.

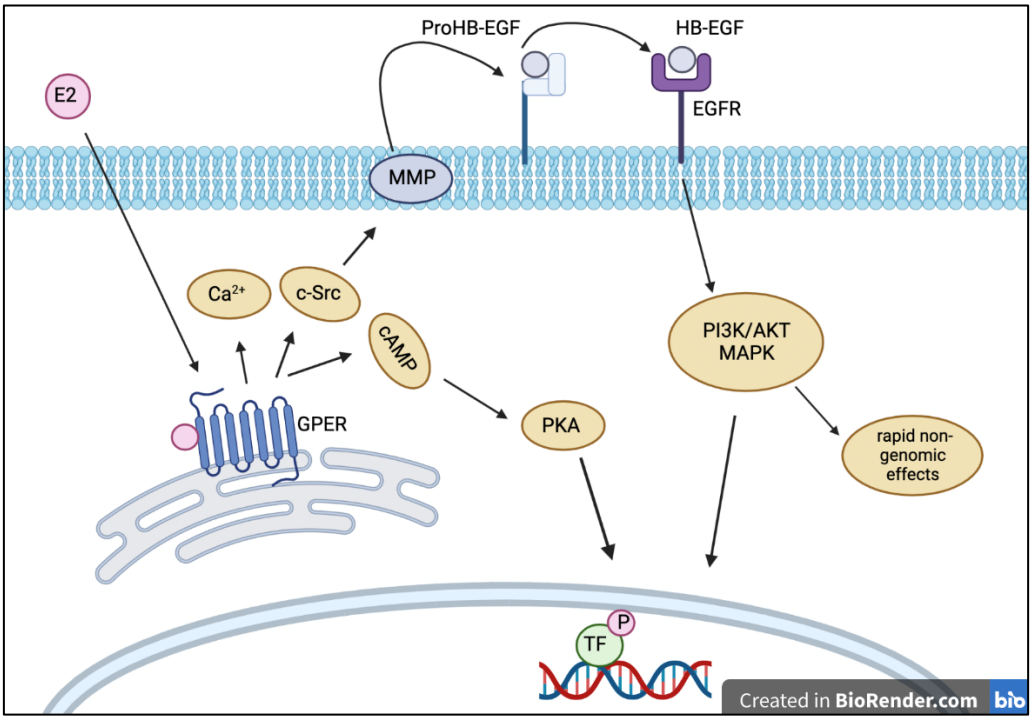


Figure 8: Genomic and non-genomic effects of GPER modified from (Prossnitz and Barton 2011)

Here upon stimulation of the GPER by E2, tyrosine kinases (c-Src) activate the matrix metalloproteinase (MMP) which in turn cleaves pro-heparin-binding-epidermal growth factor (ProHB-EGF) releasing HB-EFG which then results in a transactivation of the epidermal growth factor receptor (EGFR). Through the activation of this receptor the PI3K/Akt and MAPK pathways are induced, initiating rapid non genomic responses alongside the activation of transcription factors (Prossnitz and Barton 2011). Furthermore, the release of nucleotide cyclic adenosine monophosphate (cAMP) triggers the cAMP dependent protein kinase (PKA) which then sets the ERK signaling cascade in motion (Stork and Schmitt 2002). In summary the interplay between the stimulation of estrogen receptors and the responses elicited are regulated in a highly convoluted network of pathways.

Isoflavones and Zearalenone as ER Agonists

Apart from endogenic estrogens, exogenic compounds with structural similarities to estrogens have the potential to act upon estrogen receptors. ZEN and isoflavones represent two such compound classes due to their structural similarity to E2, as highlighted in Figure 5. Their binding to estrogen receptors has been demonstrated in various *in vitro* models, where isoflavones appear to exhibit a stronger affinity to ERβ and ZEN to ERα (Kuiper, et al. 1998). The subsequent events triggered by isoflavones and ZEN binding to estrogen receptors,

include a broad spectrum of mechanisms. ZEN has demonstrated to induce estrogenicity in the form of gene induction, cell proliferation, and activation of the ERK 1/2 pathway comparable to E2 at concentrations as low as 0.01 nM (Parveen, Zhu and Kiyama 2009). An upregulation of certain steroid hormone receptors, namely the progesterone receptor, has also been linked to ZEN, portraying a classical estrogen dependent response (Frizzell, et al. 2011). Additionally, Li et al. provided further evidence for the activation of the ERE-mediated transcription by ZEN via the luciferase reporter assay system (Li, et al. 2012). In regard to isoflavones, GEN was found to promote estrogen-dependent cell proliferation. Additionally, in combination with E2, GEN appears to interfere with E2-induced mechanisms, suggesting an interplay between the two ER isoforms (Wang, Sathyamoorthy and Phang 1996). E2 exhibits equal affinity to ERα and ERβ, consequentially being able to non-selectively recruit transcriptional coregulators in both scenarios. Meanwhile besides exhibiting a stronger binding affinity to ERβ compared to ERα, isoflavones consequentially appear to selectively trigger the transcriptional pathways of ERβ (An, et al. 2001). The activation of estrogen receptors by xenoestrogens such as isoflavones and ZEN triggers an intricate set of mechanisms resulting in a broad spectrum of endpoints, where the potential interplay between the ER isoforms is not to be disregarded.

ESR1 and *ESR2*

The translation into the receptor proteins ERα and ERβ occurs upon the transcription of the genes *ESR1* and *ESR2* respectively. Both genes are situated on two distinct chromosomes, ruling out the possibility of alterative splicing to form both isoforms. *ESR1* is located on the chromosome 6 on band q24-q27 whilst *ESR2* is found on chromosome 14 on band q22-24 (Enmark and Gustafsson 2001). The up and downregulation of genes is part of a regulatory response system to satisfy the demand for receptors, enzymes, and other proteins. The intake of isoflavones has been associated with a concentration dependent change in regulation in both estrogen receptors. Fritz et al. demonstrated a decrease in the mRNA for ERα and ERβ in the prostates of rats upon a 2-week exposure to a GEN-rich diet (Fritz, et al. 2002). *In vitro* models using primary cerebellar cell cultures from rats and mice found ZEN to increase the expression of ERα and ERβ after a 6-hour incubation time, yet the combination of ZEN with E2 or a thyroid hormone, reduced such effects (Jocsak, et al. 2019).

Role of CYP Enzymes

The constitutive family of cytochrome P450s (CYP) enzymes play an essential role in the oxidative activation and consequential detoxification of a multitude of compounds during phase I metabolism. The metabolization of estrogens lies within the scope of the CYP enzymes, with focus on the isoform *CYP1B1*. Estrogen-related tissues such as the mammary, ovary, and uterus demonstrate a high expression of *CYP1B1*, suggesting its importance in the metabolism of E2. The catechol 4-hydroxyestradiol is an E2 metabolite formed by *CYP1B1*, where a reduction in estrogenicity is achieved. This catechol generates free radicals by the means of reductive oxidative cycling, causing damage on a cellular level (Lee, et al. 2003). In *in vitro* models, the expression of *CYP1B1* was altered by exposition to E2 depending on the cell line used. In MCF-7 cells, E2 exposition resulted in an upregulation, whilst in Ishikawa cells, no such effects were observed (Tsuchiya, et al. 2004). The isoform *CYP1A1* is another CYP enzyme appearing to mediate E2 homeostasis in form of 2-hydroxylation of 17β-estradiol, again reducing the ultimate estrogenicity of E2 (Lee, et al. 2003). In estrogen responsive cell lines such as human endometrial cells and mammary carcinoma cells, the *CYP1A1* activity was reduced upon 48 h treatment with E2 (Ricci, et al. 1999). Apart from E2, evidence for other estrogenic compounds affecting the expression of CYP enzymes is accumulating. Froyen and Steinberg have found GEN to decrease basal levels of *CYP1A1* in mice upon being fed a diet rich in GEN (Froyen and Steinberg 2016). Furthermore, in MCF-7 cells, a concentration-depended kinetic inhibition of *CYP1A1* and *CYP1B1* was determined due to GEN exposure (Chan and Leun 2003). Meanwhile Yu et al. investigated the role of ZEN in combination with the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Here TCDD induced the expression of basal levels of *CYP1A1* and *CYP1B1* mRNA significantly in MCF-7 cells, where then this TCDD-induced activity was diminished by 50 nmol/L of ZEN, thus acting as a potent CYP enzyme regulator (Yu, Hua and Lib 2004). Overall, the CYP enzyme isoforms *CYP1A1* and *CYP1B1* are essential in estrogen metabolism, thus E2 assuming the dual role of substrate and modulator. This raises the question whether estrogenic compounds such as ZEN and isoflavones, as single substances or in combination, conceivably interfere in transcriptional mechanisms concerning the above mentioned CYP enzymes.

Aim

Chronic daily exposure to multiple substances simultaneously scrutinizes classical toxicological approaches concerning the assessment of single compounds. Focus is often laid on anthropogenic substances, yet a wide variety of naturally occurring compounds with toxic potential pose an equal concern. Mycotoxins represent one such natural class of toxins, classified as an emerging threat to food safety. In terms of endocrine disrupting properties, the mycotoxin ZEN demonstrates strong estrogenicity, rather than acute toxicity. Isoflavones are part of the bioactive congregate of polyphenols, and are equally associated with eliciting estrogenic responses. As ZEN and isoflavones act upon estrogenic receptors, displaying similar modes of action, whilst co-occurring in certain food and feed products, an investigation concerning plausible synergisms is deemed as relevant.

This thesis is conducted within the scope of exploring "combinatory endocrine activity of mycoestrogens and soy isoflavones in porcine feed" funded by the Austrian Research Promotion Agency (FFG) in cooperation with the company BIOMIN. Preliminary results suggested high concentrations of isoflavones along with ZEN in porcine feed. Pigs demonstrate a high bioavailability of ZEN (up to 85% upon oral administration), resulting in an evident sensitivity towards this mycoestrogen (Biehl, et al. 1993). The metabolization of ZEN to its various metabolites also plays a role in terms of sensitivity. Pigs and humans tend to biotransform ZEN to the more potent α-ZEL in contrast to broilers, who preferably form the less potent β-ZEL, hence distinguishing pigs and humans as the more sensitive species (Catteuw, et al. 2019). Adverse effects observed in various animal models, upon exposure to ZEN or its metabolites, include reproductive disorders, organ hyperplasia, as well as transgenerational effects (Kowalska, et al. 2016). Meanwhile high concentrations of isoflavones have been associated with reproductive disorders in sheep ultimately resulting in infertility. In humans, the effects of low concentrations of isoflavones remain ambiguous and subject to further research (Krizova, et al. 2019). Considering the structural resemblance of isoflavones and ZEN to E2, both phytochemicals are able to bind to estrogen receptors and hence follow a similar mode of action. Due to this mechanistic alikeness in addition to their co-occurrence in feed and food products, the question whether synergistic, additive, or antagonistic effects are found, remains to be elucidated.

Recent research has utilized the alkaline phosphatase (ALP) assay to assess the estrogenicity of ZEN and a selection of its metabolites as well as certain isoflavones. The assessment of the alkaline phosphatase activity may serve as an indicator for estrogenic activity. Upon the activation of estrogen receptors, the gene encoding for alkaline phosphatase is upregulated, hence an increased measurement of the alkaline phosphatase implies a stronger estrogenic effect. Vejdovszky et al. found ZEN and its metabolite α-ZEL to demonstrate potent estrogenic activity applying the ALP assay to the human endometrial adenocarcinoma Ishikawa cell line (Vejdovszky, et al. 2017). Based on these findings, additional research evaluated combinatory effects between ZEN and GEN, demonstrating synergism at certain concentrations and yielding in estrogenicity beyond E2 at 1 nM (Vejdovszky, Schmidt, et al. 2017). Thus, a mechanistical interplay between isoflavones and ZEN has been suggested, yet demanding further research to allow proper interpretations.

Within the framework of the "combinatory endocrine activity of mycoestrogens and soy isoflavones in porcine feed" project, the ALP assay was performed using the Ishikawa cell line to test various combinations between different isoflavones, ZEN, and ZEN metabolites. Various concentrations of the isoflavones GEN, DAI, GLY, or EQ were combined with either ZEN or α-ZEL, α-ZAL, and ZEN-14-S (Betschler 2021). In order to exclude cytotoxicity as a culprit for the observed reduction in ALP levels, cytotoxicity assays have to be conducted. In this thesis, two distinct cytotoxicity assays, "CellTiter Blue" (CTB) and "sulforhodamine B" (SRB) assay, will be utilized to demonstrate the lack of cytotoxic potential of the above-named combination. Here too, the Ishikawa cell line is the cell line of choice, in order to exclude any variability from the ALP experiments.

The ALP results indicated at certain combinations, a drastic increase in ALP activity up to a certain concentration, to be followed by an evident reduction in said activity. For example, the co-incubation of ZEN and GEN demonstrated an induced ALP activity until 1 µM of GEN, however at 10 µM of GEN the ALP activity returned to the values comparable at 0.1 µM (Betschler 2021). As a secondary aim of this thesis, real-time polymerase chain reaction (PCR) shall be utilized in an attempt to shed light on these mechanisms. The genes of interest examined include *CYP1A1, CYP1B1, ESR1*, and *ESR2*. *CYP1A1* and *CYP1B1* belong to the broad

group of cytochrome P450 enzymes, essential for the metabolization of estrogens (Lee, et al. 2003). An increased exposition to high concentrations of xenobiotic estrogens could potentially cause an upregulation of these genes and hence stimulate metabolic activity of xenoestrogens, provoking the observed decrease in ALP activity at high isoflavone concentrations. Previous research has found daidzein at concentrations of 78.5 µM to trigger an upregulation of *CYP1B1* in breast cancer cell lines (Satih, et al. 2010). Another plausible mechanism responsible for the drastic ALP activity decrease at high isoflavone concentrations is a downregulation of the genes encoding for both ER isoforms. *In vivo* data has demonstrated a decrease in ERα, yet not in ERβ upon treatment of GEN (Cotroneo, et al. 2001). Hence an elucidation of the mechanisms behind the decline in ALP activity at elevated isoflavone concentrations is anticipated by evaluating an up or downregulation of selected genes.

Materials and Method

Materials

Chemicals

Acetic acid glacial, ≥ 99.7 %	Fisher Chemical, Loughborough, UK
CellTiter-Blue® Cell Viability Assay Kit	Promega Corporation, Madison, Wisconsin, USA
Charcoal stripped FCS (CD- FCS)	Gibco, Thermo Fisher Scientific, Waltham, USA
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Dulbecco's Modified Eagle Medium /F12 (DMEM/F-12) (1x), w/o: phenol red	Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Ethanol, 96 %, VG 1 % Petrol- benzin	Brenntag Austria GmbH, Vienna, AT
Fetal Bovine Serum (FBS), South America origin	Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA
L-Glutamine	Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Minimal Essential Medium (MEM)	Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Penicillin-Streptomycin (P/S)	Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Potassium chloride (KCl)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Primers	1Hs_ALAS1_1_SG (QT00073122) HS_CYP1A1_1_SG (QT00012341) HS_CYP1B1_1_SG (QT00028714) HS_ESR1_1_SG (QT00044492)HS_ESR2_1_SG (QT00060641) Hs_HPRT1_1_SG (QT00059066)
QuantiTect® Reverse Transcription Kit	Qiagen, Hilden, DE

QuantiTect® SYBR® Green PCR Kit	Qiagen, Hilden, DE
RNase Easy Mini Kit	Qiagen, Hilden, DE
RNase AWAY	Molecular BioProducts Inc., San Diego, California, USA
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Sulforhodamine B (SRB)	Sigma Aldrich Chemie GmbH, Schnelldorf, DE
Trichloroacetic acid (TCA)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Tris-(hydroxymethyl)-amino methane	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Trypan blue solution, sterile filtered	Sigma Aldrich Chemie GmbH, Schnelldorf, DE
Trypsin, 5 000 USP-U/mg; free from salt, from porcine pancreas, lyophilized	Carl Roth GmbH + Co. KG, Karlsruhe, DE

Test Substances

17β-estradiol (E2)	Sigma Aldrich Chemie GmbH, Schnelldorf, DE
α-zearalanol (α-ZAL)	Sigma Aldrich Chemie GmbH, Schnelldorf, DE
Daidzein (DAI)	Extrasynthese, Genay Cedex, FR
Equol (EQ)	Extrasynthese, Genay Cedex, FR
Genistein (GEN)	Extrasynthese, Genay Cedex, FR
Glycitein (GLY)	Extrasynthese, Genay Cedex, FR
Hydrolyzed zearalenone (HZEN)	DSM - BIOMIN Holding GmbH, Tulln an der Donau, AT
Decarboxylated hydrolyzed zearalenone (DHZEN)	DSM - BIOMIN Holding GmbH, Tulln an der Donau, AT

Zearalenone (ZEN)	Sigma Aldrich Chemie GmbH, Schnelldorf, DE
Zearalenone-4-sulfate ammonium salt (equal to zearalenone-14-sulfate; ZEN-14-sulfate)	Santa Cruz Biotechnology, Dallas, Texas, USA

Consumables

96-well plate, black	Sarstedt AG & Co, Nümbrecht, DE
96-well plate, sterile	Sarstedt AG & Co, Nümbrecht, DE
12-well plate, sterile	Sarstedt AG & Co, Nümbrecht, DE
Biosphere® Pipette tips, sterile	Sarstedt AG & Co, Nümbrecht, DE
Cell culture flasks (75 cm^2, 175 cm^2)	Sarstedt AG & Co, Nümbrecht, DE
Cell scraper	Sarstedt AG & Co, Nümbrecht, DE
Dispenser-Tips (0.5 mL, 2.5 mL)	ratiolab GmbH, Dreieich, DE
Duran bottles (100 mL, 500 mL, 1 L, 2 L)	Schott AG, Mainz, DE
Gloves	KIMTECH™, Kimberly-Clark Worldwide Corporation, Dallas, Texas, USA
Light-duty tissue wipers	VWR International, Radnor, Pennsylvania, USA
Microscope cover glasses	24 x 24 mm, thickness 0.17 ± 0.005 mm, VWR Austria, Wien, AT
Optical adhesive film for microplates	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Pasteur pipettes	Carl Roth GmbH + Co. KG, Karlsruhe, DE
PCR 96-well plates	Peqlab VWR, Darmstadt, DE
Pipette tips (10 µL), nonsterile	VWR Austria, Wien, AT
Pipette tips (200 µL, 1000 µL, 5 mL), nonsterile	Sarstedt AG & Co, Nümbrecht, DE
Reaction tubes (0.2 mL, 0.5 mL, 1.5 mL, 2 mL, 5 mL)	Sarstedt AG & Co, Nümbrecht, DE
Serological pipettes (5 mL, 10 mL)	Sarstedt AG & Co, Nümbrecht, DE

Syringes	Henke Sass Wolf, Tuttlingen, DE
Centrifuge tubes (15 mL, 50 mL)	Sarstedt AG & Co, Nümbrecht, DE

Instruments

Autoclave	Systec DX-150, Systec GmbH, Weltenberg, DE
Balance	NewClassic MF, ML6001/01, Mettler Toledo GmbH, Gießen, DE
Centrifuge	Z 326 K, Hermle Labortechnik GmbH, Wehingen, DE Mikro 200, Hettich Zentrifugen, Tuttlingen, DE
Manual repetitive pipette	BRAND™ HandyStep™, BRAND GMBH + CO KG, Wertheim, DE
Microcentrifuge	Galaxy MiniStar, VWR, Vienna, AT
Plate spinner	Peqlab perfect Spin, Peqlab Biotechnologie GmbH, Erlangen, DE
Refrigerator/Freezer	Liebherr Premium Comfort (4°C, -20 °C, -80 °C), Liebherr, Bulle, CH
Hotplate Magnetic Stirrer	MS-H-PRO+, DLAB Scientific Inc., Ontario, California, USA
Incubator	Heracell 240 L CO_2-Incubator, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Inverse microscope	Axiovert 40C, Zeiss Objective: A-Plan, Carl Zeiss Microscopy GmbH, Jena, DE
Ice machine	MF 46, Scotsman Frimont, Mailand, IT
Laminar flow hood	Hera Safe KS 18, Thermo Fischer Scientific, Waltham, Massachusetts, USA
Multichannel pipette	10 – 100 µL (8-channel), Eppendorf Research, Hamburg, DE

	50 – 300 µL (8-channel), Labemate Pro, Corning HTL SA, Warszawa, PL
NanoDrop	NanoDrop™ 2000c Spectrophotometer, Thermo Fischer Scientific, Waltham, Massachusetts, USA
Neubauer counting chamber	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, DE
Peltier thermal cycler	DyadDisciple Bio-Rad Laboratories GmbH, California, USA
pH-meter	PC 8 + DHS, XS Instruments, Carpi MO, IT
Pipettes	10 µL, 20 µL, 100 µL, 200 µL, 1000 µL, 5 mL Eppendorf Research, Hamburg, DE Labemate Pro, Corning HTL SA, Warszawa, PL
Pipetting device	Pipetus® Akku, Hirschmann Laborgeräte, Eberstadt, DE
Plate Reader	Victor3V, 1420 Multilabel Counter, Perkin Elmer, Waltham, Massachusetts, USA Synergy H1 Plate Reader, BioTek, Winooski, Vermont, USA Cytation 3 Cell Imaging Multi-Mode Reader, BioTek, Winooski, Vermont, USA
Pump Vacuum Aspiration System	Vacusafe, INTEGRA Biosciences AG, Zizers, CH
StepOne Plus™ Instrument	Life Technologies, Waltham, Massachusetts, USA
Tabletop centrifuge	5810 R and 5417 R, Eppendorf AG, Hamburg, DE
Ultraviolet Sterilizing PCR Workstation	Peqlab Biotechnologie GmbH, Erlangen, DE

Vortexer	Lab dancer S40 VWR Deutschland, Darmstadt, DE
Water bath	GD 100 Grant Instruments, Cambridge, UK

Software

BioRender (biorender.com)	BioRender, Toronto, Ohio, USA
Microsoft® Excel® Microsoft 365	Microsoft Corporation, Redmond, Oregon, USA
NanoDrop	NanoDrop 2000c Spectrophotometer, Thermo Fischer Scientific, Waltham, Massachusetts, USA
Origin Pro® 2022b	OriginLab Corporation, Northampton, Massachusetts, USA
Plate Reader: Gen5™ Version 3.08	BioTek Instruments, Winooski, Vermont, USA
Plate Reader: Wallac 1420 Workstation	Perkin Elmer, Waltham, Massachusetts, USA
StepOne Real-Time PCR System	Thermo Fischer Scientific, Waltham, Massachusetts, USA

Cell Line

Human endometrial adenocarcinoma cells (Ishikawa)	ECACC 99040201, Public Health England, Salisbury, UK

Cell Culture Introduction

Ishikawa Cell Line

The Ishikawa cell line is considered to be a well-characterized endometrial adenocarcinoma cell line derived from a middle-aged patient of Japanese origin. Once in culture, these cells are arranged in a monolayer, where individual cells are easily distinguished. The presence of estrogen (ERα and ERβ) and progesterone receptors portray a significant attribute of this cell line in addition to their sensitivity towards receptor-activating substances. Yet, at cells above the 50th generation, these receptors were not expressed anymore. Hence, testing should be limited to cells below the 50th generation (Nishida 2002). A further important characteristic of this cell line is its ability to mimic metabolic pathways, including the modulation of estrogens. Ishikawa cells express the inducible alkaline phosphatase (ALP), which is activated by endogenous estrogens and xenoestrogens (Guevel and Pakdel 2001). As a major aspect of this project, in which this thesis is part of, estrogenicity was assessed using the estrogen-inducible ALP of the Ishikawa cells, hence the same cell line had to be utilized for the cytotoxicity assays performed in this thesis.

Cultivation of Ishikawa Cells

The cultivation of Ishikawa cells was performed following a general cell cultivation protocol. The cells were grown in cell flasks and kept in an incubator set to the following parameters: 37 °C, 5% carbon dioxide (CO_2), and 95% humidity. Depending on the number of cells required, the sizes of the cell flasks used were either the T75 (75 cm^2) or T175 (175 cm^2). In order to maintain sterile conditions, work with the cells was solely performed under a laminar flow cabinet. In this workbench, air is filtered and directed as a laminar air flow to prevent contamination of the samples in addition to hindering particles to exit the workbench, thus protecting the user. For the purpose of disinfection, the work bench was sprayed with 70% ethanol before and after usage. Furthermore, all materials transferred into the laminar flow cabinet were treated with 70% ethanol and if possible, autoclaved prior to usage. Lastly nitrile gloves were worn for the entire handling time, again to prevent contamination. The cells were solely treated with pre-warmed solutions (in a water bath set to 37°C) and the times spend outside of the incubator were kept as short as possible.

The growth of cell cultures requires appropriate media providing vital micro and macro nutrients. Ishikawa cells were propagated in Gibco Minimal Essential Medium (MEM) based on Harry Eagle´s formula containing amino acids, vitamins, ionic species, glucose, and serum proteins (Eagle 1959). As cells are highly sensitive to changes in the pH, phenol red is added to the MEM to serve as a color indicator. Between a pH of 6.2-8.2, a medium containing phenol red appears red, whilst at a lower pH it turns yellow and at a higher pH it transitions to a pink color. Cellular waste products will induce a decrease in the pH, where then the color transitioning of the medium serves as an immediate alarm (Held 2018). The physiological pH range of 7.2-7.4 offers the ideal surrounding for the majority of the cell lines cultivated (Maurer 2005). The MEM was optimized with the addition of 5% fetal bovine serum (FBS). FBS is considered to be a byproduct of the meat industry, utilized in cell culture to promote cell growth, adhesion, and proliferation (Gstraunthaler, Lindl and Valk 2013). Furthermore, to prolong microbial stability, 1% penicillin/streptomycin (P/S) was added to the medium. Penicillin aims to interfere in the building process of the cell wall, thus being effective against gram-positive bacteria. Streptomycin inhibits protein synthesis, targeting gram-negative bacteria (Thermo Fischer Scientific 2022). Lastly, the addition of 1% L-glutamine finalized the medium used for the cultivation of the Ishikawa cells.

Passaging of Ishikawa Cells

Passaging of Ishikawa cells is required to ensure constant growth and propagation of the Ishikawa cells whilst maintaining perpetual characteristics. The quintessence of cell passaging involves the transfer of cell aliquot to a fresh medium. For these types of cells, a confluency of around 80% is thought to be ideal. A density above 80% could result in a loss in proliferative activity, where this activity potentially remained to be lost throughout the following cell generations. In general, this range of confluency was achieved within 3-4 days.

In further detail, the passaging of the cells was initiated with the removal of the current medium. This was performed using a sterile glass Pasteur pipette attached to a vacuum system. As the cells adhered to the flask bottom, great care was given to not suction off the cells during the removal of the medium. Then, the cells were washed with 10 mL of phosphate-buffered saline solution (PBS), which had been previously warmed in the water

bath. The PBS consisted of 100 g sodium chloride (NaCl), 17.9 g di-sodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$), 2.54 g potassium chloride (KCl) and 2.45 g potassium dihydrogen phosphate (KH_2PO_4) dissolved in distilled water with a pH adjusted to 7.4 using 1 M NaOH. Prior to usage, the solution was sterile filtered and diluted 1:10. This washing step served to remove any metabolic waste products or dead cells which could hinder the subsequent procedure. The PBS was again suctioned off using the Pasteur pipette. In order to disintegrate the cells from the cell flask wall and to loosen the cell adhesion bonds, the protease trypsin was added on top of the cells. The volume used depended on the size of the cell flask, ranging from 2.5 mL to 3.0 mL. The cell flask was moved around slowly to ensure trypsin covering the entire cell layer and then the flask was placed again into the incubator for approximately 3 minutes. Thereafter, the cell flask was tapped strongly at the sides to facilitate the loosening of the cell bonds. Under the microscope this process was then observed to confirm the disintegration of the cell layer. Once single cells were evidently seen under the microscope and the solution assumed a cloudy color to the naked eye, 10 mL of MEM with 5% FBS and 1% P/S were added quickly using a serological pipette. This step had to follow rapidly as an overly long exposure could impose long-term damage to the cells. The medium was resuspended a few times, consequentially allowing the flask bottom to be washed from multiple angles. Finally, the suspension was transferred into a 15 mL centrifuge tube, then the cells were counted using a hemocytometer (for detailed procedure: see Counting of Ishikawa Cells).

Upon determination of the cell number, an appropriate volume of the cell suspension had to be calculated to ensure proper cell proliferation over the next 3-4 days. Depending on the number of days until the next passaging and on the size of the cell flask, either 1 000 000 cells (3 days, T175) or 850 000 cells (4 days, T175) were transferred into a new flask or the same previous flask. Each flask was reused up to 3 times, then at the 4^{th} passaging a new flask was used. Lastly to complete the passaging, fresh MEM with 5% FBS and 1% P/S had to be added. Here again the volume depended on the size of the cell flask, the larger flasks required 35 mL of medium in contrast to the smaller flask (T75) with only 10 mL. The labelling of the cell passage consisted of the passage number before freezing followed by the increasing passage number once the frozen cells were thawed. The cultivation of a cell culture was limited to approximately 40 passages, as surpassing 50 passages resulted in a loss of key characteristics.

Counting of Ishikawa Cells

As described in the previous section, the counting of cells is a necessary act to determine an appropriate volume of the cell suspension for a successful cell passaging. Furthermore, to conduct reproducible assays using cells, a consistent number of cells should be used, hence here again cell counting is important. Cell counting was performed using a Neubauer counting chamber, also described as a hemocytometer (Figure 10). This method is quick, reliable, and does not require a great deal of equipment. The counting chamber consists of two halves, each with a grid etched into the glass (as seen in Figure 11). Four large squares make up one half, each square being subdivided into 16 smaller ones. Each square is 1 mm wide with a depth of 0.1 mm, resulting in a volume of 0.1 mm^3. Thereafter, 0.1 mm^3 is then equal to a volume of 0.1 µL. Thus, once the average cell number is determined, it has to be multiplied by the factor 10^4 in order to portray the number of cells per mL.

Figure 9: Neubauer counting chamber

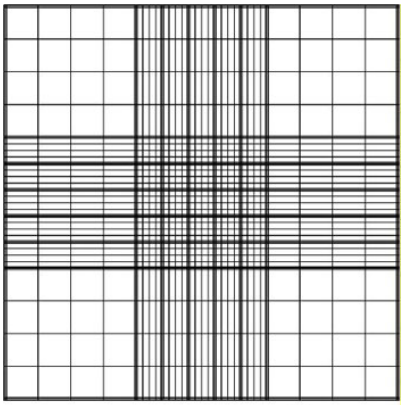


Figure 10: Gridlines counting chamber (modified from: (Labor Optik 2022)

Firstly, the counting chamber is prepared for the subsequent steps. Here 70% ethanol is used to clean the chamber with a lint-free paper. Then using one´s own breath to moisten the chamber, the cover glass is fixed on the chamber. An aliquot (20 µL) of the cell suspension was transferred into a small reaction tube, to which 80 µL trypan blue solution was added. The solution was briefly vortexed. Afterwards, 10 µL of the vortexed solution was withdrawn with a pipette and pipetted into the space between the cover glass and the chamber. This was repeated for the other side of the chamber. Under a microscope, the cells in each square were counted systematically. At this point, it should be noted that solely the white cells were taken into consideration, as the blue cells represent dead cells. This color-based distinction is

due to the trypan blue dye, which is able to penetrate the porous cell membranes of damaged cells. In healthy cells the dye cannot diffuse through the membrane, hence these cells remain white, or simply uncolored (Fang and Trewyn 2012). Once all cells were counted, the average was calculated first of each half and then of the two halves. Based on the volume of each square, following formula can be applied to determine the number of cells per mL:

$$\frac{cells}{mL} = average\ cell\ count \times 10000 \times dilution\ factor$$

In this case, the dilution factor was 5 as the suspension was diluted 20:80.

Cytotoxicity Assays

Cytotoxicity assays are *in vitro* methods performed on cells to observe changes in cell vitality upon exposure to defined concentrations of certain substances. In this thesis, cytotoxicity assays were conducted to exclude possible cytotoxic effects of the isoflavones, ZEN as well as ZEN metabolites. A perceived cytotoxicity would challenge the ALP data gathered previously, scrutinizing the estrogenicity of the above-named compounds (Betschler 2021).

Seeding of Cells for Cytotoxicity Assays

First, the seeding of cells in appropriate labware had to be achieved, in order to proceed with the cytotoxicity assays. In this case, the cells were seeded in 96-well plates, with an identical number of cells to guarantee reproducibility.

To start off the process of cell seeding, the cell suspension gained from the passaging of the cells in the 15 mL tubes were centrifuged for 10 minutes at a speed of 300*g at 37°C. After centrifugation, a cell pellet was visible at the bottom of the tube, then the remaining medium was suctioned off with the Pasteur pipette. A resuspension of the cells was done with 5 mL of a new medium named Dulbecco´s Modified Eagle Medium: Nutrient Mixture F-12, phenol red free (DMEM/F-12). A change in medium was required due to the presence of phenol red in the MEM. Phenol red has been associated with the induction of estrogenic effects in previous cell culture experiments, thus it could act as an interference (Welshons, et al. 1988). This medium also had to be enriched with growth factors in the form of fetal calf serum which had

been previously stripped off its endogenous estrogens with the help of dextran coated charcoal (CD-FCS). For this, 5% of CD-FCS and again 1% of P/S were added to the DMEM/F-12 medium to ensure proper cell growth and microbial stability. Once the resuspension had been completed, the cells were counted according to the protocol described above.

The seeding of the cells was done in 96-well plates, however here only the inner 60 wells were used, as depicted in Figure 12. The outer wells were disregarded to prevent variability of the results due to the "edge effect". This phenomenon occurs as the outer wells are subject to higher evaporation compared to the interior wells, being source of plausible discrepancies in later results (Mansoury, et al. 2021). A concentration of 100 000 cells/mL was required for well plates which were incubated for 48 hours whilst a concentration of 150 000 cells/mL was used for cells incubated for solely 24 hours. In total this would amount to 600 000 cells or 900 000 cells respectively per well plate, as 60 wells were filled with 100 µL of cell suspension. To obtain appropriate concentrations, the cell suspension was diluted with DMEM/F-12 medium upon determination of the cell number via the hemocytometer. The distribution of the cell suspension into each individual well was performed with a manual repeating pipette. In each inner well 100 µL of cell suspension was pipetted. The outer wells were filled with 100 µL of PBS. To be on the safe side, around 7 mL of the cell suspension was prepared for a single well plate, although only 6 mL were required. Once the necessary amount of well plates were seeded with the cells, the well plates were labelled and placed in the incubator at the same conditions at which the cells flasks were kept at.

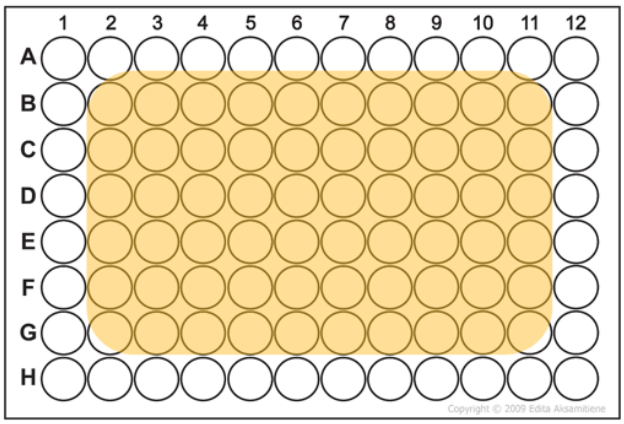


Figure 11: Schematic depiction of 96-well plate modified from (Aksamitiene 2012)

Incubation of Ishikawa Cells

In order to proceed with the cytotoxicity assays, the cells had to be exposed to the compounds of interest. Stocks of isoflavones, ZEN, and the ZEN metabolites were stored at high concentrations at -80 °C in aliquots. The compounds were diluted with DMSO. The required solutions were prepared freshly on the days of incubation to avoid any loss of activity or degradation of the compounds. The concentrations prepared were 200 times higher than the actual concentrations required, as they had to be diluted with the optimized DMEM/F-12 in order to supply the cells with nutrients during the incubation. As each measurement was carried out in technical replicates, 3 wells were incubated with the exact same solution. For a single substance mixture 346.5 µL of medium, 1.75 µL DMSO, and 1.75 µL of the test substance in DMSO were added into a reaction tube. For the assessment of combinatory effects, again 346.5 µL of medium were pipetted into a reaction tube and this time 1.75 µL of both test substances were added. In total each reaction tube contained 350 µL of the final solution. A solvent control was carried out with a solution consisting of 346.5 µL medium and 3.5 µL of DMSO. Likewise, a solution of 346.5 µL medium, 1.75 µL DMSO, and 1.75 µL E2 (200 nM) served as the positive control, resulting in a final concentration of 1 nM E2.

Once the solutions had been prepared, the seeded well plates were removed from the incubator and the medium was suctioned off with the sterile Pasteur pipette. Then 100 µL of the prepared solutions was added to each well, according to an exact scheme (see an example in the appendix, on page 112, Figure 31). Once all wells were filled, the plates were placed into the incubator again for 48 hours.

Cell Titer Blue Assay

The Cell Titer Blue (CTB) assay is an *in vitro* method based on fluorescence detection, conveying information about the cell viability and metabolic activity of the cells observed. The main principle of the CTB assay is based on the redox dye, resazurin, which is reduced by viable cells to the fluorescent resorufin end product (see Figure 13). In case of nonviability, resazurin is not converted, thus diminishing the fluorescent signal detected. This reduction in fluorescence can be interpreted as cytotoxicity. Alternatively, an increase in fluorescence could be due to cell proliferation or simply a higher metabolic activity of the cells.

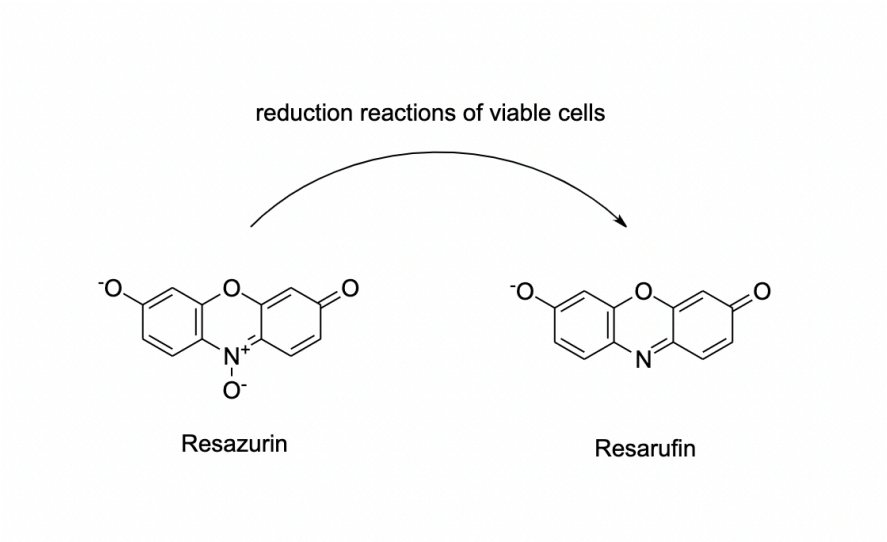


Figure 12: Reduction of resazurin to resarufin modified from (Shenoy, et al. 2017)

Figure 14 illustrates a schematic diagram encompassing each step of the CTB assay. The CTB solution was stored in aliquots in 1.5 mL reaction tubes in the freezer and the amount needed was thawed in the dark. Once it was completely thawed, it had to be diluted in a 1:10 fashion with the modified DMEM/F-12. All steps involving the handling of the CTB solution had to be performed in the dark, as the dye is light sensitive and exposure to an excessive amount of light could potentially falsify the obtained results. The incubated well plates were taken from the incubator after the incubation time and the solution was removed once more with the help of the Pasteur pipette. Then 100 µL of the diluted CTB solution was pipetted into each well using the manual repeating pipette. The outermost left row of wells (containing no cells, solely PBS) was also suctioned off, and then filled with the CTB solution. These 6 wells served as the blank value for follow-up calculations. Upon completion, the plates were placed into the incubator for 50 minutes. Meanwhile black 96-well plates were prepared, labelled, and a thermo box was organized. Following the short incubation, 80 µL of each well was transferred into the well at the same position on a black well plate. The black well plates were necessary to avoid fluorescence of neighboring wells influencing the measurements. To facilitate this process, a multi-channel pipette was used. Here great care was given to avoid the formation of any air bubbles, as these falsify the fluorescence measured. Once the transfer process was accomplished, the plates were placed into the thermo box (to avoid exposure to light) and brought to the fluorescence spectrometer. Here the excitation was set to 560 nm and the emission was measured at 590 nm.

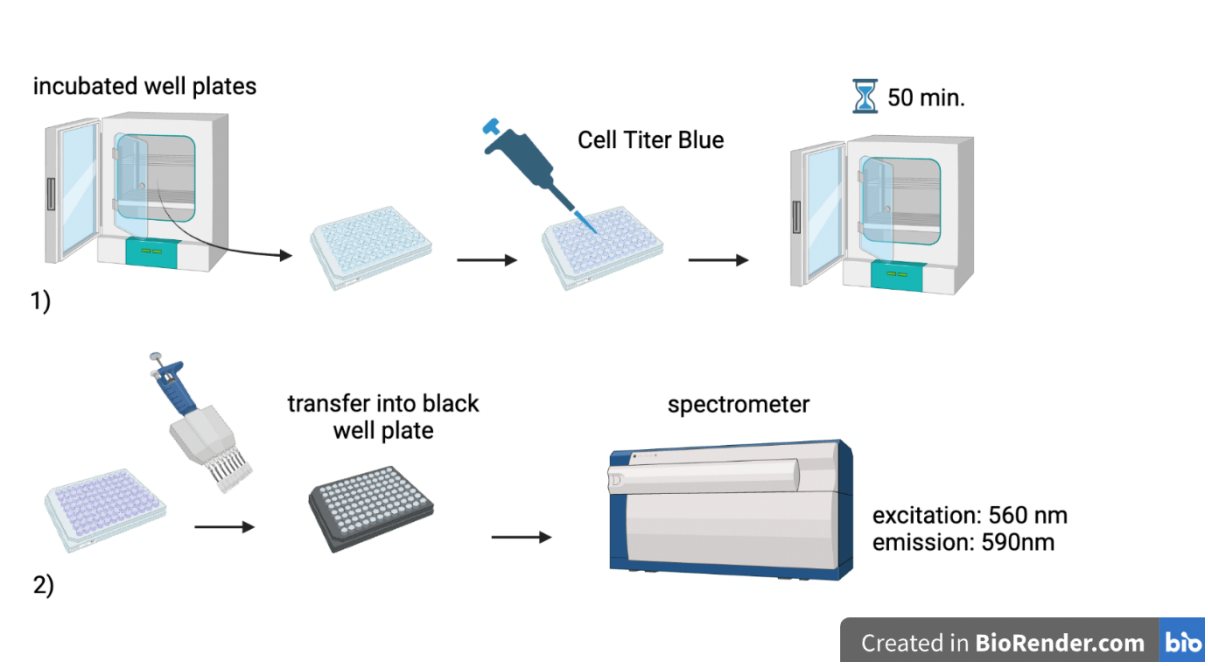


Figure 13: Schematic illustration of CTB assay

Cells are seeded in well plates and treated with selected substances for 48 h. Upon incubation, the cell medium is suctioned off and the Cell Titer Blue solution is added. The well plates are incubated for 50 minutes at 37°C. Thereafter, the well interior is transferred into black well plates. Lastly, in a spectrometer the excitation is set to 560 nm and the fluorescence is measured at 590 nm.

Lastly the mean was calculated of all three technical triplicates. For the blank value, all 6 wells were averaged and this average value was then subtracted from each triplicate average. This difference was then related to the control (1% DMSO) which was set to 100% cell viability. Hence values above 100% implied an increase in metabolic activity in contrast to values below 100% pointing towards cytotoxicity.

Sulforhodamine B Assay

Table 2: Solutions required for SRB assay

50% trichloracetic acid (TCA) (*w/v*)	12.5 g of TCA dissolved in 25 mL distilled water
0.4 % sulforhodamine B (SRB) solution (*w/v*)	4 g SRB dissolved in 1 L of 1% acetic acid
1% acetic acid (*v/v*)	20 mL acetic acid glacial diluted in 2 L of autoclaved water
Tris base	0.30 g of tris(hydroxymethyl-) aminomethane dissolved in 250 mL distilled water

The sulforhodamine B (SRB) assay is complementary to the CTB assay, yet focusing on a different parameter. In this assay the protein content is determined in each well, indirectly

offering an insight to cell cytotoxicity or proliferation. SRB is a brightly colored pink aminoxanthene dye with two sulfonic groups (illustrated in Figure 15). These sulfonic groups possess the ability to bind to basic amino acid residues. Thus, this dye is able to bind to the protein components of cells which have been previously fixed to the well plate bottom using trichloracetic acid (TCA). As this reaction follows the rules of stoichiometry, the amount of dye binding to the protein components is directly proportional to the cell mass (Vichai and Kirtikara 2006). The solutions required for this assay are noted in Table 2.

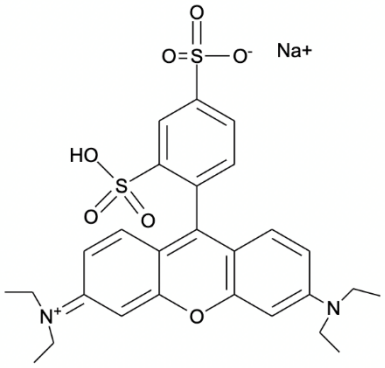


Figure 14: Structure of Sulforhodamine B

The steps of the SRB assay are depicted in Figure 16. Ultimately the SRB assay followed directly the CTB assay, as the clear 96-well plates containing the remaining incubation solution and cells, suited the purpose of the SRB assay perfectly. At first, the remaining CTB solution had to be removed. Then the cells in each well were fixed with 10 µL of a 50% TCA in distilled water solution. This and the following pipetting steps were conducted using the manual repeating pipette. The well plates were placed for 1 hour into the refrigerator at 4 °C. Upon this cold incubation, the plates were carefully rinsed twice with tap water and then left to dry in a dark surrounding. Once the plates had dried, 50 µL of the SRB solution was pipetted into each well. The dye was left to stain the proteins for 1 hour, again in a dark setting. Afterwards the dye was rinsed off with tap water twice and then twice with a 1% acetic acid solution. Here great care was given to not remove the stained proteins from the bottom of the wells. Again, the well plates were left to dry in the dark. Once the plates had dried, the SRB solution bound to the protein components of the cells was dissolved in 100 µL of an alkaline Tris base solution. The treated well plate was placed into the microplate reader with an orbital shaker and was shaken for 5 minutes before the absorbance was measured at 570 nm.

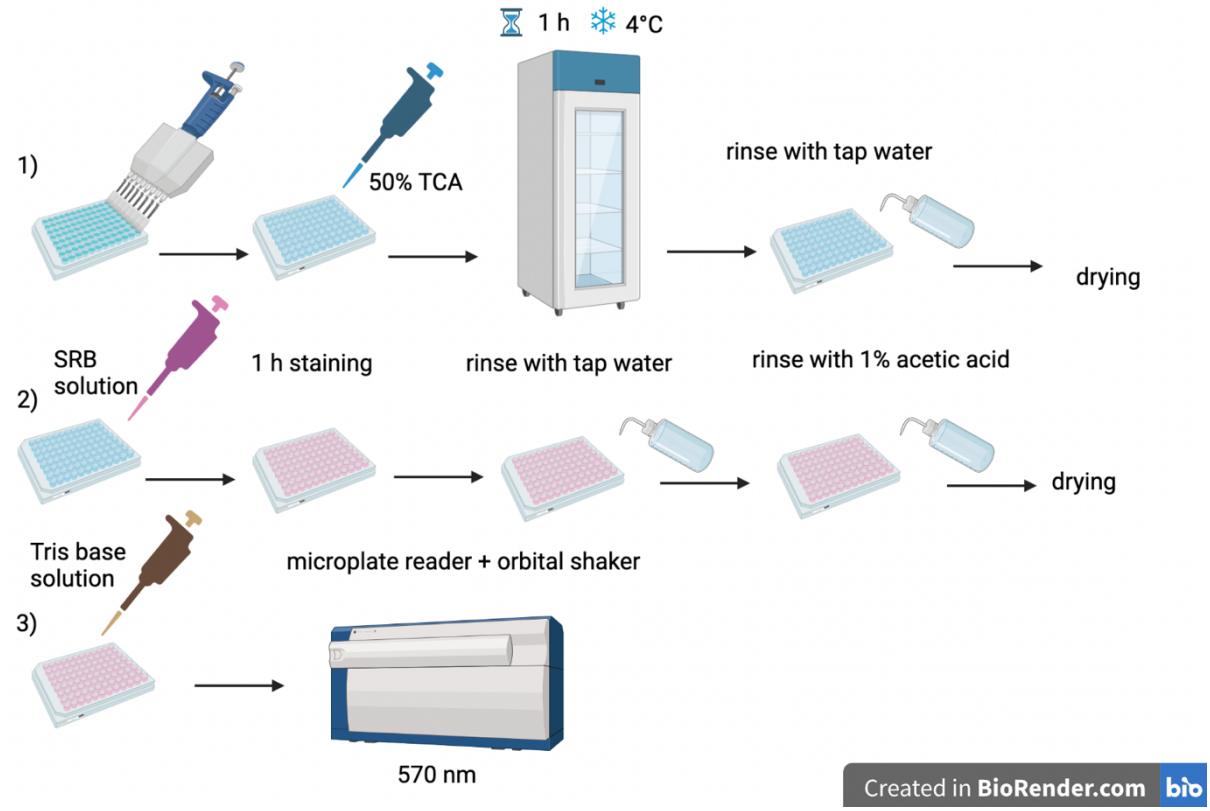


Figure 15: Schematic illustration of SRB assay

The SRB assay was performed directly after the Cell Titer Blue assay using the well plates containing the seeded and incubated Ishikawa cells. Upon suctioning off the medium, the cells are fixed with a 50% TCA solution and kept in the refrigerator for 1 hour. Then the plates are rinsed twice with tap water and left to dry in a dark place. After drying, the cells are incubated with the SRB solution for 1 hour and again rinsed twice with tap water and then twice with 1% acetic acid. The plates are again left to dry. Finally, the fixed and stained cells are treated with a Tris base solution to dissolve the dye and then placed into a microplate reader with an orbital shaker function. The absorbance is measured at 570 nm after 5 minutes of shaking.

Identical to the protocol of the CTB assay, the 6 blank values were averaged and subtracted from the averaged technical triplicates. This difference was again related to the 1% DMSO control which was here too, set to 100%. In terms of interpretation, values above 100% referred to an increase in cell mass, thus indicating possible cell proliferation. Values below 100% demonstrated a trend towards cytotoxicity. However solely values below 80% are defined as manifested cytotoxicity.

Real-time reverse-transcription PCR analysis

Method Overview

A secondary aim of this thesis comprised of a real-time (RT) reverse transcription PCR analysis of four selected genes after treatment with certain combinations of ZEN and GEN. Real-time PCR follows the general PCR process, however it uses fluorescence technology to ensure a detection of signals allowing a quantification of selected genes. Conventional PCR in its quintessence follows a three-step principle: denaturation, annealing, and elongation. With the ultimate goal to amplify specific strands of DNA, the biologic concept of DNA replication is emulated with the help of primers, free nucleotides and a thermally stable DNA polymerase. Through the application of different temperatures, the DNA is at first denatured, or rather the hydrogen bonds connecting the bases are broken. Then during annealing the primers attach to the respective regions of the DNA strands. Lastly with the help of the polymerase, the free nucleotides are attached to the primers, rebuilding a complementary DNA strand on the basis of the original DNA template. This three-step process is repeated up to 45 times, each time replicating the existing DNA templates (Hernández-Rodríguez and Ramirez 2012).

The concept of real-time PCR builds upon the principle of conventional PCR, yet resulting in immediate results without the need for further experiments. The first demonstration of this scientific accomplishment was performed in 1993 with the use of the fluorescent dye ethidium bromide which intercalates into double stranded DNA (Higuchi, et al. 1993). As the amount of double stranded DNA increases with each completed cycle, the strength of the measured fluorescence intensifies as well. In order to use RT-PCR for quantitative purposes, the threshold cycle (C_t) value is implemented and defined as the cycle at which the fluorescence measured lies significantly above the recorded baseline. Thus, the lower the C_t value, the higher the amount of initial DNA. For the purpose of normalization, housekeeping genes are utilized to overcome the differences in the amount of template DNA available in different samples and to control the inconstancy of the efficiency during the reverse transcription in reverse transcription PCR (Loftis and Reeves 2012). The reverse transcription process refers to the conversion of RNA to double stranded DNA which can then undergo the actual PCR process. The mRNA is often extracted when the gene of interest is transcribed into mRNA and is used to build certain proteins such as receptors. Here the

amount of mRNA transcribed indirectly offers information on possibly how many proteins were to be translated, without the necessity to perform assays focusing on the actual build proteins. The enzyme reverse transcriptase is applied to perform this conversion from mRNA to DNA, where it possesses both DNA polymerase and RNase H activity. The latter enzyme is responsible for the degradation of the RNA, which is important to avoid competition in the following PCR reactions (Langton 2013). In summary, extracted mRNA from samples is converted to double stranded DNA which can then inaugurate the actual real-time PCR process resulting in DNA amplicons.

Seeding of Cells

In this experiment the samples represented Ishikawa cells, exposed to a selected combinations of ZEN and GEN, seeded in 12-well plates. The seeding of the cells followed the same protocol intended for the cytotoxicity assay (see chapter: Seeding of Cells for Cytotoxicity Assays), however the number of cells seeded varied. 150 000 cells were seeded for 48 hours growth or 100 000 cells for 72 hours. The cells were seeded in a 12-well plate where 1 mL of the cell suspension with the appropriate concentration was added to each well.

Incubation

For the incubation of the cells, the solutions had to be again prepared freshly. The highly concentrated stock solutions kept in the freezer were thawed and diluted with DMSO to achieve concentrations 200 times higher than the final concentration used for the incubation. For the single incubation substances, 693 µL assay medium with 3.5 µL DMSO and 3.5 µL of diluted substance was mixed in a reaction tube. For the combinatory solutions comprising of two different substances, 693 µL assay medium was combined with 3.5 µL each of the respective compounds of interest. The solvent control was achieved by mixing 693 µL assay medium with 7 µL DMSO. E2 (1 nM) served as the positive control, using 3.5 µL of a 200 nM solution combined with 3.5 µL of DMSO and 693 µL assay medium. Once all solutions were prepared, the seeded cells in the 12-well plates were taken from the incubator. The liquid was suctioned off using a sterile glass Pasteur pipette. Then, 600 µL of each appropriate solution was added to a in prior defined well. Then the well plate was again placed into the incubator to allow an incubation of either 24 or 48 hours.

RNA Extraction

Table 3: Solutions required for RNA extraction

1% β-mercaptoethanol	22 µl of β-mercaptoethanol diluted in 2.2 mL of RLT buffer solution

Table 4: RNeasy Plus Mini Kit contents

gDNA Eliminator Mini Spin Columns (uncolored) (each in a 2 ml Collection Tube)
RNeasy Mini Spin Columns (pink) (each in a 2 ml Collection Tube)
Collection Tubes (1.5 ml)
Collection Tubes (2 ml)
Buffer RLT Plus
Buffer RW1
Buffer RPE (concentrate)
RNase-Free Water
Quick-Start Protocol
Modified from (Qiagen 2019)

The extraction of mRNA of the incubated cells is a prerequisite for the continuation with the real-time PCR analysis. The RNeasy MiniKit from Qiagen (contents listed in Table 4) was used to achieve this extraction where the principle is based upon ion exchange chromatography. Through lysing, homogenizing, and treatment with a buffer solution containing guanidine-thiocyanate, RNases are effectively denatured. Through the addition of ethanol, binding to the silica-based membrane of the spin columns is facilitated. A series of washing steps ensures an RNA solution of high purity (Qiagen 2019).

The first step involved the preparation of a fresh 1% β-mercaptoethanol (see Table 3 for preparation) in RLT buffer solution. Again, all work was performed under a laminar flow cabinet and was previously treated with RNase decontamination solution. RNase contamination has the potential to break down mRNA, impeding subsequential experiments.

The incubated 12-well plate was taken out of the incubator and the medium in each well was removed using a 5 mL pipette. Then 5 mL of PBS was added to each well to ensure washing of the cells. The PBS was removed from the first well and discarded. Then, 350 µL of the RLT buffer solution with the 1% β-mercaptoethanol was pipetted into the well. Using a cell scraper, the cells were scraped off the bottom of the well and then pipetted into a labelled reaction tube. In the tube the solution was resuspended multiple times. The same procedure was repeated for each well. Insulin syringes with a diameter of 0.9 mm were used to resuspend the cell-solution in each reaction tube where resuspension was performed approximately 20 times. Following this step, 350 µL of a 70% ethanol solution was added to each reaction tube and again resuspended using a pipette. Lastly, 700 µL of this solution from each reaction tube was transferred into a 2 mL "RNeasy mini spin column" and then centrifuged for 1 minute at 10 000**g* at room temperature. The eluate was then discarded. Following this step, 700 µL of the RW1 buffer was added to each mini spin column and then again centrifuged at the same conditions used before. Here too, the eluate was discarded. Another washing step followed where twice 500 µL of RPE buffer solution as added to each column and each time the columns were centrifuged and the eluate was removed. Finally, the drying of the columns was achieved by centrifuging them without the addition of a further buffer. Then each mini spin column was stuck into a 1.5 mL elution tube and 30 µL of RNase free water was added to each column. Then the mini spin columns-elution tube complex was centrifuged once more at 10 000**g* for 1 minute. Afterwards, the mini spin columns were discarded and the elution tubes containing the extracted mRNA were immediately placed on ice.

Determining RNA Concentration

Once the RNA was extracted using the protocol described in the section above, the RNA concentration had to be determined in order to ensure a sufficient amount of mRNA for the PCR procedure. This measurement was performed using a NanoDrop™ spectrophotometer. In the appropriate computer program, the nucleic acid template was selected on the basis of RNA. Then RNase free water was measured and set as a blank. Following this, a new RNase free water sample was measured to ensure that the blank has been set correctly. Then, each sample was vortexed, measured and the RNA concentrations were recorded. In order to

warrant for the purity of the RNA, the ratio between 260 nm to 280 nm had to be between 1.9 and 2.1 Once the RNA concentrations of all samples were determined, another RNase free water sample was measured.

Transforming RNA into DNA via Reverse Transcription

Table 5: Genomic DNA elimination reaction components

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA, up to 1 µg	Variable
RNase-free water	Variable

Modified from (Qiagen 2016)

Table 6: Reverse-transcription reaction components

Component	Volume/reaction
Reverse-transcription master mix Quantiscript Reverse Transcriptase	1 µl
Quantiscript RT Buffer, 5x	4 µl
RT Primer Mix	1 µl
Template RNA	14 µl

Modified from (Qiagen 2016)

As the actual PCR process can only be performed using DNA, the extracted RNA from the samples had to undergo a transformation into the required DNA. As stated previously, this was achieved with the help of the reverse transcriptase enzyme and here the “QuantiTect Reverse Transcription Kit” from Qiagen was utilized (components described in Table 5 and Table 6). This kit encompasses two separate steps: the removal of genomic DNA followed by the actual reverse transcription. The presence of genomic DNA can be considered as a contamination which could then in consequence hinder the PCR process. The reverse transcription process, aiming to produce complementary DNA (cDNA), whilst utilizing the enzyme “Quantiscript Reverse Transcriptase” is illustrated in Figure 17 (Qiagen 2009).

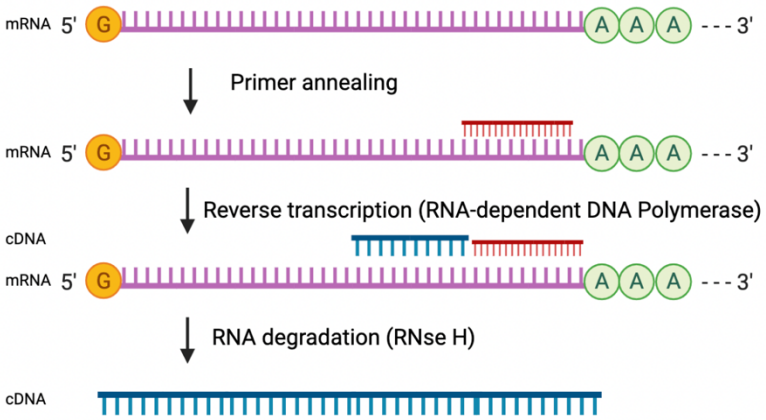


Figure 16: cDNA synthesis modified from (Qiagen 2009)

The synthesis of cDNA is achieved by the annealing of appropriate primers to the mRNA followed by the actual reverse transcription where an RNA-dependent DNA polymerase builds DNA based on the RNA template. Lastly, RNA degradation finishes up the process by removing the RNA.

To start off, the appropriate volume of the samples containing RNA has to be calculated to achieve a concentration of 1000 ng RNA per 0.2 mL reaction tube. The dilution was performed using RNase free water yet the final volume in each 0.2 mL tube was set to exactly 12 µL. Then, 2 µL of genomic DNA (gDNA) wipeout buffer was added to each tube and the fingers were used to snipped against the tube before using a micro centrifuge. After centrifuging, the samples were placed into a thermal cycler for 2 minutes at 42°C. Meanwhile a master mix consisting of 1 µL reverse transcriptase (RT), 1 µL RT Primer, and 4 µL RT buffer per sample was prepared as a batch solution for all samples. Lastly, 6 µL of this master mix was then added to each sample tube and each tube was once more snipped with the fingers, centrifuged, and placed into the thermal cycler at a temperature program of 42°C for 15 min followed by 2 minutes of 95°C. This temperature gradient allows for the synthesis of cDNA and the subsequent inactivation of the enzyme at the higher temperature. Then the samples were either stored at -20°C or kept on ice for further use.

Real Time PCR

During real time PCR the cDNA synthesized previously is amplified and quantitively measured using a fluorescent dye. In this protocol, SYBR Green is the type of fluorescent dye utilized to intercalate into the double stranded DNA. The appropriate primers for the genes *CYP1A1*, *CYP1B1*, *ESR1*, and *ESR2* were placed on ice including the housekeeping genes *HPRT-1* and *ALAS1*. Additionally, SYBR Green was also placed on ice, however it was covered in order to ensure darkness. Then a master mix of each primer type was prepared with 6 µL RNase free water per well, 2 µL primer per well, and 10 µL SYBR Green per well. In a 96-well PCR plate, 18 µL of the respective master mix was pipetted in duplicates into the individual wells. The cDNA of each sample was then diluted with RNase free water (60 µL) to reach a concentration of 20 ng/µL. Then 2 µL of the cDNA was pipetted into the respective wells, according to a previously established scheme. The PCR well plate was carefully sealed with a transparent sticky film and then placed into a plate spinner. Following this step, the PCR plate was positioned into the thermocycler. The software "Step One" was booted up and a temperature program was selected consisting of three distinct phases. The initial phase was set to 15 minutes of 95 °C. The cycling stage involved 40 cycles of 15 seconds at 94 °C (denaturation), 30 seconds at 55 °C (annealing), and 30 seconds at 72 °C (elongation). The melt curve stage included 15 seconds at 95 °C, 1 minute at 60 °C and heating in 0.5 °C steps to reach 94 °C.

Once the temperature program was completed, the raw data of all C_t values was extracted and analyzed. The housekeeping genes were used to normalize the data whilst a mean and standard deviation was calculated of all replicates. All in all, data on the up or downregulation of the analyzed genes was generated.

The raw data accumulated from the real-time PCR data was also at first processed using Microsoft® Excel 365. As each gene´s C_t value was measured as a technical duplicate, here too a mean value had to be at first determined. As described previously, the C_t values of the two selected housekeeping genes were used to normalize the C_t values for the remaining genes. To achieve this, a series of calculations had to be performed which are summarized below:

	C_t1	C_t2	Mean value (M)	Mean value (M)
Housekeeping Gene 1 (HK1)	a	b	M_{ab}	M_{abcd}
Housekeeping Gene 2 (HK1)	c	d	M_{cd}	
Gene of Interest (GoI)	e	f	M_{ef}	

$$1\Delta C_t = e - M_{ab}$$

$$2\Delta C_t = f - M_{cd}$$

$$1\Delta\Delta C_t = 1\Delta C_t - (M_{ef} - M_{abcd})$$

$$2\Delta\Delta C_t = 2\Delta C_t - (M_{ef} - M_{abcd})$$

$$2^{-\Delta\Delta Ct} = 2^{-1\Delta\Delta CT}$$

$$2^{-\Delta\Delta Ct} = 2^{-2\Delta\Delta CT}$$

$$Final\ normalized\ C_t\ value\ for\ GoI\text{:}\ \frac{2^{-1\Delta\Delta CT} + 2^{-2\Delta\Delta CT}}{2}$$

Once all C_T values for each gene of interest were normalized, the mean values of all replicates were calculated. Outliers were exempted following the method of Nalimov and were not considered in the final mean and standard deviation calculations. Again, using Origin Pro® 2022b, a student´s *t*-test with a significance level of 5% was applied, in order to determine a statistical significance between the solvent control and the single substances or selected combinations of ZEN and GEN.

Statistical Evaluation

In order to generate substantial data, the cytotoxicity measurements concerning the various combinations of isoflavones, ZEN, and ZEN metabolites, were conducted in technical triplicates in addition to a minimum of five independent biological replicas. The raw data was processed using Microsoft® Excel 365. The mean value was determined of each technical replicate. Then, the mean values for the biological replicates were calculated upon eliminating outliers based on the method of Nalimov and verifying normality following Shapiro-Wilk. Eliminated outliers were exempted from calculations concerning the mean and standard deviation values.

Further statistical analysis was performed using the software “Origin Pro® 2022b”. To determine the statistical significance between the solvent control (DSMO) and the various combinations or single substances measured, a one-way Student´s *t*-test was utilized. Here a significance level of 5% was applied. To allow visualization of the data, bar graphs and heatmaps were created.

Results and Discussion

Cytotoxicity

As stated above, the cytotoxicity data included 5 biological replicates for each investigated combination and single substance. As the SRB assay directly followed the CTB assay, the number of replicates of each assay is identical to each other and shall be summarized as "cytotoxicity assays". In combination with the efforts of Andrea Betschler, said replicates were achieved and all data together were able to be statistically analyzed. The data regarding the cytotoxicity and estrogenicity of the combinations has been published successfully in 2022 (Grgic et al. 2022). In Table 7 below, a visual representation is depicted describing the number of replicates performed in total of each combination, in addition to highlighting the combinations performed by the author of this thesis. Due to reasons of confidentiality, the results encompassing combinations with HZEN and DHZEN shall not be further discussed in this work.

Table 7: Overview of biological replicates performed

	GEN	DAI	GLY	EQ
ZEN	5/5	5/5	5/5	5/5
α-ZAL	1/5	1/5	3/5	3/5
α-ZEL	0/5	0/5	0/5	0/5
HZEN	5/5	3/5	2/5	4/5
DHZEN	3/5	2/5	3/5	3/5
ZEN-14-Sulfate	2/5	2/5	2/5	2/5

An overview offering an insight to the exact number of replicates performed concerning the cytotoxicity assays. The format "x/y" refers to "x" representing the number of replicates executed by the author itself whilst "y" equals to the total number of replicates. The difference between y and x expresses the number of replicates performed by Andrea Betschler.

The concentrations selected for ZEN and its metabolites as well as the isoflavones depended on the bioavailability found in previous literature. As one of the main targets of this project was to investigate the estrogenicity of the single substances in addition to the binary combinations, concentrations reflecting physiologically relevant plasma levels were aimed at. Various studies analyzed ZEN concentrations in urine and serum samples in specified

populations such as young girls entering puberty precociously or cancer patients. Massart et al. found an average serum concentration of ZEN in young girls to be at 2.93 nM (Massart, et al. 2008). A further study analyzed urine samples of Tunisian women, where 19% of the samples contained α-ZAL at concentrations ranging from 2.39 to 9.96 nM. Meanwhile ZEN was detected in 2.4% of the samples and α-ZEL remained undetected (Belhassen, et al. 2014). The concentration of ZEN and its metabolites selected to range from 0.001-10 nM mirror the bioavailability found in specified populations where estrogenicity plays a prominent role. For the isoflavones, GEN was reported to be physiologically active at 0.5-10 µM (Ariyani, et al. 2018). Plasma concentrations of GEN were found to range between 0.19-2.96 µM in adults consuming a traditionally soy-rich diet (Borras, et al. 2006). For the isoflavone GLY, the lowest concentration of 0.001 µM was eliminated and instead a higher concentration of 20 µM was introduced. GLY has been reported to be physiologically less active compared to the other isoflavones, thus a higher concentration was used (Sakamoto, et al. 2010). As a positive control E2 was utilized at a concentration of 1 nM reflecting premenopausal levels (Uifalean, et al. 2016).

Cytotoxicity of Single Compounds

For each biological replicate performed, the single substances tested were included in order to offer a direct comparison between singular and combinatory effects. Hence, for each ZEN metabolite 20 biological replicates exist whilst for every isoflavone there is data from 25 biological replicates. The results regarding this data are displayed below in respective bar graphs. The results involving the ZEN metabolites HZEN and DHZEN are left out due to reasons of confidentiality.

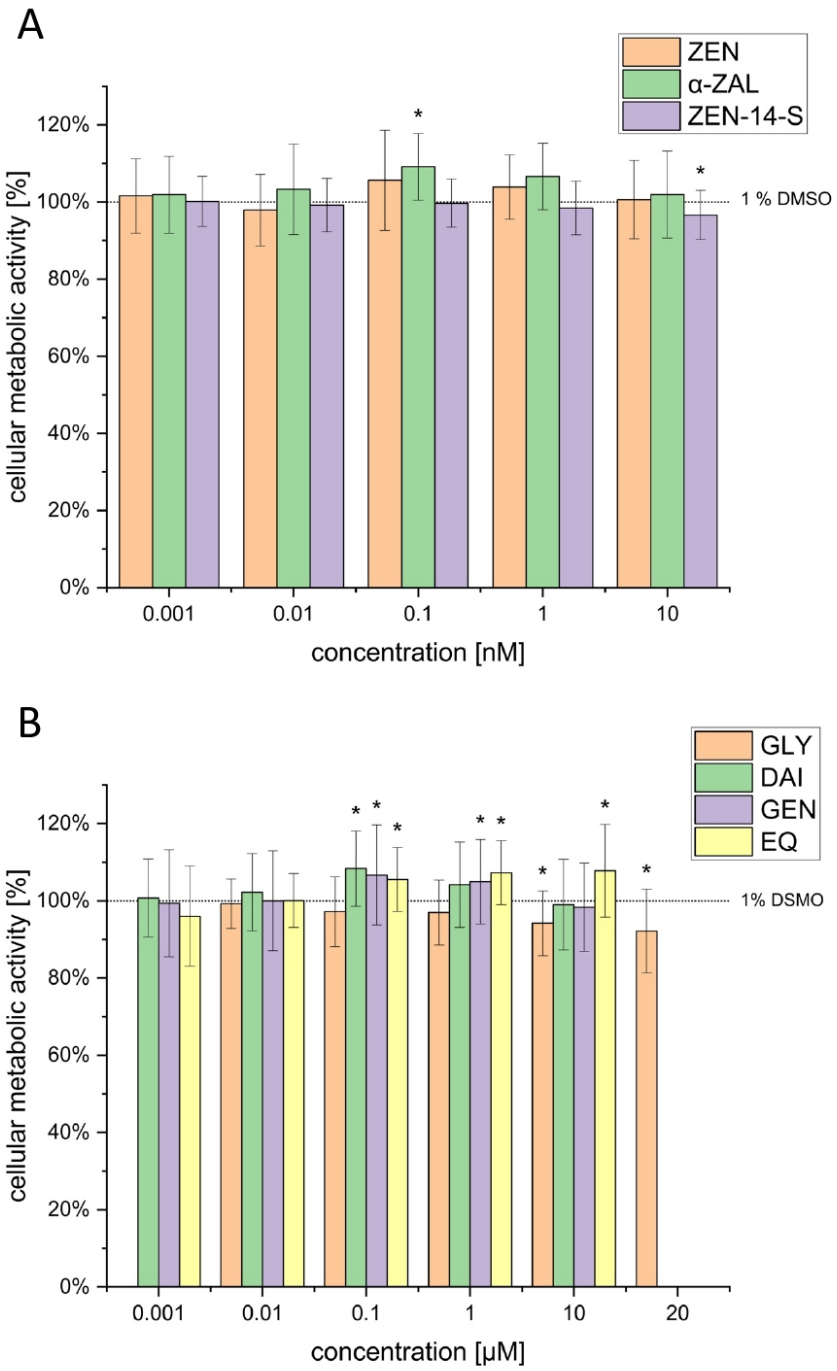


Figure 17: Effects of ZEN, ZEN metabolites, and isoflavones on cellular metabolic activity

The effect on cell viability was determined via the Cell Titer Blue assay upon 48 h of incubation with respective compounds. In Figure 18A the effect of ZEN, α-ZAL, and ZEN-14-S was determined on the cell viability, where each mean value consists of a total of 20 biological replicates derived from technical triplicates. Figure 18B displays the effects of the isoflavones on cell viability, however here 25 biological replicates were available. The values were normalized by the solvent control (1% DMSO), which is accordingly depicted as a horizontal line at a cell viability of 100%. Outliers were identified following the method of Nalimov and were exempted from further calculations. Error bars depict the calculated standard deviations of each set of results. The asterix (*) labels a significant difference to the solvent control of 1% DMSO, based on the application of a one-way student´s t-test ($p < 0.05$). In Figure 18B, the initial GLY concentration used was 0.01 ranging up to 20 µM.

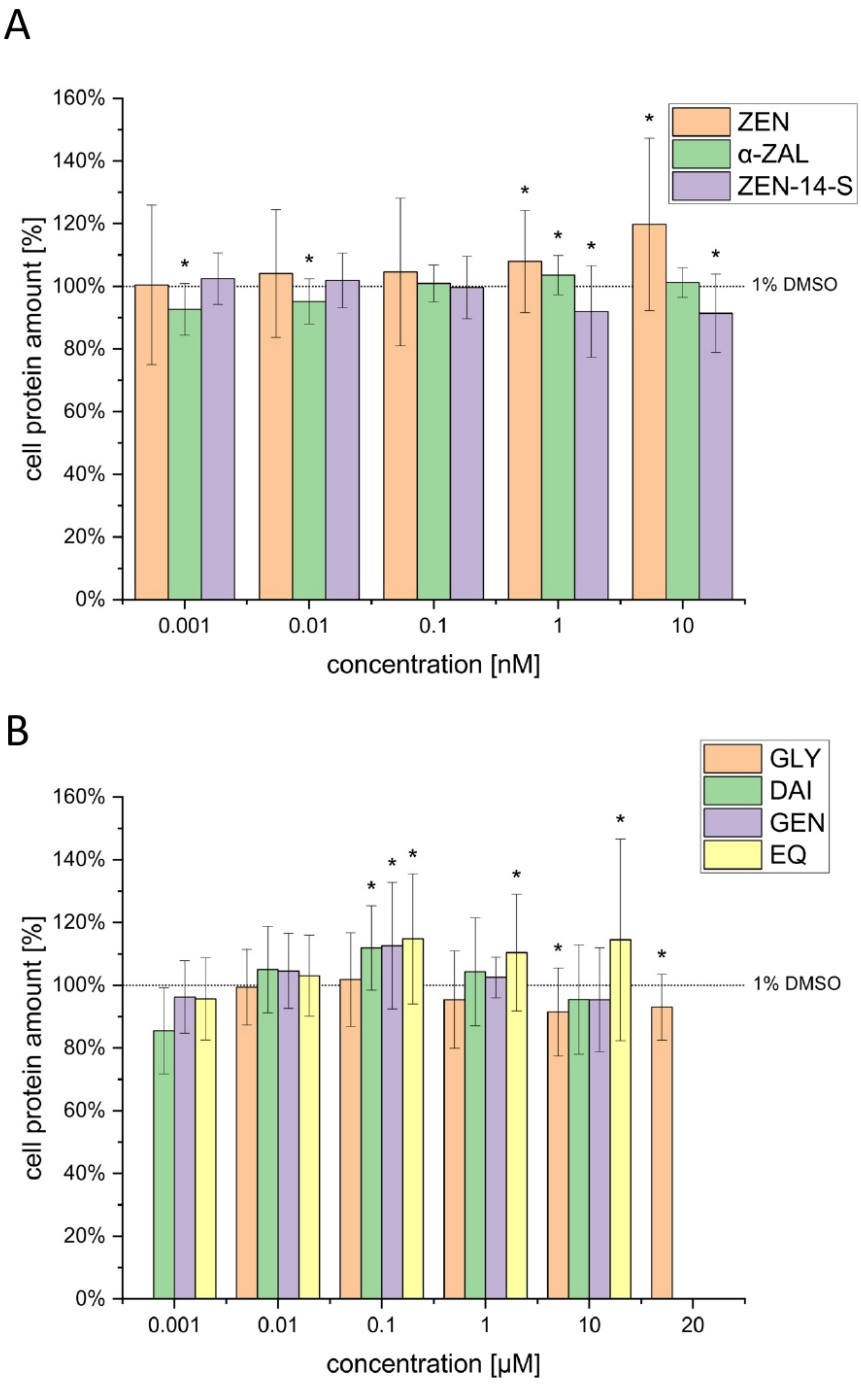


Figure 18: Effect of ZEN, ZEN metabolites, and isoflavones on cell protein amount

The effect on the cell protein amount was determined via the SRB assay upon 48 h of incubation with respective compounds. In Figure 19A the effect of ZEN, α-ZAL, and ZEN-14-S was determined on the cell protein amount, where each mean value consists of a total of 20 biological replicates derived from technical triplicates. Figure 19B displays the effects of the isoflavones on the cell protein amount, however here 25 biological replicates were available. The values were normalized by the solvent control (1% DMSO), which is accordingly depicted as a horizontal line at a cell viability of 100%. Outliers were identified following the method of Nalimov and were exempted from further calculations. Error bars depict the calculated standard deviations of each set of results. The asterix (*) labels a significant difference to the solvent control of 1% DMSO, based on the application of a one-way student´s t-test ($p < 0.05$). In Figure 19B, the initial GLY concentration used was 0.01 ranging up to 20 µM

In general, the values of all compounds analyzed and of both assays implemented, suggest no cytotoxicity on the Ishikawa cells. All values lie within the range of the solvent control, with only a handful of compounds at certain combinations displaying a significant difference to said control. A recent review published in 2022 summarized a variety of cytotoxicity assays performed on various cell lines of several mycotoxins. The IC_{50} value for ZEN was found to range around 10 µM to 170 µM , depending on the cell line, type of assay, and incubation time (Skrzydlewski, Twaruzek and Grajewski 2022). A further study assessed the cytotoxicity of ZEN and α-ZAL on HepG2 cells incubated for 72 hours at concentrations ranging from 5-100 µM whilst using the neutral red assay. Here the IC_{50} values were found to be 38 µM for ZEN and 120 µM for α-ZAL (Marin, et al. 2019). Hence, a cytotoxicity observed at the selected low concentrations was not expected for the Ishikawa cells. Abassi et al. conducted a study in 2016 assessing plausible proliferation of human colon carcinoma cells (HCT116) at low concentrations including 1 and 10 nM using the MTT assay. Different incubation end points were selected ranging from 24 hours to 120 hours. At the lowest concentration of 1 nM, following 120 hours of exposure, ZEN seemed to induce a two-fold increase in cell proliferation, where this effect was noticeably reduced by an increasing ZEN concentration (Abassi, et al. 2016). Postulated mechanisms behind induced cell proliferation include the involvement of extracellular pathways involving ERK1/2, where ZEN has demonstrated potential to trigger these pathway (Parveen, Zhu and Kiyama 2009). In colon cancer cells (HT29), 3 µM ZEN has induced cell proliferation via the GPER. The authors found ZEN to promote proliferation markers such as cAMP and phosphorylation of ERK1/2, however the cell lines containing a p53 mutation were more susceptible to said proliferative effects (Lo, et al. 2021). In Figure 19A, at ZEN concentrations of 1 nM and 10 nM, there seems to be a significant induction of the cell protein amount, pointing towards cell proliferation. Yet the incubation times were limited to 48 hours, thus making a direct comparison difficult. Overall, Figure 18A and 19A point towards a non-cytotoxic potential of ZEN, α-ZAL, and ZEN-14-sulfate at concentrations ranging from 0.001 nM to 10 nM.

Figure 18B and 19B depict the effect of the isoflavones on the cell viability and cellular protein content. At concentrations of 0.1 µM and 1 µM a tendency towards an increase in cell viability and protein amount is found, suggesting a slight proliferative effect. Choi and Kim demonstrated similar results using DAI and GEN at concentrations of 1 µM and 10 µM on

MCF-7 cells. The authors postulated this effect to be due to the estrogenic-like activity (Choi and Kim 2013). An older study from 1998 also found GEN to induce cell proliferation after 96 hours in MCF-7 cells within a concentration range of 0.01 µM to 1 µM. Again, the involvement of estrogenic receptors was hypothesized to be responsible for the observed effects. The authors analyzed the increased expression of the pS2 gene, which is in general found to be regulated by estrogens in MCF-7 cells. Hence an activated expression of this gene would link the involvement of estrogen receptors to the proliferative effects observed by GEN (Hsieh, et al. 1998). Maggiolini et al. investigated the effects 1 µM GEN and E2 had on the MAPK pathway in addition to ERα-mediated mechanism on human breast cancer cells. GEN mirrored the activity of E2, where they were able to induce rapid upregulation of the transcription factor c-*fo*s via GPR30-mediated mechanisms. This early upregulation of said transcription factor seemed to be achieved without the involvement of the ERα and results in immediate regulation of the cell cycle, including proliferative effects of the breast cancer cells (Maggiolini, et al. 2004). The effect of EQ and GEN upon 96 hours of incubation on the cell proliferation of MCF-7 cells was investigated by Liu et al. in 2010. The authors found the cell proliferation to peak at 10 µM, with EQ demonstrating a slightly stronger effect and higher concentrations eliciting the opposite effect. Through the use of inhibitors such as the ICI 182,780 (ICI) and Uo126, which act antagonistically against estrogen receptors and MEK1/2 respectively, the involvement of the ERK1/2 pathway in addition to the ERα-mediated-pathway was suggested concerning the observed cell proliferation (Liu, et al. 2010). A study performed on Ishikawa cells, demonstrated a very slight reduction in cell number (5-15%) when treated with 10 µM DAI metabolites or EQ upon 48 hours of incubation (Lehman, et al. 2005). All in all, the existing literature seems to suggest a tendency towards cell proliferation at certain concentrations, however once surpassing a limit, the opposite holds true.

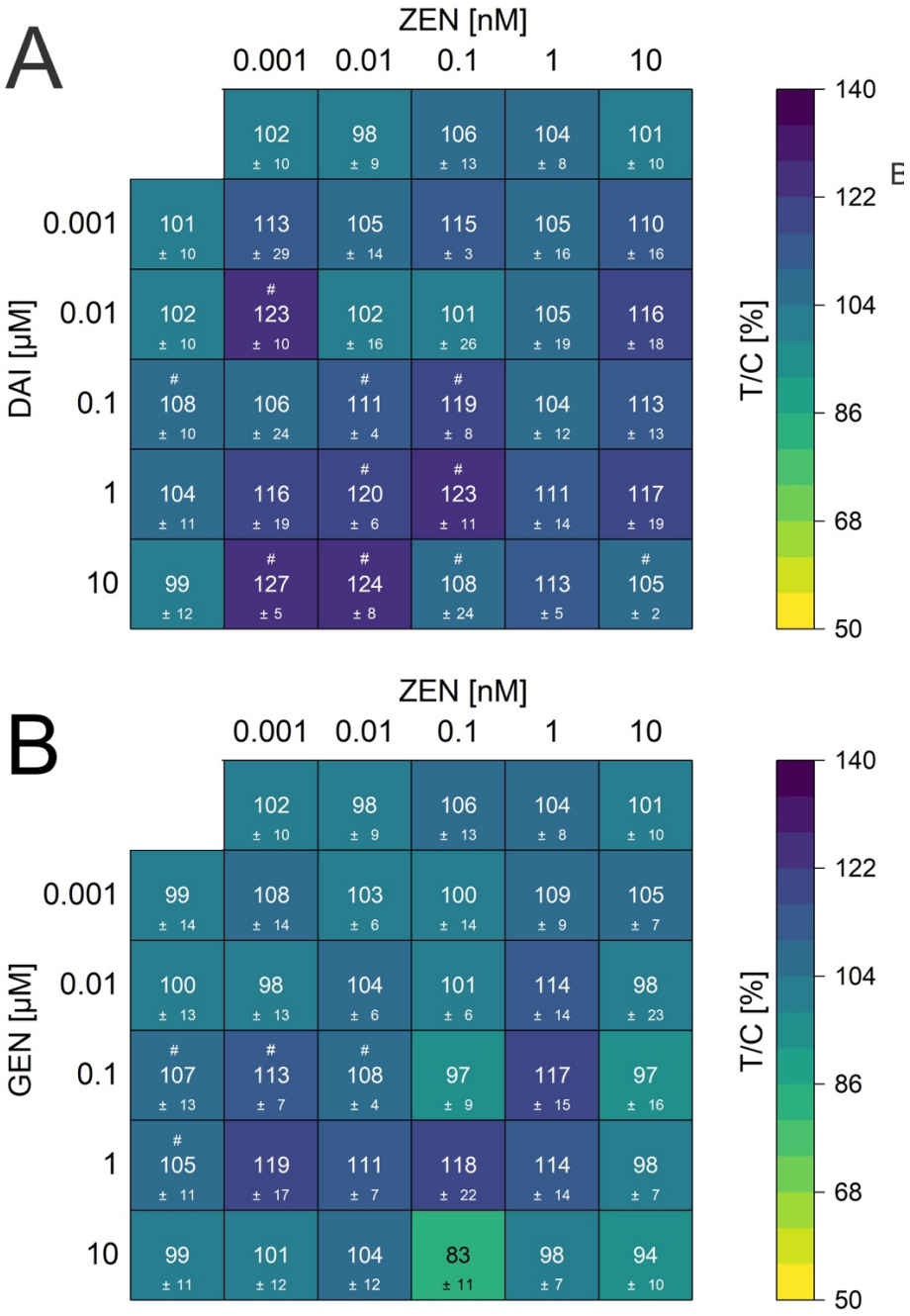
As many studies regarding the effect of estrogenicity of isoflavones are conducted on breast cancer cells, a direct comparison is impeded. Breast cancer cells such as MCF-7 are classified as hormone-sensitive cells and contain estrogen receptors, similar to Ishikawa cells (Papoutsi, et al. 2009). Tchoumtchoua et al. conducted a study focusing on the estrogenic activity of certain isoflavonoids, including DAI and GLY at 10 µM for 96 hours, using three different cell lines to assess cell viability via the MTT assay. Noteworthily, the Ishikawa cells demonstrated no significant changes in the number of viable cells in comparison to the MCF-7 cells, which

did experience a cell proliferation upon incubation with DAI or GLY. Additionally, the ER antagonist ICI diminished this increase cell number, whilst on the Ishikawa cells no effect was observed (Tchoumtchoua, et al. 2016). A plausible explanation to justify this difference might be a difference in the ratio between ERα to ERβ. During breast cancer, ERβ expression is frequently lost, thus breast cancer cell lines such as the MCF-7, do not express both estrogen receptor isoforms equally (Grober, et al. 2011). Ishikawa cells on the other hand do express both isoforms, however also the ERα isoform predominates significantly (Nishida 2002).

Cytotoxicity of Binary Combinations

In the following sections the different cytotoxicity results of the binary combinations of ZEN, α-ZAL, or ZEN-14-S with the isoflavones (GEN, DAI, GLY, and EQ) are displayed. Heatmaps were created in order to allow proper visualization of the data. For the values of the single substances, the mean values of all replicates were utilized.

Combinations of ZEN with isoflavones



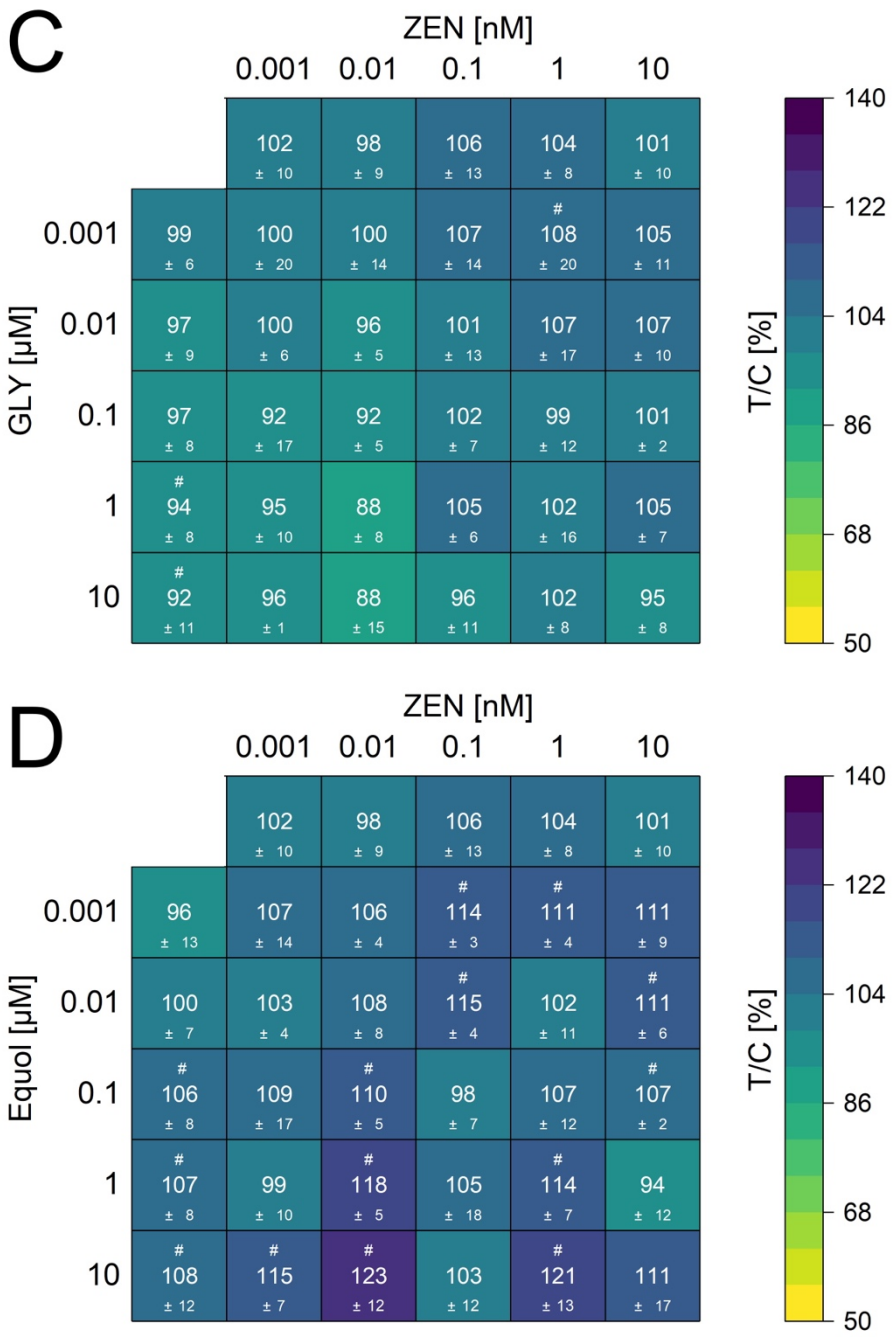
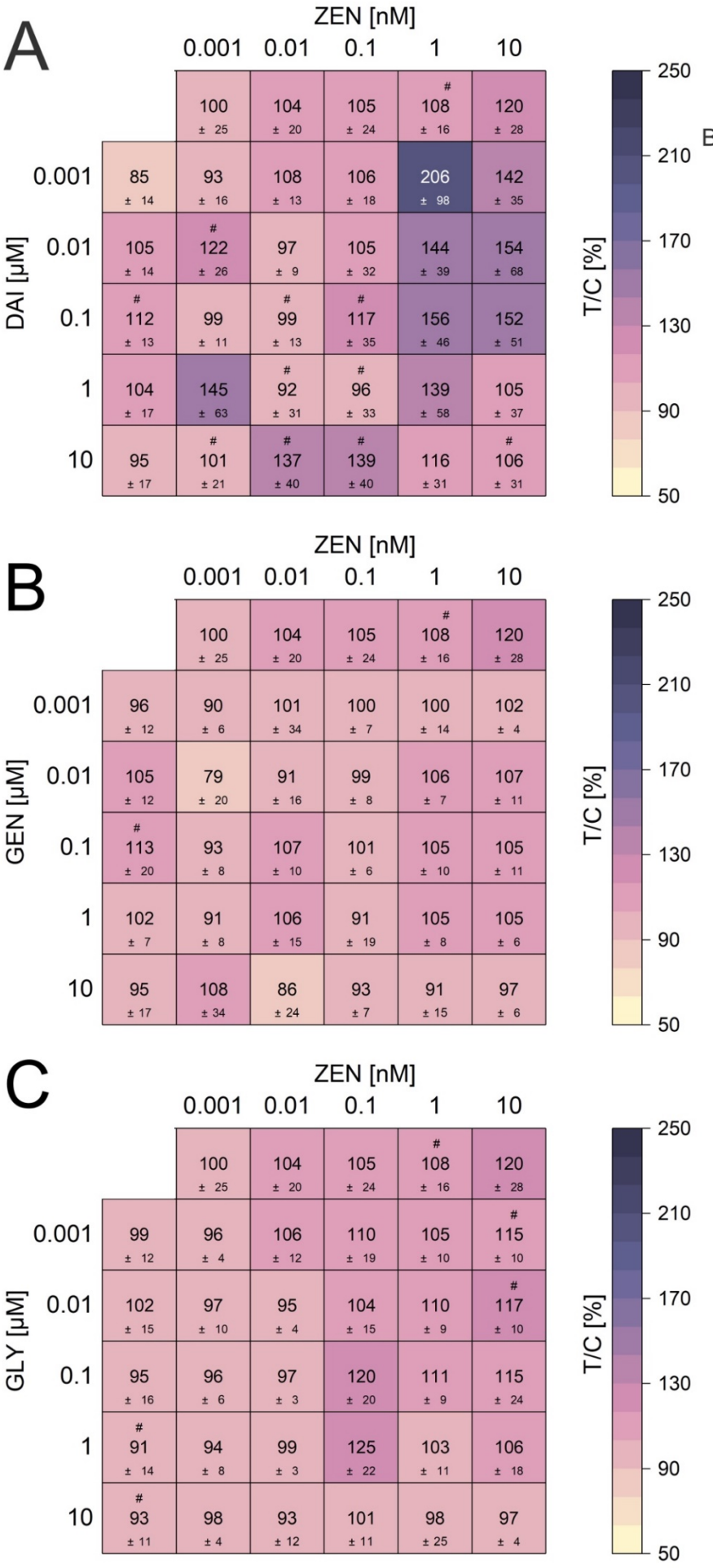


Figure 19: Effects of ZEN and isoflavones on cell viability

Heatmaps displaying binary combinations of A) ZEN and DAI, B) ZEN and GEN, C) ZEN and GLY, D) ZEN and EQ on cell viability [%] determined via the Cell Titer Blue (CTB) assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of ZEN [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.


A
ZEN [nM]
0.001 0.01 0.1 1 10
DAI [µM]
0.001 0.01 0.1 1 10
100 ± 25 | 104 ± 20 | 105 ± 24 | # 108 ± 16 | 120 ± 28
85 ± 14 | 93 ± 16 | 108 ± 13 | 106 ± 18 | 206 ± 98 | 142 ± 35
105 ± 14 | # 122 ± 26 | 97 ± 9 | 105 ± 32 | 144 ± 39 | 154 ± 68
112 ± 13 | 99 ± 11 | # 99 ± 13 | # 117 ± 35 | 156 ± 46 | 152 ± 51
104 ± 17 | 145 ± 63 | # 92 ± 31 | # 96 ± 33 | 139 ± 58 | 105 ± 37
95 ± 17 | # 101 ± 21 | # 137 ± 40 | # 139 ± 40 | 116 ± 31 | # 106 ± 31
T/C [%]
250 210 170 130 90 50
B
ZEN [nM]
0.001 0.01 0.1 1 10
GEN [µM]
0.001 0.01 0.1 1 10
100 ± 25 | 104 ± 20 | 105 ± 24 | # 108 ± 16 | 120 ± 28
96 ± 12 | 90 ± 6 | 101 ± 34 | 100 ± 7 | 100 ± 14 | 102 ± 4
105 ± 12 | 79 ± 20 | 91 ± 16 | 99 ± 8 | 106 ± 7 | 107 ± 11
113 ± 20 | 93 ± 8 | 107 ± 10 | 101 ± 6 | 105 ± 10 | 105 ± 11
102 ± 7 | 91 ± 8 | 106 ± 15 | 91 ± 19 | 105 ± 8 | 105 ± 6
95 ± 17 | 108 ± 34 | 86 ± 24 | 93 ± 7 | 91 ± 15 | 97 ± 6
T/C [%]
250 210 170 130 90 50
C
ZEN [nM]
0.001 0.01 0.1 1 10
GLY [µM]
0.001 0.01 0.1 1 10
100 ± 25 | 104 ± 20 | 105 ± 24 | # 108 ± 16 | 120 ± 28
99 ± 12 | 96 ± 4 | 106 ± 12 | 110 ± 19 | 105 ± 10 | # 115 ± 10
102 ± 15 | 97 ± 10 | 95 ± 4 | 104 ± 15 | 110 ± 9 | # 117 ± 10
95 ± 16 | 96 ± 6 | 97 ± 3 | 120 ± 20 | 111 ± 9 | 115 ± 24
91 ± 14 | 94 ± 8 | 99 ± 3 | 125 ± 22 | 103 ± 11 | 106 ± 18
93 ± 11 | 98 ± 4 | 93 ± 12 | 101 ± 11 | 98 ± 25 | 97 ± 4
T/C [%]
250 210 170 130 90 50

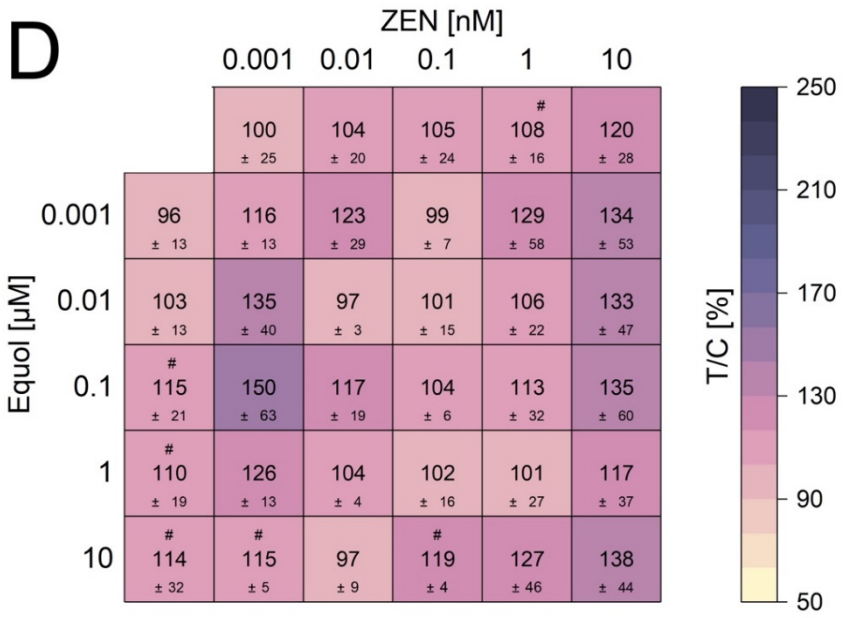


Figure 20: Effects of ZEN and isoflavones on cell protein amount

Heatmaps displaying binary combinations of A) ZEN and DAI, B) ZEN and GEN, C) ZEN and GLY, D) ZEN and EQ on cell protein amount [%] determined via the SRB assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of ZEN [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.

None of the binary combinations of ZEN and either of the isoflavones demonstrated cytotoxicity. The calculated mean values all lie within the range of the solvent control, at a cell viability or cell protein content of 100%. However, at certain high isoflavones concentrations and low ZEN concentrations there seems to be a slight increase in cell viability. For example, as illustrated in Figure 20A, at a DAI concentration of 10 µM and at ZEN concentration of 0.001 and 0.01 nM, a significant increase in cell viability up to 127% was observed. The respective SRB results, reported a significant elevated cell protein amount, yet here the high standard deviations only allow cautious interpretation. The same trend can be observed between ZEN and EQ, where again a higher cell viability seems to match an augmentation in cellular protein levels. No effect at all was seen in the results for GLY and ZEN. GEN and ZEN seemed to display an increase in cell viability at a GEN concentration of 0.1 µM and 1 µM with the lower ZEN concentrations, yet not all of these results were tested to be significant. Additionally, the SRB results do not indicate an increase in the cell protein

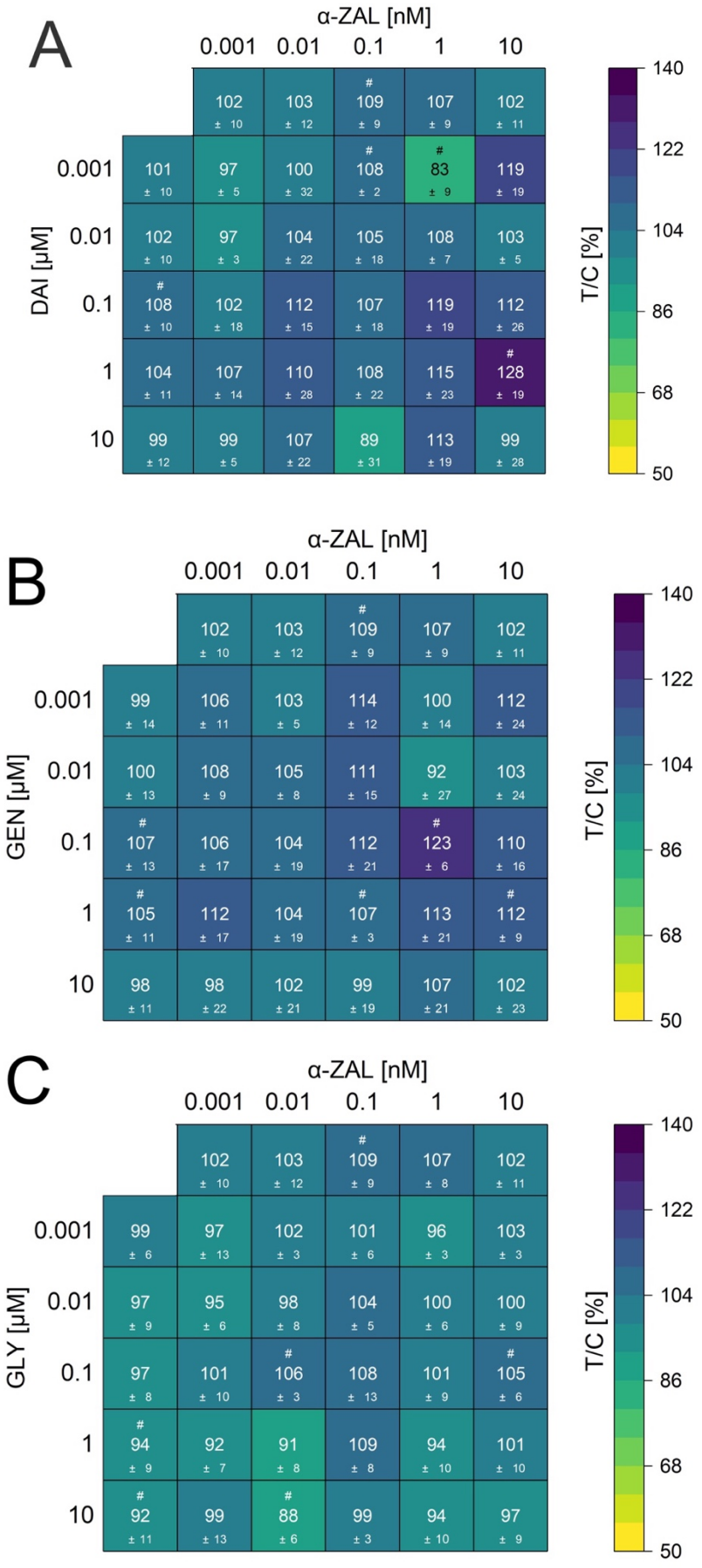
amount. It must be noted that the CTB assay assesses cell viability via the means of a reduction reaction. Thus, it is a form of metabolic activity of the cells and an increase in the measured absorbance could just as well point towards an increase in metabolic activity. Due to this possibility to misinterpret an augmentation of the absorbance immediately as cell proliferation, the SRB assay offers some insight to whether more cellular protein was produced. Together these two different endpoints ideally allow a more conclusive statement concerning cell proliferation. At times, the SRB results concerning ZEN and the isoflavones displayed large standard deviations. This was due to a series of washing steps which took time to perfection. As these combinations were the first ones performed, the largest deviations are to be seen here, where then at the latter combinations the reliability of results improved.

In past and present literature, combinatory effects between ZEN and isoflavones has not been frequently studied. A similar study conducted by Vejdovszky et al. investigated changes in the cellular protein amount via the SRB assay on Ishikawa cells in order to exclude cytotoxic effects impacting estrogenicity. The authors tested combinations of ZEN and GEN ranging from 0.001-20 µM to 0.001 nM-1 µM respectively. At a concentration of 1 µM of GEN and 0.001-0.1 nM of ZEN, a significant increase in the cellular protein level was observed compared to the solvent control, indicating a similar trend as in the results seen in Figure 21 above (Vejdovszky, Schmidt, et al. 2017). A different study utilized MCF-7 cells which were incubated with various combinations of ZEN and GEN for 72 hours. Here the authors found concentrations of 16 and 32 µM of GEN to repress cell viability to around 90% and 60% respectively, whilst ZEN at 10 nM and 20 nM induced cell viability to around 110%. Furthermore, combinations of ZEN (10 nM) and GEN (32 µM) or ZEN (10 nM) and GEN (16 µM) still demonstrated a lower cell viability than ZEN solely induced, suggesting GEN to reduce proliferative effects. This effect was then further investigated where a plausible mechanistical explanation concerned the involvement of GEN initiating a reduction in the expression of the anti-apoptotic *bcl-2* gene and upregulating the pro-apoptotic *bax* gene. An incubation solely with ZEN reversed these effects, allowing cell proliferation. Furthermore, as ZEN has an affinity for the ERα whilst GEN preferentially binds to ERβ, there might be an interplay between these two different receptors. Generally, ERα is known to act upon cell proliferation where ERβ is speculated to be of inhibitory nature (Wang, et al. 2010). Looking at the heatmap concerning the cell viability results of ZEN and GEN (Figure 20B), a tendency towards a

decrease in cell viability is suggested at the highest concentration (10 µM) of GEN when compared directly to the metabolic activity achieved at the the concentration of 1 µM of GEN.

As stated previously, A. Betschler investigated the estrogenicity of the exact combinations of this thesis, where the data sets regarding the cytotoxicity and estrogenicity are thought to complement each other. The exclusion of cytotoxic effects strengthens the ALP results as cytotoxicity cannot be held accountable for observed reductions in estrogenicity. The ALP results generated tend to follow similar tendencies as seen in the heatmaps displayed above. The highest estrogenicity is found at the higher isoflavone (1 µM) and higher ZEN concentrations of 1-10 nM. Yet at the highest isoflavone concentration of 10 µM, a fall in estrogenicity was observed. EQ had the most potent effect in inducting an increase in ALP levels in combination with ZEN, but also DAI and GEN displayed such effects, however at a lower level. Synergism was observed between low levels of isoflavones such as 0.1 µM of EQ with 0.001-1 nM of ZEN (Betschler 2021). The induction of estrogenicity was more prominent than any of the observed cell proliferative effects, yet the incubation time of solely 48 hours was not chosen to investigate cell proliferation. An intriguing abrupt decrease in the levels of ALP measured between GEN and ZEN at the highest concentration raised multiple questions to the mechanisms behind this observation. An attempt to elucidate this is discussed in the upcoming qRT-PCR analysis section.

Combinations of α-ZAL with isoflavones



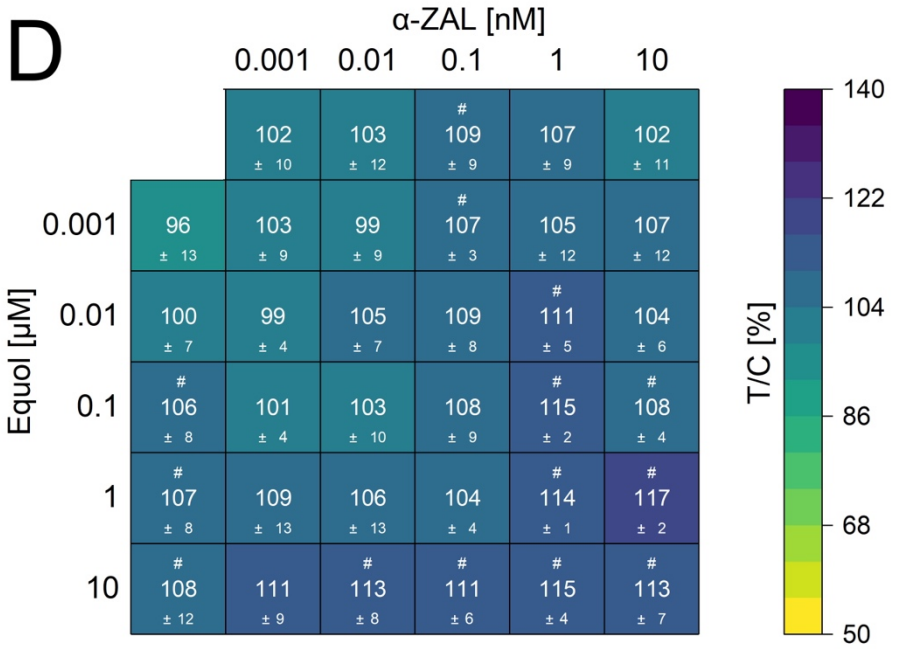
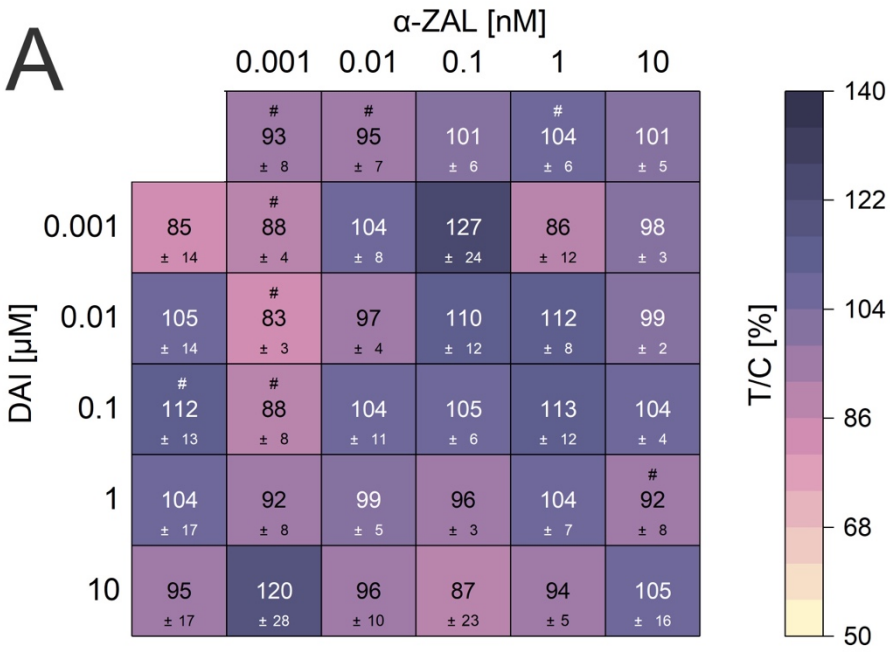
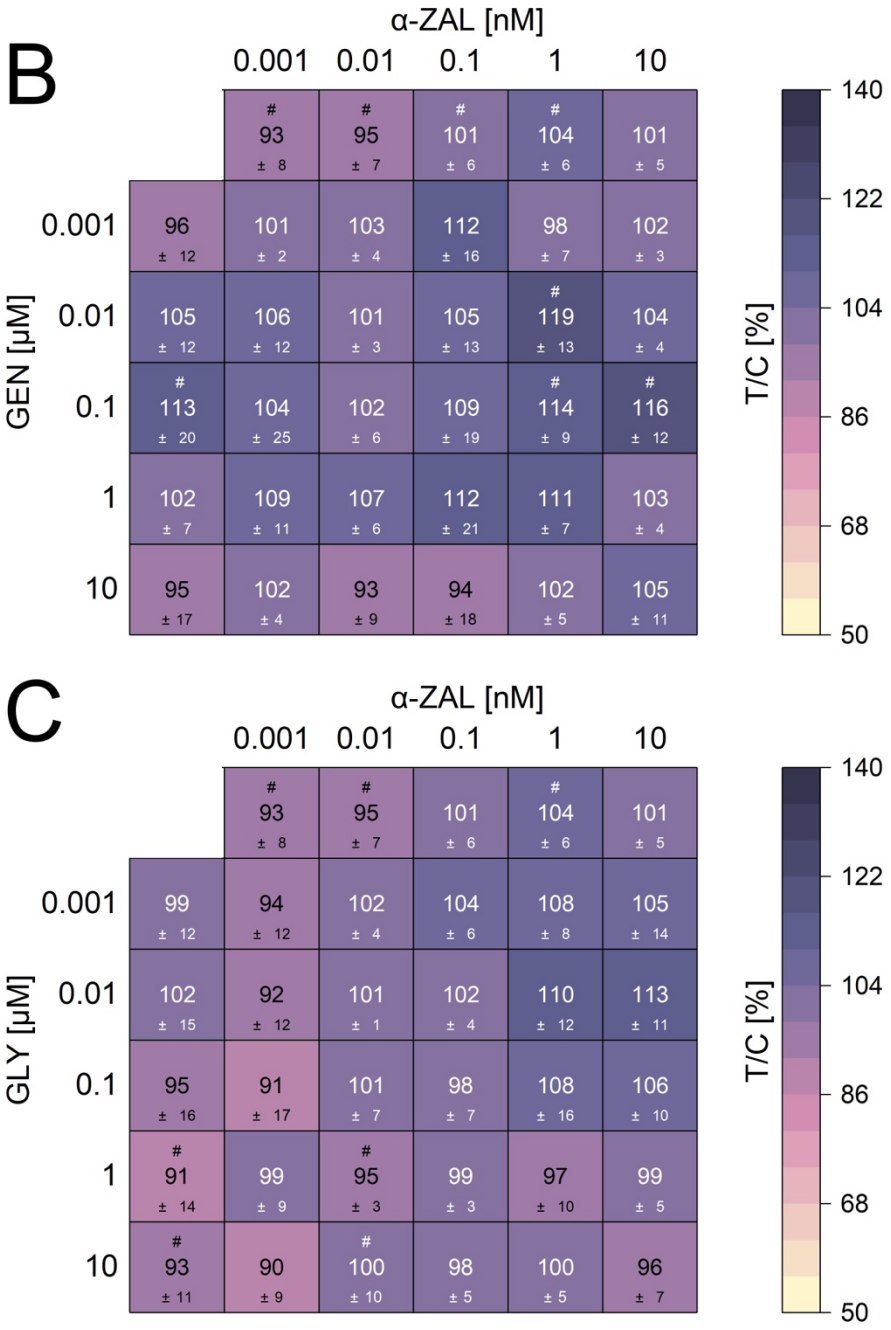


Figure 21: Effect of α-ZAL and isoflavones on cell viability

Heatmaps displaying binary combinations of A) α-ZAL and DAI, B) α-ZAL and GEN, C) α-ZAL and GLY, D) α-ZAL and EQ on cell viability [%] determined via the Cell Titer Blue (CTB) assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of α-ZAL [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.




B
α-ZAL [nM]
0.001
0.01
0.1
1
10
GEN [µM]
0.001
0.01
0.1
1
10
93 ± 8
95 ± 7
101 ± 6
104 ± 6
101 ± 5
96 ± 12
101 ± 2
103 ± 4
112 ± 16
98 ± 7
102 ± 3
105 ± 12
106 ± 12
101 ± 3
105 ± 13
119 ± 13
104 ± 4
113 ± 20
104 ± 25
102 ± 6
109 ± 19
114 ± 9
116 ± 12
102 ± 7
109 ± 11
107 ± 6
112 ± 21
111 ± 7
103 ± 4
95 ± 17
102 ± 4
93 ± 9
94 ± 18
102 ± 5
105 ± 11
T/C [%]
140
122
104
86
68
50
C
α-ZAL [nM]
0.001
0.01
0.1
1
10
GLY [µM]
0.001
0.01
0.1
1
10
93 ± 8
95 ± 7
101 ± 6
104 ± 6
101 ± 5
99 ± 12
94 ± 12
102 ± 4
104 ± 6
108 ± 8
105 ± 14
102 ± 15
92 ± 12
101 ± 1
102 ± 4
110 ± 12
113 ± 11
95 ± 16
91 ± 17
101 ± 7
98 ± 7
108 ± 16
106 ± 10
91 ± 14
99 ± 9
95 ± 3
99 ± 3
97 ± 10
99 ± 5
93 ± 11
90 ± 9
100 ± 10
98 ± 5
100 ± 5
96 ± 7
T/C [%]
140
122
104
86
68
50

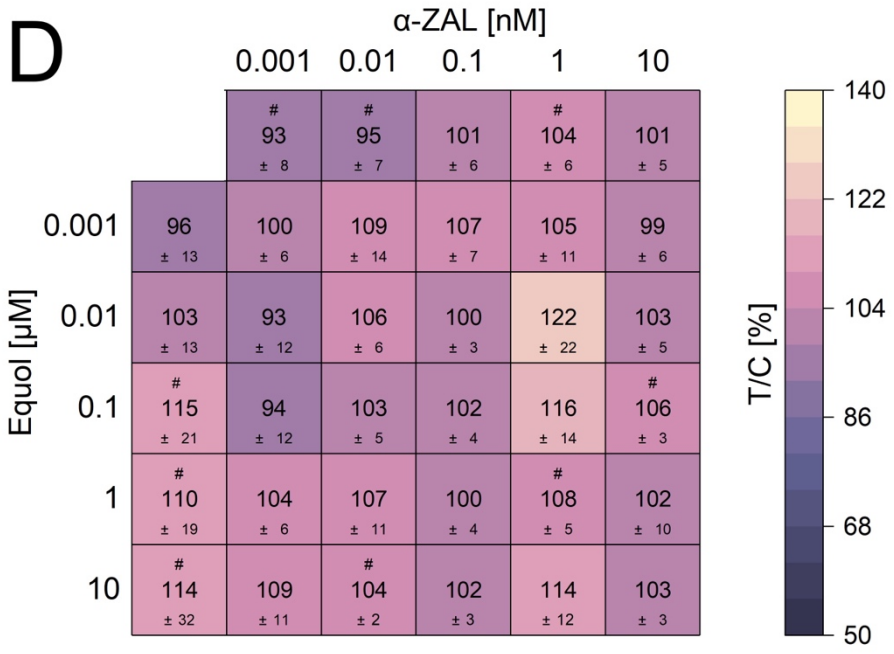


Figure 22: Effect of α-ZAL and isoflavones on cell protein amount

Heatmaps displaying binary combinations of A) α-ZAL and DAI, B) α-ZAL and GEN, C) α-ZAL and GLY, D) α-ZAL and EQ on cell viability [%] determined via the SRB assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of α-ZAL [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.

In Figure 22 and 23, combinations with α-ZAL and the respective isoflavones are displayed. Here, as with the binary combinations with ZEN, no cytotoxicity was observed throughout all various combinations and concentrations tested. Also similar to the results observed with ZEN, there seems to be an increase in cell viability at higher concentrations of the isoflavones GEN, EQ, and DAI. Yet only at specific concentrations this variation in cell viability was significantly different to the solvent control. For example, α-ZAL at 1 nM with 0.1 µM of GEN or EQ demonstrated such significant differences. The results gathered from the SRB assays demonstrated a similar trend, however here the results were rarely significant from the solvent control. Here too, the possibility of solely an increase in metabolic activity could explain the increase in absorbance measured during the CTB assay.

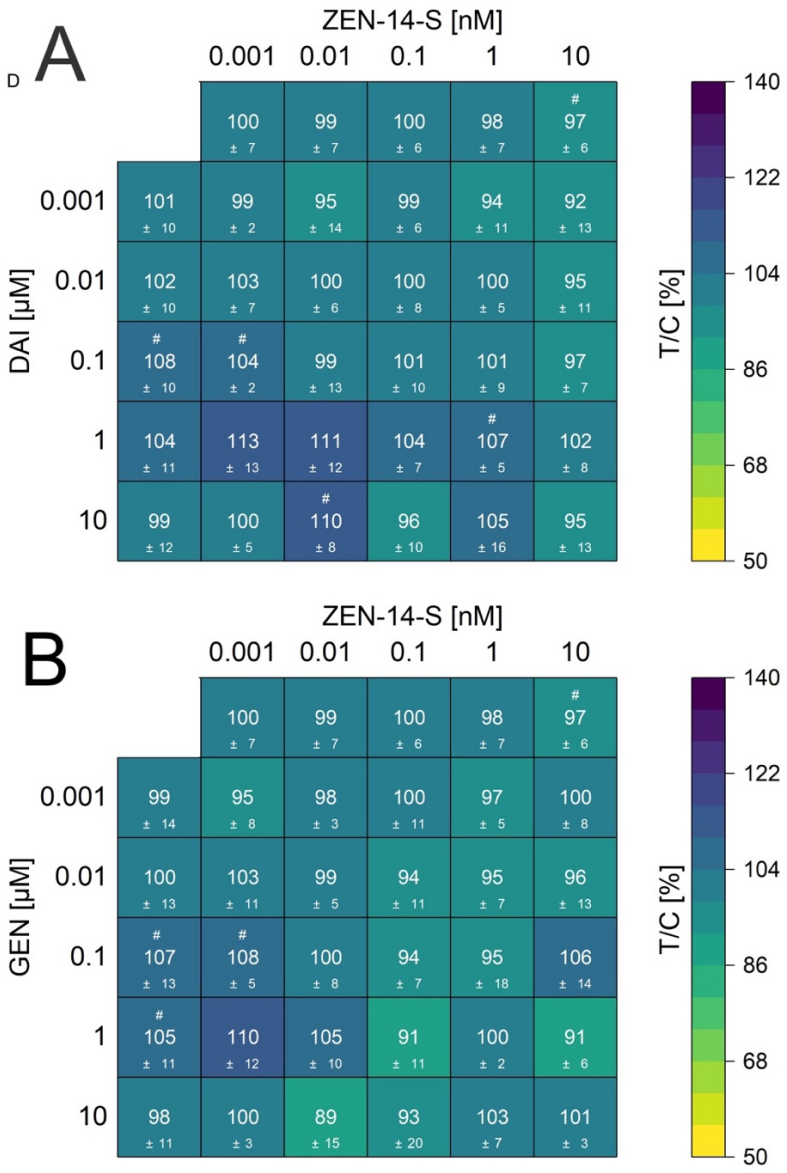
Data from previous studies regarding α-ZAL in binary combination with isoflavones is rare and hardly comparable. Preexisting studies have the notion to focus on single substances as the focus on ZEN metabolites still represents a moderately new field of research. The cell viability of MCF-7 cells upon incubation with α-ZAL for 24 hours with concentrations ranging from 0.001–10 000 nM has been previously studied utilizing the MTT assay. The results demonstrated no cytotoxicity as cell survival ranged between 95% to 100%, mirroring the results displayed above (Molina-Molina, et al. 2014).

As α-ZAL is a reductive metabolite of ZEN and has demonstrated a higher estrogenicity in cell culture studies compared to the parent compound ZEN. Nevertheless, α-ZEL demonstrates the strongest estrogenicity. Furthermore, α-ZAL is legitimized growth promoting hormone in the United States, implemented in animal husbandry, with the goal to improve the output of meat (Mukherjee, et al. 2014). The ability to induce cell proliferation is a prerequisite characteristic of growth promotors. Cell proliferation, similar to cytotoxicity, can also be assessed by means of *in vitro* cell assays such as the MTT assay. Previous investigations have studied the effect of α-ZAL compared to other ZEN metabolites as well as ZEN on cell proliferation using MCF-7 cells via the MTT assay. Concentrations of 0.1 pM to 0.1 µM of the mycoestrogens were used and the cells were incubated for 5 days. As a result, induction of cell proliferation was determined due to α-ZAL and ZEN, where these effects were of equal magnitude, achieving proliferative effects of around 1.8. Meanwhile incubation with α-ZEL demonstrated a higher cell proliferative effect of 2.6. To determine proliferative effects, the effect of the mycotoxin was divided by the effect of the non-treated control. To further elucidate the importance of estrogenic activity behind the observed cell proliferation, tamoxifen (an estrogen antagonist) was co-incubated with the various mycoestrogens. As a major finding, cell proliferation was reduced, thus asserting estrogen receptors to play a role in the mechanisms of ZEN and α-ZAL induced cell proliferation (Minervini, et al. 2005). In a similar endeavor to investigate the differences between ZEN and α-ZAL in terms of their estrogenicity, the ALP assay was applied to Ishikawa cells. Here too, the EC_{50} of both mycoestrogens was comparable to each other (5.8×10^{-11} M and 3.0×10^{-11} M respectively), eliciting the same magnitude of response. Whereas here too, the EC_{50} value of the metabolite α-ZEL was found to be lower at a concentration of 6.6×10^{-12} M (Guevel and Pakdel 2001). Thus, if the results from the previous section (Figure 20) are compared with the results

displayed in this section (Figure 22), the similarities between the obtained cell viability values reflects previous observations.

Regarding the data generated by A. Betschler, again the highest estrogenicity was found at the higher concentrations of the isoflavones (0.1-1 µM) and α-ZAL (1-10 nM). The induction beyond the effect of E2 was barely demonstrated, with some exceptions such as GEN at 1 µM and α-ZAL at 1 nM. Synergism was observed at lower concentrations of isoflavones such as DAI at 0.001 µM and α-ZAL at 0.01-0.1 nM (Betschler 2021). Here too, the highest ALP levels coincide with the highest cell viability measured, yet again the differences between cell viabilities were less profound. In order to investigate the proliferative effects of the estrogenic substances, longer incubation times would be required, however this was beyond the scope of this thesis.

Combinations of ZEN-14-Sulfate with isoflavones



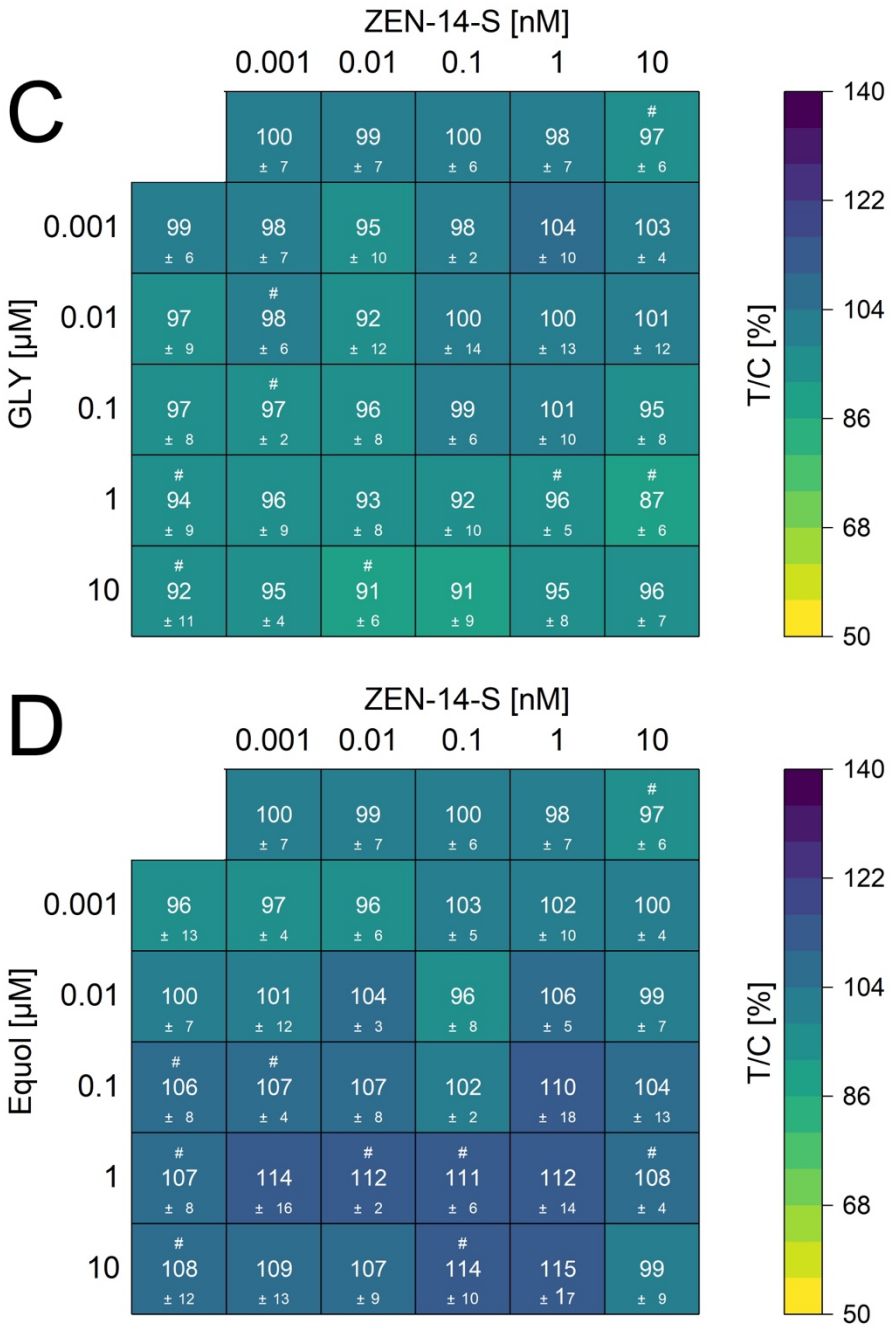
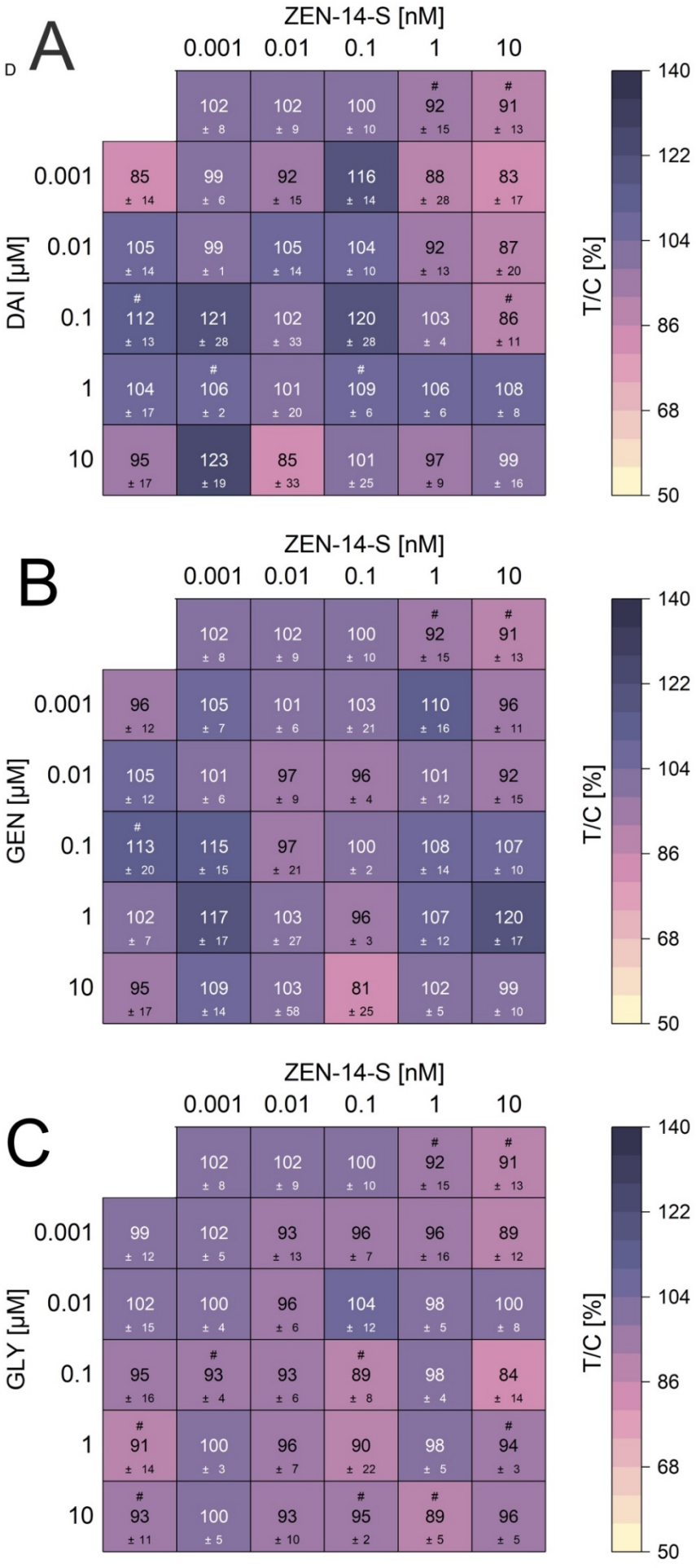


Figure 23: Effect of ZEN-14-S and isoflavones on cell viability

Heatmaps displaying binary combinations of A) ZEN-14-S and DAI, B) ZEN-14-S and GEN, C) ZEN-14-S and GLY, D) ZEN-14-S and EQ on cell viability [%] determined via the Cell Titer Blue (CTB) assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of ZEN-14-S [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.


A
ZEN-14-S [nM]
0.001 0.01 0.1 1 10
DAI [µM]
0.001 0.01 0.1 1 10
102 ± 8 | 102 ± 9 | 100 ± 10 | # 92 ± 15 | # 91 ± 13
85 ± 14 | 99 ± 6 | 92 ± 15 | 116 ± 14 | 88 ± 28 | 83 ± 17
105 ± 14 | 99 ± 1 | 105 ± 14 | 104 ± 10 | 92 ± 13 | 87 ± 20
112 ± 13 | 121 ± 28 | 102 ± 33 | 120 ± 28 | 103 ± 4 | # 86 ± 11
104 ± 17 | # 106 ± 2 | 101 ± 20 | # 109 ± 6 | 106 ± 6 | 108 ± 8
95 ± 17 | 123 ± 19 | 85 ± 33 | 101 ± 25 | 97 ± 9 | 99 ± 16
T/C [%]
140 122 104 86 68 50
B
ZEN-14-S [nM]
0.001 0.01 0.1 1 10
GEN [µM]
0.001 0.01 0.1 1 10
102 ± 8 | 102 ± 9 | 100 ± 10 | # 92 ± 15 | # 91 ± 13
96 ± 12 | 105 ± 7 | 101 ± 6 | 103 ± 21 | 110 ± 16 | 96 ± 11
105 ± 12 | 101 ± 6 | 97 ± 9 | 96 ± 4 | 101 ± 12 | 92 ± 15
113 ± 20 | 115 ± 15 | 97 ± 21 | 100 ± 2 | 108 ± 14 | 107 ± 10
102 ± 7 | 117 ± 17 | 103 ± 27 | 96 ± 3 | 107 ± 12 | 120 ± 17
95 ± 17 | 109 ± 14 | 103 ± 58 | 81 ± 25 | 102 ± 5 | 99 ± 10
T/C [%]
140 122 104 86 68 50
C
ZEN-14-S [nM]
0.001 0.01 0.1 1 10
GLY [µM]
0.001 0.01 0.1 1 10
102 ± 8 | 102 ± 9 | 100 ± 10 | # 92 ± 15 | # 91 ± 13
99 ± 12 | 102 ± 5 | 93 ± 13 | 96 ± 7 | 96 ± 16 | 89 ± 12
102 ± 15 | 100 ± 4 | 96 ± 6 | 104 ± 12 | 98 ± 5 | 100 ± 8
95 ± 16 | # 93 ± 4 | 93 ± 6 | # 89 ± 8 | 98 ± 4 | 84 ± 14
91 ± 14 | 100 ± 3 | 96 ± 7 | 90 ± 22 | 98 ± 5 | # 94 ± 3
93 ± 11 | 100 ± 5 | 93 ± 10 | # 95 ± 2 | # 89 ± 5 | 96 ± 5
T/C [%]
140 122 104 86 68 50

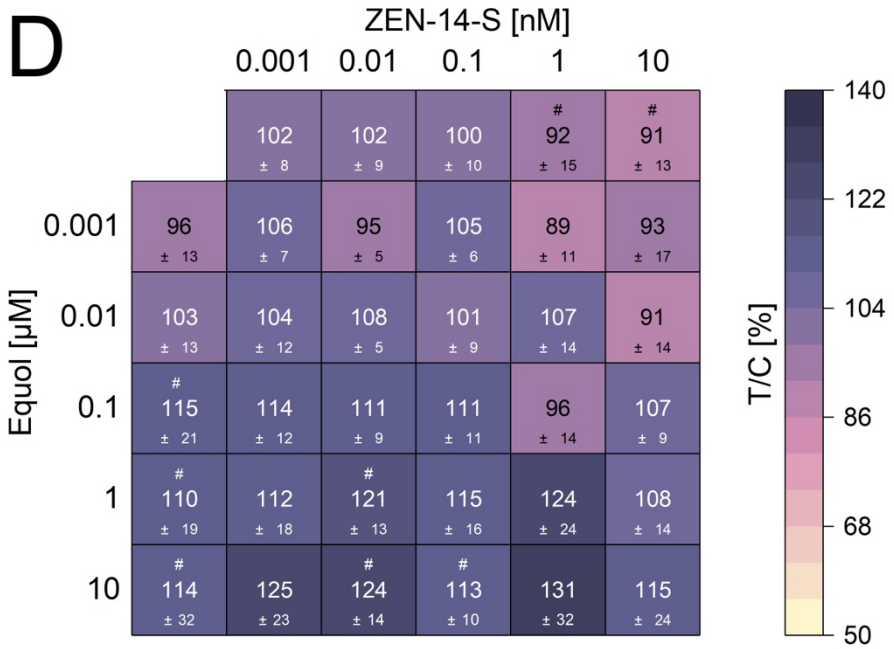


Figure 24: Effect of ZEN-14-S and isoflavones on cell protein amount

Heatmaps displaying binary combinations of A) ZEN-14-S and DAI, B) ZEN-14-S and GEN, C) ZEN-14-S and GLY, D) ZEN-14-S and EQ on cell viability [%] determined via the SRB assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone [µM] and the y-axis indicates concentration of ZEN-14-S [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the solvent control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.

ZEN-14-S is considered to be a phase II metabolic product of ZEN. In general, the conjugation reactions occurring during phase II metabolism result in detoxification and elimination of a compound. Looking at the heatmaps concerning ZEN-14-S in Figure 24 and 25, once more no cytotoxicity whatsoever was observed. As seen with the previous binary combinations, at certain concentrations there seems to be an ascent in cell viability and the cell protein amount, yet these values rarely significantly differ from the solvent control. Namely at the highest concentrations of EQ, such an increase in said endpoints is demonstrated.

In past and present literature, data on binary combinations with this specific conjugate is scarce and difficult to come by. A decrease in the estrogenicity of ZEN-14-S, compared to ZEN and α-ZAL, has been demonstrated by the utilization of an E-screen assay on MCF-7 cells. The authors hypothesized that the blocking of the phenolic C14 hydroxy group is blocked, thus losing its estrogenic capacity (Drzymala, et al. 2015). The toxicity of ZEN-14-S has been

screened employing the nematode *Caenorhabditis elegans,* which demonstrates a similar metabolic activity to higher animals. During the brood size assay, the effect elicited by ZEN-14-S was surprisingly within the range of ZEN. Nevertheless, the production of oxidative stress was greater by ZEN than the conjugated compound. The deconjugation of ZEN-14-S to its parent compound ZEN was not observed in this study, where the authors suggested this to be due to a missing capacity of the worm (Keller, et al. 2018). Comparing the results between ZEN-14-S with ZEN and α-ZAL, no immediate discrepancies are visible. Figure 26 below attempts to illustrate a comparison between the three mycoestrogens with the isoflavone EQ (at higher concentrations), to visualize the homogeneity of the results. EQ alone is able to induce cell viability significantly, yet it seems as in combination with the mycotoxins, this effect is strengthened (Figure 27). Nevertheless, the incubation time of 48 hours, did not aim to investigate proliferative effect, as this would have required a longer incubation. Hence, solely conclusions towards observed tendencies can be drawn.

Lastly, glancing again at the data of A. Betschler, she found ZEN-14-S to act non-estrogenically as a single substance, yet in combinations with the isoflavones, namely EQ and DAI, an induction in the ALP levels up to 108% and 104 % respectively was observed. Similar to the cytotoxicity results, EQ at a concentration of 1 µM paired with 1 or 10 nM of ZEN-14-S, resulted in a significant induction (Betschler 2021). Intriguingly, endogenous estrogens are sulfated and thus experience an inactivation due to their inability to bind to estrogen receptors. In serum and female tissues, estrogens are primarily present in their sulfated form and can be activated following the "sulfatase pathway". Enzymes, such as the steroid sulfatase, are required to cleave off the sulfate group, hence resulting in a reactivation of the estrogen due to their renewed ability to bind to respective estrogen receptors (Secky, et al. 2013). Cell types such as MCF-7 cells are equipped with low levels of steroid sulfatases, allowing the assumption of a detachment of sulfate groups of xenoestrogens, such as ZEN-14-S, occurring in the cells itself, as part of a cellular regulatory pathway (Drzymala, et al. 2015). To offer a further example regarding xenobiotics: the synthesized xenoestrogen bisphenol A (BPA) in its sulfated entity has demonstrated to act as a substrate to estrone sulfatase in MCF-7 cells, thus revealing its full estrogenic potential (Stowell, et al. 2006). Flasch et al. investigated metabolic products of GEN and ZEN in MCF-7 cells, where cells underwent 48, 66, or 70 hours of proliferation prior to 24, 6, or 2 hours of incubation respectively. This

allowed an equal time end-point of 72 hours for cell harvest and extraction. The authors demonstrated sulfation of both compounds within this time frame, providing further evidence for the metabolic activities happening within cells (Flasch, et al. 2022). Meanwhile Ishikawa cells have also been shown to express sulfatases, which were able to convert estrone sulfate to estrone and consequentially to the active estradiol, yet the magnitude of this conversion was quite low compared to other endometrial cell lines (Hevir-Kene and Rižner 2015). Therefore, cellular metabolic pathways could give rise to the plausibility of ZEN-14-S to be converted to the active form of ZEN, thus offering an explanation to how a theoretically inactive estrogenic compound could exert slight estrogenic effects, when paired with appropriately activating compounds. This estrogenicity could then in turn suggest the observed tendencies towards cell proliferation, however here again the limitations regarding the low incubation times remain. A further plausible explanation could be found within the stability of ZEN-14-S, which naturally underlies degradation processes. However, as the stock solutions were kept at appropriately low temperatures and prepared freshly prior to each incubation, a certain stability is assumed. Furthermore, the majority of ZEN-14-S has been demonstrated to withstand a baking process including kneading, fermentation, and baking, hence advocating a certain stability under mild conditions (Bryła, et al. 2020).

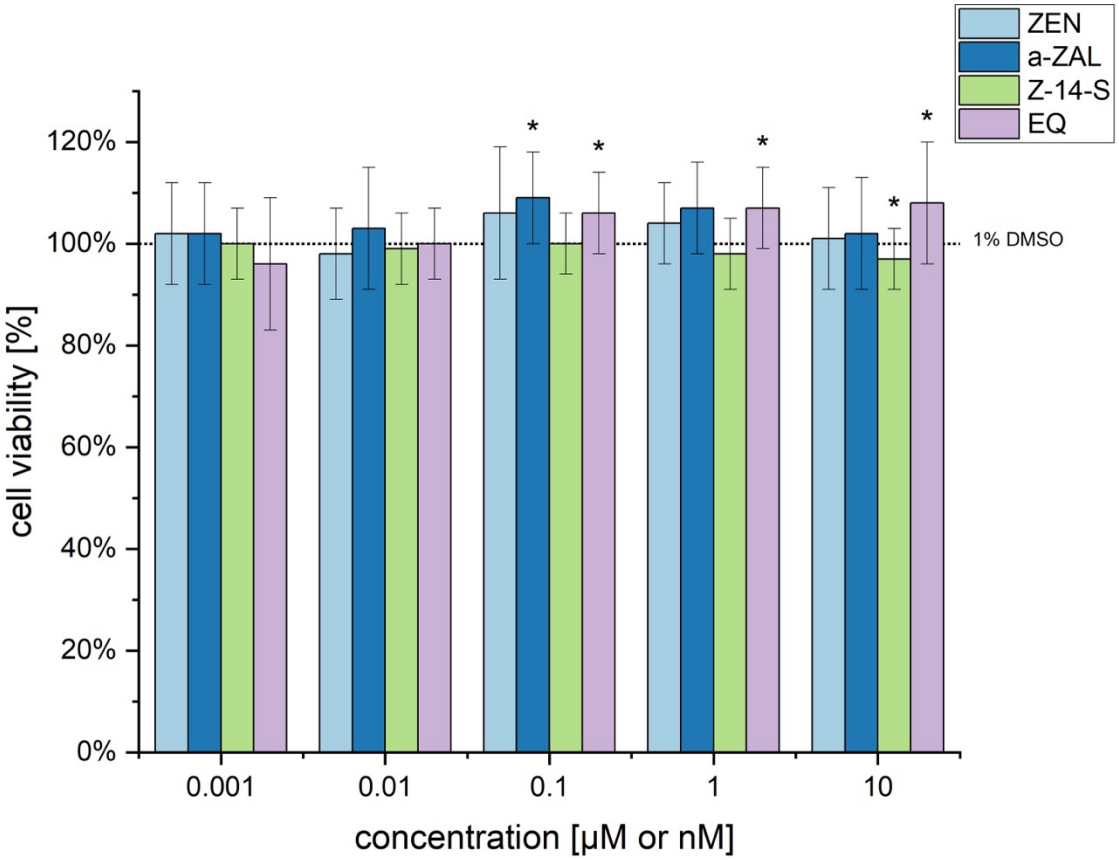


Figure 25: Selected single substances on cell viability - a direct comparison

Bar graph illustrating effect of ZEN and its metabolites in addition to EQ on cell viability [%] determined via the CTB assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of the isoflavone EQ [µM] and ZEN and its metabolites [nM]. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.

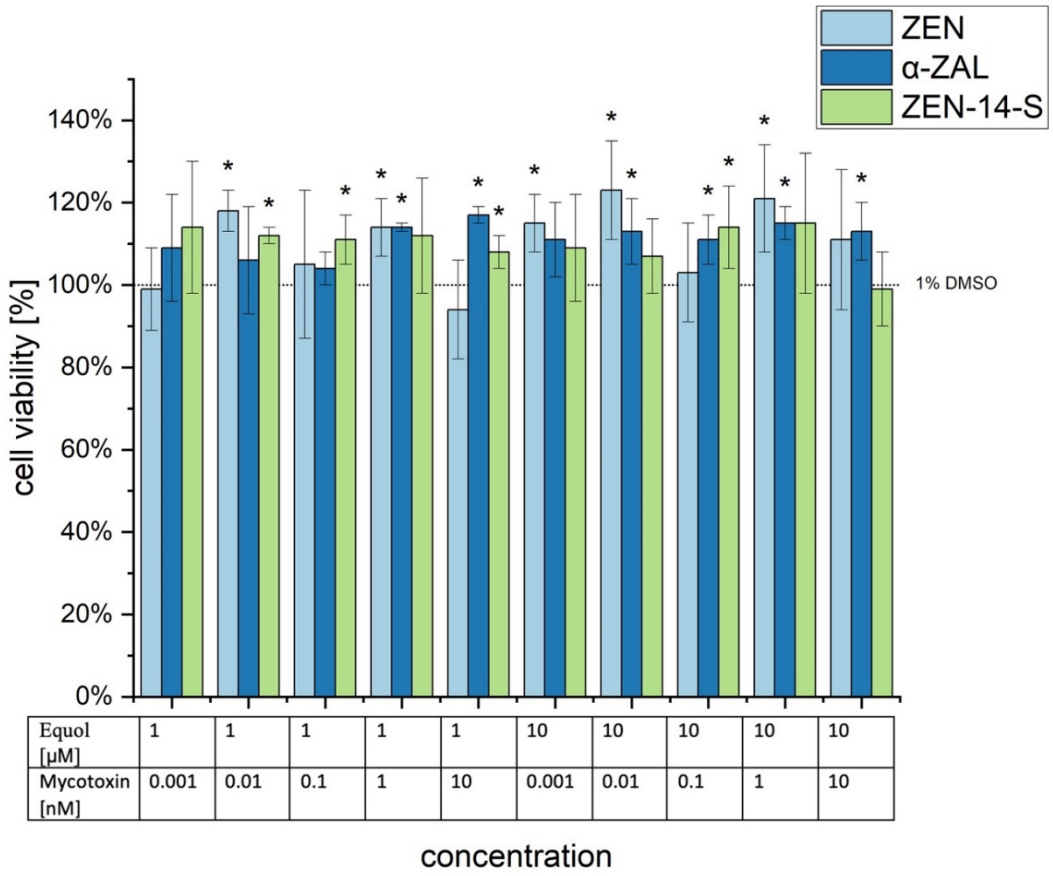


Figure 26: Binary effects of ZEN and its metabolites with EQ on cell viability - a direct comparison

Bar graph illustrating effect of ZEN and its metabolites in combination with EQ on cell viability [%] determined via the CTB assay upon 48 h incubation of Ishikawa cells. The x-axis indicates concentration of isoflavone EQ [µM] and ZEN and its metabolites [nM] at selected concentrations. Results are depicted as mean value ± standard deviation of at least 4 biological replicates. Each biological replicate consists of a mean value of 3 technical replicates. All mean values refer to the solvent control of 1% DMSO, which was set to 100% cell viability. Outliers are calculated via Nalimov and excluded from calculations. Significance compared to the control is indicated as # ($p < 0.05$), determined using a one sample student´s t-test.

From a holistic approach, the relevance of understanding the toxicity of ZEN-14-S lies within is capacity to be deconjugated by microorganisms in the gastrointestinal tract, becoming part of the phenomenon of "masked mycotoxins". Plants and fungi, as part of their own detoxification process, can produce ZEN-14-S out of the parent compound ZEN, which then can then be found in different cereals such a muesli or crackers (Vendl, et al. 2010). Dall´Erta et al. described deconjugation to occur rapidly under digestive conditions and microbial fermentation using an *in vitro* digestive assay (Dall'Erta, et al. 2013). Upon oral administration of ZEN-14-S in pigs, the analyte itself was not detected in feces or urine samples, however the detection of ZEN suggested hydrolysis reactions to be the culprit of these observations (Binder, et al. 2017). Recent EFSA evaluations have thus declared the toxicity of ZEN conjugates to be similar to ZEN and has taken these modified forms into consideration in the established TDI (EFSA 2017).

qRT-PCR Analysis Results

As a secondary part of this thesis, real-time PCR was performed in order to elucidate certain mechanisms on a gene expression level. As described previously, A. Betschler found a drastic decrease in estrogenicity, between 1 and 10 µM of GEN combined with ZEN (see Figure 28), α-ZAL, and also ZEN-14-S. EQ and DAI also demonstrated a similar trend, yet not as profound (Betschler 2021).

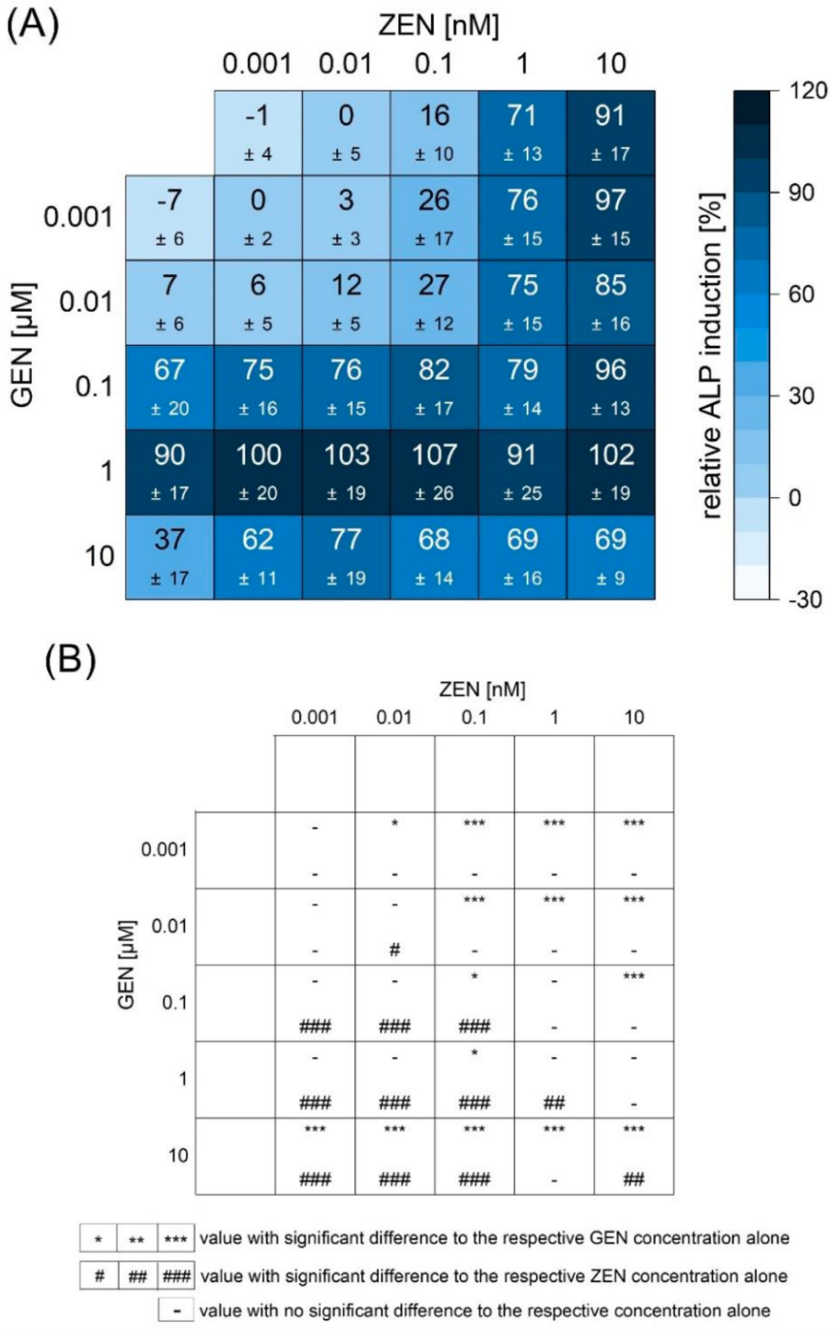


Figure 27: Combined effects of ZEN and GEN on ALP activity modified from (Betschler 2021)

In an attempt to explain this evident decrease in ALP induction, the genes *CYP1B1*, *CYP1A1*, *ESR1,* and *ESR2* were analyzed upon incubation with ZEN and GEN. The CYP enzymes play an important role in the metabolism of estrogens, where an increased expression of such enzymes could potentially lead to a higher inactivation of estrogens, thus lowering the estrogenicity observed. On the other hand, a decrease in estrogen receptors would result in the same effect, as estrogenic activity is exerted through the bonding of estrogens to their receptors. The higher concentrations of ZEN and GEN were selected, where they were tested individually in addition to their binary combination. The incubation time of the compounds was set to 48 hours; however, a single replicate was performed for an incubation time of 24 hours to provide a tendency. For each single compound or combination, except for ZEN combined with E2, a total of 5 replicates were performed. The combination with ZEN and E2 only had 3 replicates. The solvent control remained to be 1% of DMSO whilst 1 nM E2 was used as a positive control. Significant differences between the solvent control and the compounds were identified using a student´s *t*-test. As an internal control, the housekeeping genes *HPRT-1* and *ALAS1* were used. Both genes have been previously used in publications regarding Ishikawa cells, suggesting their suitability as stable genes (Melissa, Phelim and Navaratna 2017; Johnson, et al. 2007). The summarized results are displayed in the sections below while the data is found in the appendix (Table 9).

ESR1 and *ESR2* Results

Figure 29 illustrate the results gathered from 48 hours of incubation with the respective compounds. Unfortunately for the binary combinations, as can be seen at the first glance, the standard deviations are extremely high. This might be due to handling errors or difficulties, which were not overcome in the course of this thesis. Overall, no real induction was to be found in both genes, except for E2 inducing *ESR2* and a very slight induction of *ESR1* at 10 µM of GEN (1.45 ± 0.17). However, a two-fold increase is generally considered to be a pronounced induction, which was not the case of 10 µM of GEN. Interestingly, the combination of E2 with ZEN lead to a significant decrease of *ESR2* (0.42 ± 0.11) and suggested a decrease of *ESR1* (0.69 ± 0.31), although here it was not significant. All in all, this data does not support the theory of 10 µM of GEN and 10 nM of ZEN directly downregulating the mRNA levels of either estrogen receptors. In regard to 1 µM paired with 10 nM of ZEN, it could potentially point towards a tendency to upregulate *ERS1*, where then at the higher GEN concentration, this

upregulation is rescinded, resulting in the decrease in ALP levels observed. Furthermore, an interplay between the two different estrogen receptors is not to be excluded, as they are co-expressed in Ishikawa cells. ERβ has demonstrated to counteract certain actions imposed by ERα, especially regarding the induction of genes coding for proliferative actions (Ström, et al. 2004). Additionally, isoflavones have demonstrated a higher affinity to ERβ compared to ERα whereas ZEN prefers the ERα. Specifically, GEN has shown to present a 20-30-fold higher affinity to ERβ (Uifalean, et al. 2016). Thus, a decrease in the observed estrogenicity at the highest concentration of GEN, could also be due to an upregulation of ERβ, which in turn counteracts the estrogenic actions of ERα.

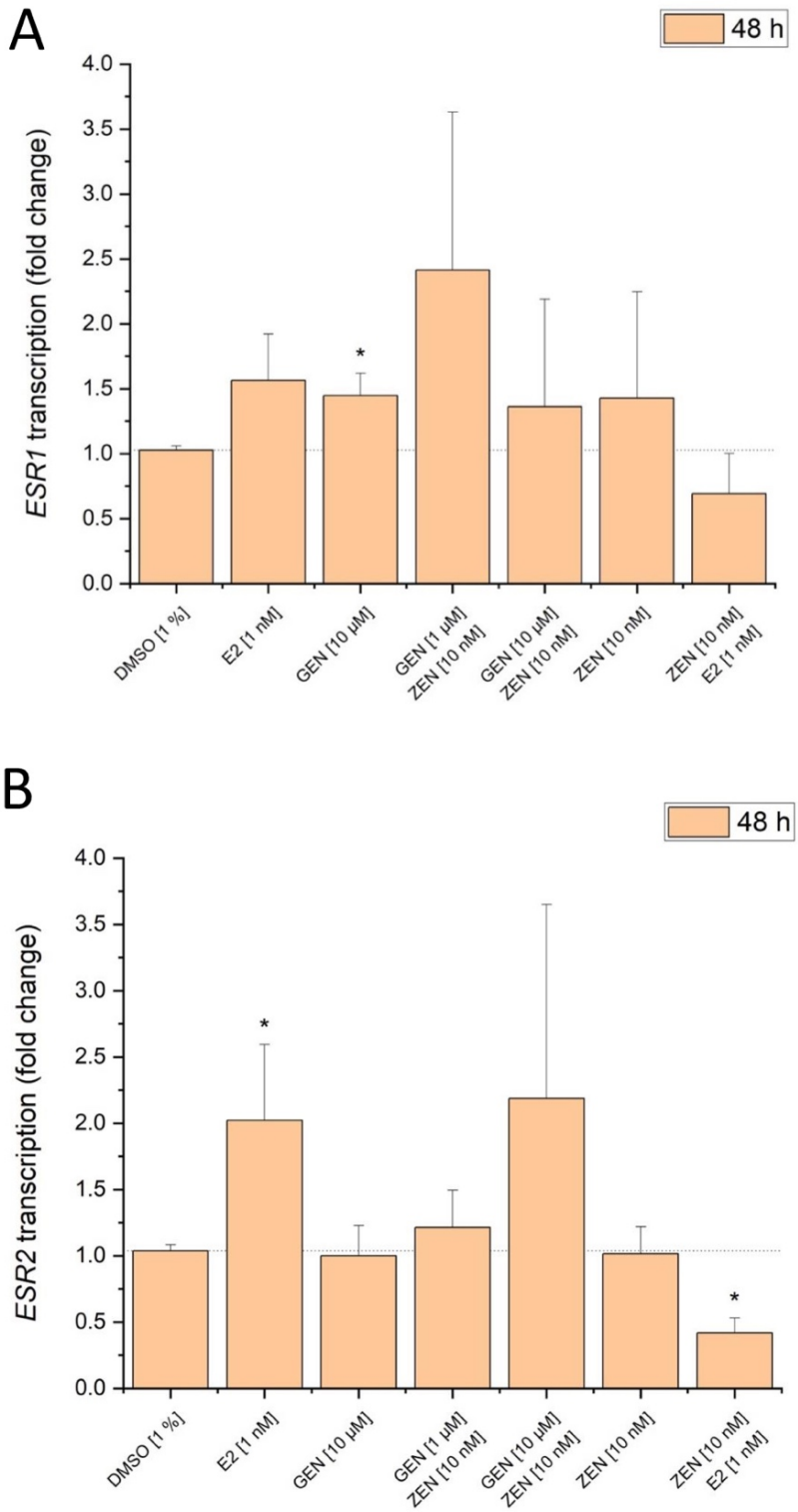


Figure 28: Effect of ZEN and GEN on ESR1 and ESR2 transcription fold change

*The single and binary effect of ZEN and GEN on A) the ESR1 and B) the ESR2 gene determined via qRT-PCR upon 48 h incubation of Ishikawa cells. Results were normalized on basis of the housekeeping genes HPRT1 and ALAS1. Data is presented as a mean ± standard deviation of at least 4 biological replicates for all combinations except ZEN with E2. Solely 2 biological replicates were used for ZEN and E2. Outliers were excluded according to the method of Nalimov. Significances compared to the control of 1% DMSO are indicated as * ($p<0.05$), determined using a student´s t-test.*

The effect of phytoestrogens has been previously investigated on mRNA levels of ERα and ERβ. Often, cell proliferation and an upregulation of ERα is looked at in the same context, as ERα is thought to initiate proliferative effects. The effect of GEN on MCF-7 cell proliferation via the regulation of ERα was studied by a group of authors in 2013. They found ERα to be downregulated in a concentration dependent manner, however the lowest concentration started at 50 µM GEN, hence limiting comparisons to the concentration (10 µM) tested within this thesis (Choi, Jung and Kim 2013). A similar study, again utilizing MCF-7 cells, examined the effect of a long-term exposure of 1 µM of GEN and 0.1 nM of E2 over the timespan of 6 days on the mRNA levels of estrogen receptors. Here, the authors found a significant reduction induced by GEN and E2, however the authors did not differentiate between ERα and ERβ. The at the time, present state of art called for northern blotting technology, probing the RNA samples solely for general ER mRNA bands, not allowing discrimination between the types of ER (Wang, Sathyamoorthy and Phang 1996). A different study attempted to clarify the role of GEN on the estrogen receptor expression in the rat prostate upon feeding rats with diets high in GEN. Once the rats were sacrificed, serum levels were determined to be 0.167 µM of total GEN for the group receiving 25 mg GEN/kg diet. In this context, total GEN refers to the sum of unconjugated and conjugated GEN in contrast to free GEN, which would refer solely to the aglycone or unconjugated GEN. For the rats who were fed 250 mg GEN/kg diet, the blood serum levels were at around 1.908 µM. The dorsolateral prostate was homogenized and served as the sample for the following assays. Succeeding a PCR analysis, ERα was downregulated significantly by each group. ERβ on the other hand was solely significantly downregulated by the group receiving the GEN-richer diet (Fritz, et al. 2002). Here too comparisons to the generated data are limited as the study designs differ greatly. *In vivo* experiments consider the entire metabolism with all its different pathways and possibilities, whereas *in vitro* cell culture methods simply target a few selected mechanisms. Furthermore, the prostate represents a different organ than the endometrium with a different sensitivity to estrogens. Nevertheless, such studies offer an insight to how physiologically relevant exposures may interfere with the expression of estrogen receptors.

The effect of ZEN on the mRNA expression of ERα and ERβ has also been investigated in a variety of publications. Gajęcka et al. scrutinized the effect of dietary low doses of ZEN on the immunohistochemical mRNA expression of estrogen receptors in intestinal epithelium of pre-

pubertal gilts. Following successive exposure, the gilts were sacrificed and samples were taken from the different segments of the intestine. The results suggested an induction of ERα in the small intestine and colon whilst a weakening of the ERβ expression was observed. However, in the intestine, ERβ is the more dominant receptor (Gajęcka, et al. 2020). A further publication regarding pre-pubertal gilts, investigated the effects of ZEN in combination with an isoflavone-rich diet, on the reproductive hormones and estrogen receptor expression. Here too, the gilts were fed with the appropriate diets and then slaughtered after 21 days. Uterine samples were taken to undergo further analysis. The major findings of this study included that both ZEN and the isoflavones (including a mixture of GEN, DAI, and GLY) were able to induce the ERα/ERβ expression. Furthermore, a low dosage of ZEN (0.5 mg/kg) combined with the isoflavone diet was able to increase the estrogenic effects observed compared to the sole ingestion of ZEN. Yet at a high dosage of ZEN (2.0 mg/kg) paired with the isoflavone diet, this increased induction was rescinded, suggesting competitive binding to the estrogen receptors to consequentially weaken the effects elicited (Wang, et al. 2010). Again, the same limitations as described above apply, as comparisons between *in vitro* with *in vivo* studies remains elusive. Nevertheless, these studies highlight the potential of both xenoestrogens, ZEN and GEN, prompting changes in the expressions of both ERs. The exact impacts would be highly depended on the exposure concentration, cell type, and length of exposure.

CYP1A1 and *CYP1B1* Results

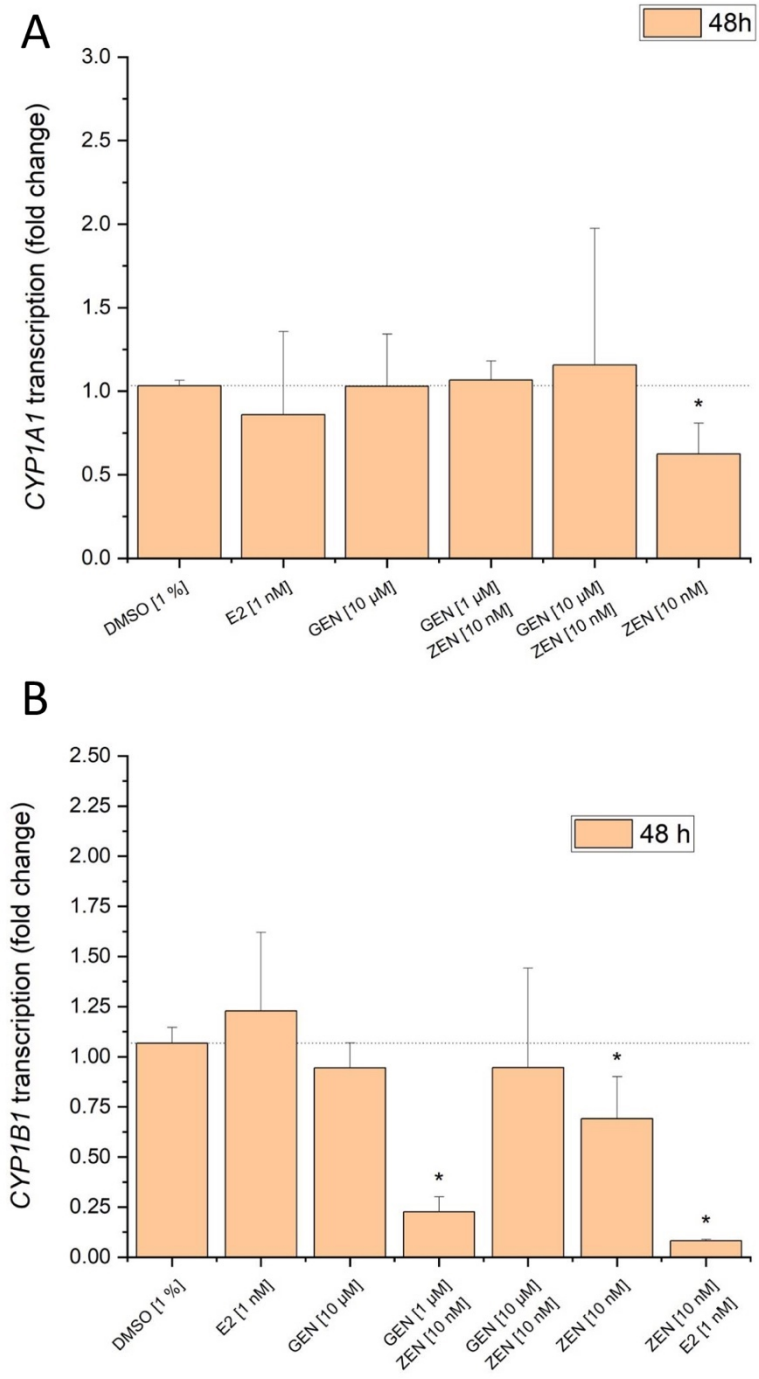


Figure 29: Effect of ZEN and GEN on CYP1A1 and CYP1B1 transcription fold change

*The single and binary effect of ZEN and GEN on A) the ESR1 and B) the ESR2 gene determined via qRT-PCR upon 48 h incubation of Ishikawa cells. Results were normalized on basis of the housekeeping genes HPRT1 and ALAS1. Data is presented as a mean ± standard deviation of at least 4 biological replicates for all combinations except ZEN with E2. Solely 2 biological replicates were used for ZEN and E2. Outliers were excluded according to the method of Nalimov. Significances compared to the control of 1% DMSO are indicated as * ($p<0.05$), determined using a student´s t-test.*

Figure 30A and 30B illustrate the results of the *CYP1A1* and *CYP1B1* expression gathered from 48 hours of incubation with the respective compounds. The results concerning *CYP1A1* do not indicate significant differences compared to the solvent control, except for 10 nM of ZEN. ZEN caused a significant, however slight reduction (0.62 ± 0.18) in the transcription of this gene. Intriguingly, E2 itself demonstrated no induction of *CYP1A1* by itself. The primer for this gene did not seem to work as effectively towards the last replicates performed, thus unfortunately there are no results regarding the combination of ZEN and E2. Meanwhile the combination of 1 µM GEN with 10 nM ZEN, induced a significant decrease in the *CYP1B1* transcription (0.23 ± 0.08). Furthermore 10 nM of ZEN in addition to ZEN and E2 had a similar significant effect of fold changes being respectively 0.69 ± 0.21 and 0.08 ± 0.01. As a decrease in the transcription of *CYP1B1* at 1 µM GEN with 10 nM ZEN could potentially imply a decreased metabolization of the xenoestrogens, resulting in a higher observed estrogenicity. Additionally, at the higher concentration of GEN paired with ZEN, no such transcriptive reduction is demonstrated, therefore this could point towards an increased rate of metabolized xenoestrogens, hence offering an explanation to the drop of ALP levels seen in Figure 28.

For these enzymes, cell culture studies have been performed previously upon ZEN or isoflavones exposition, but not in combination. Yu et al. investigated the role of ZEN in combination with the environmental pollutant TCDD. TCDD induced the expression of basal levels of *CYP1A1* and *CYP1B1* mRNA significantly in MCF-7 cells, where then this TCDD-induced activity was diminished by 50 nM of ZEN, thus acting as a potent CYP enzyme regulator. Yet here *CYP1A1* experienced an increase in mRNA levels in addition to an increased measured activity, whereas CYP1B1 solely experienced an increase in activity (Yu, Hua and Lib 2004). In the results generated in this thesis, *CYP1B1* seemed to be affected more profoundly, however the concentrations and cell line utilized differed. Moreover, the ratio between *CYP1A1* and *CYP1B1* is a relevant parameter concerning the toxicity of the metabolic byproducts generated from endogenous estrogens. *CYP1A1* is known to produce primarily 2OH-E2 whilst *CYP1B1* produces the genotoxic 4OH-E2 catechol. Thus, an upregulation of *CYP1B1* could lead to an increased production of the toxic catechol, asserting adverse effects (Coumoul, et al. 2001). In a similar study, 7,12-dimethylbenz[*a*]anthracene (DMBA) was used

to induce the expression of both enzymes in MCF-7 cells, where then 25 µM of GEN reduced these induced levels significantly. To inhibit the enzymatic activity, lower concentrations of 15.35 and 0.68 µM for *CYP1A1* and *CYP1B1* respectively were required (Chan and Leun 2003). A further study again examined the effect of a DMBA induced *CYP1A1* and *CYP1B1* expression in non-cancerous MCF-10A cells. Here only 1 µM of GEN was required to reduce the induced expression of *CYP1A1* whilst 5 µM of GEN were required for the reduction of *CYP1B1* (Leung, et al. 2009). All in all, the data from previous literature demonstrates the plausibility of the xenoestrogens to assert an effect on both CYP enzymes where the extend of the impact is highly dependent on the surrounding situation.

The regulating mechanisms behind the expression of *CYP1A1* and *CYP1B1* is thought to rely heavily on the aryl hydrocarbon receptor (AhR) pathway. Briefly, this cytosolic receptor is activated via ligand-binding and then undergoes a conformational change which promotes its translocation into the nucleus. There it then binds to a nuclear translocator and forms the AhR complex (AhRC), consequentially binding to its matching xenobiotic responsive elements (XRE) and initiating the transcription of certain genes such as *CYP1A1* and *CYP1B1* (Santes-Palacios, et al. 2016). Isoflavones such as GEN, DAI, and EQ have demonstrated agonistic and antagonistic effects on the AhR, thus it is conceivable that these phytoestrogens take part in the regulation of the phase I enzymes via the described pathway (Medjakovic, Mueller and Jungbauer 2010). A different theory implies a crosstalk between the estrogen receptors and the aryl hydrocarbon receptors, where estrogens limit the activation of the AhR through a series of degradation and depletion processes (Dunlap, et al. 2017). Specifically, ERα has been demonstrated to repress the expression of *CYP1A1* in the presence of E2 (Marques, Laflamme and Gaudreau 2013). ZEN preferentially binds to ERα, where this activation could then potentially regulate *CYP1A1*, the results displayed below demonstrating such decrease in enzyme expression. Nevertheless, further research is required to elucidate the role of isoflavones and mycoestrogens in the regulation of either of the CYP enzymes in addition to clarifying the function of the ERs in such mechanisms.

Conclusion

The primary aim of this thesis was to elucidate the cytotoxic potential of ZEN, α-ZAL, and ZEN-14-S in binary combinations with the selected isoflavones GEN, DAI, GLY, and EQ in physiologically relevant concentrations in Ishikawa cells. Previously, synergistic estrogenic effects have been determined for specific combinations within the scope of this project utilizing the ALP assay, where certain combinations demonstrated a profound increase in induced ALP activity (Betschler 2021). Overall, cytotoxicity prompted by the investigated substances is to be excluded, as higher concentrations of mycotoxins or isoflavones would have been required to illicit drastic cell viability decreases. Thus, alterations in the estrogenicity observed was not due to cytotoxic potential.

Meanwhile at specific concentrations of the investigated compounds, proliferative potential was suggested. Higher concentrations of EQ, GEN, and DAI demonstrated cell viability to reach up to a maximum of 128% (10 nM of α-ZAL and 1 µM of DAI). GLY did not seem to affect the cell viability in any of the binary combinations effectively. The cell protein amount was measured simultaneously in order to offer two distinct end points, strengthening statements asserted in the context of cell cytotoxicity and proliferation. The results regarding the cell protein amount also indicated a trend towards increasing cell amount, such as seen in the combination of 0.1-10 nM of ZEN and 10 µM of EQ, however the results were generally less profound and significant, compared to the solvent control. Furthermore, the incubation time was set to 48 hours, which is insufficient to properly assay cell proliferation. All in all, a trend towards proliferative capacity can be described, yet concrete evidence would require a different experimental setup.

As a drastic decrease of estrogenicity was observed at the highest GEN concentration in combination with ZEN, an attempt to offer mechanistical explanations on a molecular level was made by utilizing qRT-PCR technology. The combination of 1 µM paired with 10 nM of ZEN indicated the potential towards an upregulation of *ERS1*, where this upregulation was repealed at 10 µM paired with 10 nM. The results for *ERS2* suggested the opposite effect. As *ERS1* is responsible for the transcription of ERα whereas *ERS2* transcribes ERβ, an interplay between these two receptors might be of importance. ERβ has been described to act

antagonistically to actions provoked by ERα, notably regarding the induction of genes controlling cell proliferation, which are in turn frequently linked to estrogenicity (Ström, et al. 2004). Furthermore, ZEN and GEN differ in their affinities to each receptor, where ZEN prefers the ERα whilst GEN favors binding to ERβ (Uifalean, et al. 2016). A different approach encompassed the importance of the two CYP enzymes responsible for the metabolization of estrogens. *CYP1A1* failed to demonstrate any effect in transcription fold change upon incubation with the selected compounds. On the other hand, *CYP1B1* experienced a deregulation at 1 µM GEN with 10 nM ZEN, implying a decrease metabolization of the available xenoestrogens which in turn could result in the increase of estrogenicity at these concentrations. In turn, at 10 µM GEN with 10 nM ZEN, this reduction in transcription was rescinded, suggesting a higher degradation of estrogens compared to the concentration before, thus reducing total estrogenicity.

Nevertheless, a multitude of limitations to the results described above apply. The standard deviations concerning the qRT-PCR results were high, especially regarding the results of the binary combinations. Hence the reproducibility of the data is under scrutiny and further replicates would be required to clarify the significance of the effects observed. Moreover, caution is required to restrain from an oversimplification of such complicated molecular pathways. Crosstalk between the estrogen receptors and CYP enzymes is subject of current research in addition to the involvement of the aryl hydrocarbon receptor. The presence of the GPER and the various pathways estrogens can exert their functions through, should not be neglected. Lastly this research focuses solely on Ishikawa cells in the time frame of a 48-hour incubation, thus limiting the observations to these parameters. To further expand knowledge of mycoestrogens in combination with isoflavones, numerous studies varying in their experimental design will have to be conducted.

This thesis as part of an extensive project, aimed to promote understanding towards combinatory effects of ZEN and isoflavones, where data is still rare and limited to a handful of studies. Meanwhile the data concerning the cytotoxicity and estrogenicity generated in the scope of this project has been published contributing to the establishment of fundamental research (Grgic, et al. 2022). Compounds emulating hormones have increasingly become subject of research due to their ubiquitous and diverse nature, influencing us on a daily basis.

Certain exposure settings, such as the monotone feed of livestock, contain notable amounts of ZEN and isoflavones. Due to their similar mode of action, an interplay between mycoestrogens and isoflavones is not to be excluded. Solely ZEN and its metabolite are encompassed in regulatory measures. Thus, an enhancement of the actions of ZEN, as a product of binary combinations with other xenoestrogens, escapes previous risks assessment. This thesis was conducted in an attempt to aid in clarification concerning plausible combinatory effects between mycoestrogens and isoflavones.

Lastly, as a final note, changing climatic conditions encourage the growth of certain fungi, hence a steady increase of mycotoxin contamination in food and feed is anticipated. Hereafter, as part of preventive measures, further research regarding the toxicological assessments of combinations of mycotoxins with each other, or with distinct compounds with similar modes of action, is required.

Summary

Mycotoxins are considered to be fungal metabolites with a broad spectrum of characteristics and toxicological properties. Zearalenone (ZEN) classifies as a type of mycotoxin, being produced by various *Fusarium* species, frequently contaminating agricultural products such as corn and soy. Commonly found metabolites of ZEN are α-zearalanol (α-ZAL), zearalenone-14-sulfate (ZEN-14-S) or α-zearalenol (α-ZEL), where in this thesis the first two were investigated alongside of ZEN. Isoflavones such as daidzein (DAI), genistein (GEN), and glycitein (GLY), are secondary plant metabolites, mainly found in soy and soy product. Equol (EQ) is a bacterial metabolite of DAI, yet is frequently affiliated with the previously named isoflavones due to its similar properties. ZEN and the isoflavones are found to be estrogenic compounds as their structural similarity to 17β-estradiol allows binding to respective estrogen receptors (ERs) and hence triggers activation of a series of estrogen-dependent pathways. The relevance of co-occurrence of isoflavones, ZEN, and ZEN metabolites in food and feed products represents the main objective of this investigation with focus on possible synergism due to the structural resemblances of the compounds.

In this thesis the cytotoxicity assays Cell Titer Blue (CTB) and sulforhodamine B (SRB) were applied in order to investigate the cytotoxic potential of the isoflavones, ZEN, and ZEN metabolites as single substances and as binary combinations. Both assays were performed on Ishikawa cells with an incubation duration of 48 hours. For DAI, GEN, and EQ concentrations ranging from 0.001-10 µM were applied whereas the ZEN and ZEN metabolite concentrations ranged from 0.001–10 nM. For GLY concentrations of 0.01–20 µM were used. Furthermore, quantitative real-time reverse transcriptase PCR (RT-PCR) was utilized to determine the role of *CYP1A1*, *CYP1B1*, *ESR1*, and *ESR2* in the metabolization of GEN and ZEN at high concentrations (1–10 µM and 1–10 nM respectively).

The results of the cytotoxicity assays concluded no cytotoxic potential of any of the applied combinations or as single substances. The observed cytotoxicity did not reach below 80% compared to the control in any of the conducted experiments. On the other hand, slight proliferative potential was suggested at certain combinations with a maximum of 128% at 10 nM of α-ZAL and 1 µM of DAI. However, as these investigations were not set up to assay

proliferative potential, conclusions are limited. The RT-PCR results were overshadowed by high standard deviations, here too limiting final statements concerning the role of the selected genes. Further research would be necessary to elucidate mechanisms involved at high concentrations of isoflavones paired with ZEN and its metabolites.

As a final remark, this work strengthens previous investigations by excluding cytotoxic potential of the studied compounds at the physiological relevant concentrations. Co-occurrence of isoflavones, ZEN, and ZEN metabolites poses a pressing conundrum to be further investigated as similar modes of actions give rise to synergistic concerns. Monotone agricultural feed comprising largely of soy and corn portray ideal scenarios of enhanced toxicological properties, providing an example where consideration single-substance toxicity might be insufficient.

Zusammenfassung

Mykotoxine gelten als pilzliche Stoffwechselprodukte mit einem breiten Spektrum an Eigenschaften und toxikologischen Eigenschaften. Zearalenon (ZEN) wird als eine Art Mykotoxin klassifiziert, das von verschiedenen *Fusarium*-Arten produziert wird und häufig landwirtschaftliche Produkte wie Mais und Soja kontaminiert. Häufig gefundene Metaboliten von ZEN sind α-Zearalanol (α-ZAL), Zearalenon-14-sulfat (ZEN-14-S) oder α-Zearalenol (α-ZEL), wobei in dieser Arbeit die ersten beiden neben ZEN untersucht wurden. Isoflavone wie Daidzein (DAI), Genistein (GEN) und Glycitein (GLY) sind sekundäre Pflanzenstoffe, die hauptsächlich in Soja und Sojaprodukten vorkommen. Equol (EQ) ist ein bakterieller Metabolit von DAI, wird jedoch aufgrund seiner ähnlichen Eigenschaften häufig mit den zuvor genannten Isoflavonen in Verbindung gebracht. Es wurde festgestellt, dass ZEN und die Isoflavone Östrogene Verbindungen sind, da ihre strukturelle Ähnlichkeit mit 17β-Estradiol die Bindung an entsprechende Östrogenrezeptoren (ERs) ermöglicht und somit die Aktivierung einer Reihe von östrogenabhängigen Signalwegen auslöst. Die Relevanz des gleichzeitigen Vorkommens von Isoflavonen, ZEN und ZEN-Metaboliten in Lebens- und Futtermitteln stellt das Hauptziel dieser Untersuchung dar, wobei der Schwerpunkt auf einem möglichen Synergismus aufgrund der strukturellen Ähnlichkeiten der Verbindungen liegt.

In dieser Arbeit wurden die Zytotoxizitätsassays Cell Titer Blue (CTB) und Sulforhodamine B (SRB) eingesetzt, um das zytotoxische Potential der Isoflavone, ZEN und ZEN-Metaboliten als Einzelsubstanzen und als binäre Kombinationen zu untersuchen. Beide Assays wurden an Ishikawa-Zellen mit einer Inkubationsdauer von 48 Stunden durchgeführt. Für DAI, GEN und EQ wurden Konzentrationen im Bereich von 0,001–10 µM angewendet, während die ZEN- und ZEN-Metabolitenkonzentrationen im Bereich von 0,001–10 nM lagen. Für GLY wurden Konzentrationen von 0,01–20 µM verwendet. Darüber hinaus wurde quantitative Echtzeit-Reverse-Transkriptase-PCR (RT-PCR) verwendet, um die Rolle von *CYP1A1*, *CYP1B1*, *ESR1* und *ESR2* bei der Metabolisierung von GEN und ZEN in hohen Konzentrationen (1–10 µM bzw. 1–10 nM) zu bestimmen).

Die Ergebnisse der Zytotoxizitätsassays ergaben kein zytotoxisches Potenzial der angewendeten Kombinationen oder der Einzelsubstanzen. Die beobachtete Zytotoxizität

erreichte in keinem der durchgeführten Experimente weniger als 80 % im Vergleich zur Kontrolle. Andererseits wurde bei bestimmten Kombinationen mit einem Maximum von 128% bei 10 nM α-ZAL und 1 µM DAI ein leichtes proliferatives Potential suggeriert. Da diese Untersuchungen jedoch nicht darauf ausgerichtet waren, das proliferative Potenzial zu untersuchen, sind die Schlussfolgerungen begrenzt. Die RT-PCR-Ergebnisse wurden von hohen Standardabweichungen überschattet, was auch hier die endgültigen Aussagen zur Rolle der ausgewählten Gene einschränkte. Weitere Forschung ist notwendig, um die Mechanismen aufzuklären, die bei hohen Konzentrationen von Isoflavonen in Kombination mit ZEN und seinen Metaboliten beteiligt sind.

Als abschließende Bemerkung stärkt diese Arbeit vorherige Untersuchungen, indem sie das zytotoxische Potenzial der untersuchten Verbindungen bei den physiologisch relevanten Konzentrationen ausschließt. Das gleichzeitige Vorkommen von Isoflavonen, ZEN und ZEN-Metaboliten stellt ein dringendes Problem dar, das weitere Versuche benötigt, da ähnliche Wirkungsweisen Anlass zu synergistischen Wirkungsweisen geben. Monotone landwirtschaftliche Futtermittel, die größtenteils aus Soja und Mais bestehen, stellen ideale Szenarien für verstärkte toxikologische Wirkweisen dar und liefern ein Beispiel dafür, wo die Betrachtung der Einzelstofftoxizität möglicherweise nicht ausreicht.

References

Abassi, Haila, Imen Ayed-Boussema, Sarah Shirley, Salwa Abid, Hassen Bacha, and Olivier Micheau. 2016. "The mycotoxin zearalenone enhances cell proliferation, colony formation and promotes cell migration in the human colon carcinoma cell line HCT116." *Toxicology Letters* 1-7.

Aksglaede, Lise, Anders Juul, Henrik Leffers, Niels E. Skakkebæk, and Anna-Maria Andersson. 2006. "The sensitivity of the child to sex steroids: possible impact of exogenous estrogens." *Human Reproduction Update* 341–349.

Aksamitiene, Edita. "Templates". 2012. Accessed 29 August, 2022. http://www.cellsignet.com/media/templ.html

Al-Jaal, Belqes Ahmad, Morana Jaganjac, Andrei Barcaru, Peter Horvatovich, and Aishah Latiffa. 2019. "Aflatoxin, fumonisin, ochratoxin, zearalenone and deoxynivalenol biomarkers in human biological fluids: A systematic literature review, 2001–2018." *Food and Chemical Toxicology* 211-228.

Amandusson, Asa, and Anders Blomqvist. 2013. "Estrogenic influences in pain processing." *Frontiers in Neuroendocrinology* 329-349.

Amararathna, Madumani, Michael R. Johnston, and H. P. Vasantha Rupasinghe. 2016. "Plant Polyphenols as Chemopreventive Agents for Lung Cancer." *International Journal of Molecular Science* 1352-1366.

An, Jinping, Christina Tzagarakis-Foster, Tiffany C. Scharschmidt, Noureddine Lomri, and Dale C.Leitman. 2001. "Estrogen Receptor β-Selective Transcriptional Activity and Recruitment of Coregulators by Phytoestrogens." *Journal of Biological Chemistry* 17808-17814.

Ariyani, Winda, Toshiharu Iwasaki, Wataru Miyazaki, Lu Yu, Shigeki Takeda, and Noriyuki Koibuchi. 2018. "A Possible Novel Mechanism of Action of Genistein and Daidzein for Activating Thyroid Hormone Receptor-Mediated Transcription." *Toxicological Sciences* 417–427.

B. Balakrishnan, A. P. Ponnampalam, E. B. Thorstensen, and M. D. Mitchell. 2010. "Transplacental Transfer and Biotransformation of Genistein in Human Placenta." *Placenta* 506-511.

Behr, Maximilian, Jörg Oehlmann, and Martin Wagner. 2011. "Estrogens in the daily diet: *in vitro* analysis indicates that estrogenic activity is omnipresent in foodstuff and infant formula." *Food Chemical Toxicology* 2681–2688.

Belhassen, Hidaya, Inmaculada Jiménez-Díaz, Ridha Ghali, Hichem Ghorbel, Jose Manuel Molina-Molina, Nicholas Olea, and A. Hedili. 2014. "Validation of a UHPLC–MS/MS method for quantification of zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone in human urine." *Journal of Chromatography B* 68-74.

Bencze, Szilvia, Katalin Puskás, Gyula Vida, Ildikó Karsai, Krisztina Balla, Judit Komáromi, and Ottó Veisz. 2017. "Rising atmospheric CO_2 concentration may imply higher risk of Fusarium mycotoxin contamination of wheat grains." *Mycotoxin Research* 229–236.

Bennett, Joan W., and Maren Klich. 2003. "Mycotoxins." *Clinical Microbiology Reviews* 497–516.

Betschler, Andrea. 2021. *Combinatory estrogenic effects of mycoestrogens and isoflavones.* Master Thesis, Münster: WWU Münster.

Biehl, Michael L., Daniel B. Prelusky, Gary. D. Koritz, Kenneth. E. Hartin, William. B. Buck, and Locksley H. Trenholm. 1993. "Biliary Excretion and Enterohepatic Cycling of Zearalenone in Immature Pigs." *Toxicology Applied Pharmacology* 152−159.

Binder, Sabina B., Heidi E. Schwartz-Zimmermann, Elisabeth Varga, Gerlinde Bichl, Herbert Michlmayr, Gerhard Adam, and Franz Berthiller. 2017. "Metabolism of Zearalenone and Its Major Modified Forms in Pigs." *Toxins* 56.

Björnström, Linda, and Maria Sjöberg. 2005. "Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes." *Molecular Endocrinology* 833–842.

Bliss, C. I. 1939. "The toxicity of poisons applied jointly." *Annals of Applied Biology* 585–615.

Bolaños, Rafael, Angélica Del Castillo, and José Francia. 2010. "Soy isoflavones versus placebo in the treatment of climacteric vasomotor symptoms: systematic review and meta-analysis." *Menopause* 660-666.

Borras, Consuelo, Juan Gambini, M. Carmen Gomez-Cabrera, Juan Sastre, Federico V. Pallardo, Giovanni E. Mann, and Jose Vina. 2006. "Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFκB." *The FASEB Journal* 2136-2138.

Botana, Luis M., and Maria J. Sainz. 2015. *Climate Change and Mycotoxins.* Berlin/Boston: De Gruyter.

Bryła, Marcin, Edyta Ksieniewicz-Wozniak, Agnieszka Waskiewicz, Tomoya Yoshinari, Krystyna Szymczyk, Grazyna Podolska, Romuald Gwiazdowski, and Krzysztof Kubiak. 2020. "Transformations of Selected Fusarium Toxins and Their Modified Forms During Malt Loaf Production." *Toxins* 385.

Fleck, Stefanie C., Mona I. Churchwell, Daniel R. Doerge, and Justin G. Teeguarden. 2016. "Urine and serum biomonitoring of exposure to environmental estrogens II: Soy isoflavones and zearalenone in pregnant women." *Food and Chemical Toxicology* 19-27.

Carballo, Dionisia, Noelia Pallarés, Emilia Ferrer, Francisco J. Barba, and Houda Berrada. 2021. "Assessment of Human Exposure to Deoxynivalenol, Ochratoxin A, Zearalenone and Their Metabolites Biomarker in Urine Samples Using LC-ESI-qTOF." *Toxins* 530.

Catteuw, Amelie, Nathan Broekaert, Siegrid De Baere, Marianne Lauwers, Elke Gasthuys, Bart Huybrechts, Alfons Callebaut, *et al.* 2019. "Insights into *In Vivo* Absolute Oral Bioavailability, Biotransformation, and Toxicokinetics of Zearalenone, α-Zearalenol, β-Zearalenol, Zearalenone-14-glucoside, and Zearalenone-14- sulfate in Pigs." *Journal of Agriculture and Food Chemistry* 3448-3458.

Chan, Ho Yee, and Lai K. Leun. 2003. "A potential protective mechanism of soya isoflavones against 7,12-dimethylbenz[a]anthracene tumour initiation." *British Journal of Nutrition* 457–465.

Chen, Meinan, Yanhua Rao, Yi Zheng, Shiqing Wei, Ye Li, Tong Guo, and Ping Yin. 2014. "Association between Soy Isoflavone Intake and Breast Cancer Risk for Pre- and Post-Menopausal Women: A Meta-Analysis of Epidemiological Studies." *PloS One* e89288-e89288.

Choi, Eun Jeong, and Gun-Hee Kim. 2013. "Antiproliferative activity of daidzein and genistein may be related to ERα/c-erbB-2 expression in human breast cancer cells." *Molecular Medicine Reports* 781-784.

Choi, Eun Jeong, Jae Yeon Jung, and Gun-Hee Kim. 2013. "Genistein inhibits the proliferation and differentiation of MCF-7 and 3T3-L1 cells via the regulation or ERα expression and induction of apoptosis ." *Experimental and Therapeutic Medicine* 454-458.

Chou, T. C., and P. Talalay. 1984. "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors." *Advances in Enzyme Regulation* 27–55.

Colletti, Alessandro, Andrea Attrovio, Luisa Boffa, Stefano Mantegna, and Giancarlo Cravotto. 2020. "Valorisation of By-Products from Soybean (*Glycine max* (L.) Merr.) Processing." *Molecules* 2129.

Committee on Toxicity. 2003. *Phytoestrogens and Health: COT Report.* Food Standards Agency. Accessed August 31, 2022. https://cot.food.gov.uk/sites/default/files/cot/phytoreport0503.pdf

Cotroneo, S., Michelle, Jun Wang, Isam-Eldin A. Eltoum, and Coral A. Lamartiniere. 2001. "Sex steroid receptor regulation by genistein in the prepubertal rat uterus." *Molecular and Cellular Endocrinology* 135-145.

Coumoul, Xavier, Monique Diry, Cedric Robillot, and Robert Barouki. 2001. "Differential Regulation of Cytochrome P450 1A1 and 1B1 by a Combination of Dioxin and Pesticides in the Breast Tumor Cell Line MCF-7." *Endocrinology* 3942–3948.

Crain, D. Andrew, Sarah J. Janssen, Thea M. Edwards, Jerrold Heindel, Shuk-Mei Ho, Patricia Hunt, Taisen Iguchi, *et al.* 2008. "Female reproductive disorders: the roles of endocrinedisrupting compounds and developmental timing." *Fertility and Sterility* 911–940.

Dall'Erta, Andrea, Martina Cirlini, Margherita Dall'Asta, Daniele Del Rio, Gianni Galaverna, and Chiara Dall'Asta. 2013. "Masked Mycotoxins Are Efficiently Hydrolyzed by Human Colonic Microbiota Releasing Their Aglycones." *Chemical Research in Toxicology* 305–312.

Drzymala, Sarah S., Jennifer Binder, Antje Brodehl, Martin Penkert, Mark Rosowski, Leif-Alexander Garbe, and Matthias Koch. 2015. "Estrogenicity of novel phase I and phase II metabolites of zearalenone and cis-zearalenone." *Toxicon* 10-12.

Dunlap, Tareisha L., Caitlin E. Howell, Nita Mukand, Shao-Nong Chen, Guido F. Pauli, Birgit M. Dietz, and Judy L. Bolton. 2017. "Red Clover Aryl Hydrocarbon Receptor (AhR) and Estrogen Receptor (ER) Agonists Enhance Genotoxic Estrogen Metabolism." *Chemical Research in Toxicology* 2084-2092.

Eagle, Harry. 1959. "Amino Acid Metabolism in Mammalian Cell Culture." *Science* 432-437.

ECHA. 2021. *Registered substances.* June 2022. Accessed August 31, 2022. https://echa.europa.eu/information-on-chemicals/registered-substances

EFSA Scientific Committee. 2019. *Guidance on harmonised methodologies for human health,animal health and ecological risk assessment of combinedexposure to multiple chemicals.* EFSA Journal. https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2019.5634

EFSA. 2004. "Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Zearalenone as undesirable substance in animal feed." *The EFSA Journal* 1-35. https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2004.89

EFSA. 2011. "Scientific Opinion on the risks for public health related to the presence of zearalenone in food." *EFSA Journal* (EFSA Journal) 2197. https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2011.2197

EFSA. 2015. "Risk assessment for peri- and post-menopausal women taking food supplements containing isolated isoflavones." *EFSA Journal* (EFSA) 4246. https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2015.4246

EFSA. 2016. "Appropriateness to set a group health-based guidance value for zearalenone and its modified forms." *The EFSA Journal* 4425 *https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2016.4425*

EFSA. 2017. "Panel on contaminants in the food chain (2017) Risks for animal health related to the presence of zearalenone and its modified forms in feed." *EFSA Journal* 4851. https://efsa.onlinelibrary.wiley.com/doi/full/10.2903/j.efsa.2017.4851

EFSA. n.d. *Food Supplements.* Accessed August 31, 2022. https://www.efsa.europa.eu/en/topics/topic/food-supplements#eu-framework.

Eisenbrand, Gerhard. 2007. "Isoflavones as phytoestrogens in food supplements and dietary foods for special medical purposes." *Molecular Nutrition & Food Research* 1305-1312.

Enmark, E., and Jan-Åke Gustafsson. 2001. "Oestrogen receptors – an overview." *Journal of Internal Medicine* 133–138.

EPA. 2000. *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures.* Risk Assessment, Washington, D.C.: EPA. https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=20533

EU Council Regulation (EEC) No. 2009, 315/93. "of 8 February 1993 laying down Community procedures for contaminants in food." EU.

EuroMix. 2017. *A tiered strategy for risk assessment of mixtures of multiple chemicals.* Accessed August 31, 2022. https://www.euromixproject.eu/.

European Commission. 2006. "Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC)." *Official Journal of the European Union. https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32006H0576&from=EN*

Fang, I-Ju, and Brian Trewyn. 2012. "Chapter three - Application of Mesoporous Silica Nanoparticles in Intracellular Delivery of Molecules and Proteins." *Methods in Enzymology* 41-59.

Flasch, Mira, Christoph Bueschl, Giorgia Del Favero, Gerhard Adam, Rainer Schuhmacher, Doris Marko, and Benedikt Warth. 2022. "Elucidation of xenoestrogen metabolism by non-targeted, stable isotope-assisted mass spectrometry in breast cancer cells." *Environment International* 106940.

Franke, Adrian A., and Laurie J. Custer. 1996. "Daidzein and genistein concentrations in human milk after soy consumption." *Clinical Chemistry* 955-964.

Frankenfeld, Clara L. 2021. "Isoflavones." In *Dietary Polyphenols*, by Francisco A. Tomás-Barberá, Antonio González-Sarrías and Rocío García-Villalba, 199-222. John Wiley & Sons, Inc.

Fritz, Wayne A., Jun Wang, Isam-Eldin Eltoum, and Coral A. Lamartiniere. 2002. "Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate." *Molecular and Cellular Endocrinology* 89-99.

Frizzell, C., D. Ndossi, S. Verhaegen, E. Dahl, G. Eriksen, M. Sørlie, E. Ropstad, M. Muller, C.T. Elliott, and L. Connolly. 2011. "Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis." *Toxicology Letters* 210-217.

Froyen, Erik, and Francene Steinberg. 2016. "Genistein decreases basal hepatic cytochrome P450 1A1 protein expression and activity in Swiss Webster mice." *Nutrition Research* 430-439.

G.Howes, Laurence, Jan B. Howes, and David C. Knight. 2006. "Isoflavone therapy for menopausal flushes: A systematic review and meta-analysis." *Maturitas* 203-211.

Gajęcka, Magdalena, Michał Dąbrowski, Iwona Otrocka-Domagała, Paweł Brzuzan, Anna Rykaczewska, Katarzyna Cieplińska, Marzena Barasińska, Maciej T. Gajęcki, and Łukasz Zielonka. 2020. "Correlations between exposure to deoxynivalenol and zearalenone and the immunohistochemical expression of estrogen receptors in the intestinal epithelium and the mRNA expression of selected colonic enzymes in pre-pubertal gilts." *Toxicon* 75-93.

Gökmen, Vural. 2015. *Acrylamide in Food.* Academic Press.

Grgic, Dino, Andrea Betschler, Rebeka Früholz, Barbara Novak, Elisabeth Varga, and Doris Marko. 2022. "Estrogenic in vitro evaluation of zearalenone and its phase I and II metabolites in combination with soy isoflavones." *Archieves of Toxicology*

Grgic, Dino, Elisabeth Varga, Barbara Novak, Anneliese Müller, and Doris Marko. 2021. "Isoflavones in Animals: Metabolism and Effects in Livestock and Occurrence in Feed." *Toxins* 836.

Grober, O., M. Mutarelli, G. Giurato, M. Ravo, L. Cicatiello, M. R. De Filippo, L. Ferraro, *et al.* 2011. "Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for targe gene regulation." *BMC Genomics* 33-36.

Gstraunthaler, Gerhard, Toni Lindl, and Jan van der Valk. 2013. "A plea to reduce or replace fetal bovine serum in cell culture media." *Cytotechnology* 791–793.

Guevel, Remy Le, and Farzad Pakdel. 2001. "Assessment of oestrogenic potency of chemicals used as growth promoter by *in-vitro* methods." *Human Reproduction* 1030-1036.

Han, Xiao, Bingxin Huangfu, Tongxiao Xu, Wentao Xu, Charles Asakiya, Kunlun Huang, and Xiaoyun He. 2022. "Research Progress of Safety of Zearalenone: A Review." *Toxins* 386.

Health Canada. 2017. *Regulatory Proposal PRO2017-01, Cumulative Risk Assessment Framework.* Ottawa: Health Canada. https://www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management/public/consultations/regulatory-proposals/2017/cumulative-risk-assessment-framework/document.html

Held, Paul. 2018. *Using the Cytation 5 Cell Imaging Microplate Reader to Monitor Cell Culture Status.* Accessed August 31, 2022. https://www.biotek.com/resources/application-notes/using-phenol-red-to-assess-ph-in-tissue-culture-media/.

Hernández-Rodríguez, Patricia, and Arlen Gomez Ramirez. 2012. "Polymerase Chain Reaction: Types, Utilities and Limitations." In *Polymerase Chain Reaction*, by Patricia Hernández-Rodríguez and Arlen Gomez Ramirez, 157-172. IntechOpen.

Hevir-Kene, Neli, and Tea Lanišnik Rižner. 2015. "The endometrial cancer cell lines Ishikawa and HEC-1A, and the control cell line HIEEC, differ in expression of estrogen biosynthetic and metabolic genes, and in androstenedione and estrone-sulfate metabolism." *Chemico-Biological Interactions* 309-319.

Higuchi, Russell, Carita Fockler, Gavin Dollinger, and Robert Watson. 1993. "Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions." *Nature Biotechnology* 1026–1030.

Hodgson, Ernest. 2004. *A Textbook of Modern Toxicology.* Hoboken, New Jersey: John Wiley & Sons, Inc.

Hsieh, Ching-Yi, Ross C. Santell, Sandra Z. Haslam, and William G. Helferich. 1998. "Estrogenic Effects of Genistein on the Growth of Estrogen Receptor-positive Human Breast Cancer (MCF-7) Cells *in Vitro* and *in Vivo*." *Cancer Research* 3833-3838.

Hussain, D., C. Kolli, and R. Sethi. 2015. *Toxicology : The Past, Present, and Future of Basic, Clinical and Forensic Medicine.* New York: Nova Science Publishers.

Inbaraj, Baskaran Stephen, and Bing Huei Chen. 2013. "Isoflavones in Food and Ingestion in the Diet." In *Isoflavones : Chemistry, Analysis, Function and Effects*, by Victor R. Preedy, 28-42. RSC Publishing.

Jocsak, Gergely, Eniko Ioja, David Sandor Kiss, Istvan Toth, Zoltan Barany, Tibor Bartha, Laszlo V. Frenyo, and Attila Zsarnovszky. 2019. "Endocrine Disruptors Induced Distinct Expression of Thyroid and Estrogen Receptors in Rat versus Mouse Primary Cerebellar Cell Cultures." *Brain Sciences* 359.

Johnson, Suzanne M., Manijeh Maleki-Dizaji, Jerry A. Styles, and Ian N. H. White. 2007. "Ishikawa cells exhibit differential gene expression profiles in response to oestradiol or 4-hydroxytamoxifen." *Endocrine-Related Cancer* 337–350.

Keller, Julia, Antje Borzekowski, Hajo Haase, Ralph Menzel, Liliane Rueß, and Matthias Koch. 2018. "Toxicity Assay for Citrinin, Zearalenone and Zearalenone-14-Sulfate Using the Nematode *Caenorhabditis elegans* as Model Organism." *Toxins* 284.

Keller, Raymond B. 2009. *Flavonoids: Biosynthesis, Biological Effects and Dietary Sources.* Nova Science Publishers, Inc.

Kovalsky, Paula, and Christiane Gruber-Dorninger. 2021. *Zearalenone Compendium.* Getzersdorf: BIOMIN Holding GmbH.

Kowalska, Karolina, Dominika Ewa Habrowska-Górczyńska, Agnieszka Wanda, and Piastowska-Ciesielska. 2016. "Zearalenone as an endocrine disruptor in humans." *Environmental Toxicology and Pharmacology* 141-149.

Krizova, Ludmilla, Katerina Dadakova, Jitka Kasparovska, and Tomas Kasparovsky. 2019. "Isoflavones." *Molecules* 1076.

Kuiper, George G. J. M., Josephine M. Lemmen, Bo Carlsson, J. Christopher Corton, Stephen H. Safe, Paul T. Van der Saag, Bart Van Der Burg, and Jan-Ake Gustafsson. 1998. "Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor b." *Endcrinology.*

Labor Optik. 2022. *Zählkammer Neubauer - Improved.* Accessed July 28, 2022. http://www.zaehlkammer.de/deutsch/neubauer.improved.html.

Langton, Phil. 2013. *Essential Guide to Reading Biomedical Papers.* Wiley-Blackwell.

Lee, Anthony J., May Xiaoxin Cai, Paul E. Thomas, Allan H. Conney, and Bao Ting Zhu. 2003. "Characterization of the Oxidative Metabolites of 17β-Estradiol and Estrone Formed by 15 Selectively Expressed Human Cytochrome P450 Isoforms." *Endocrinology* 3382–3398.

Lee, Michael R. 2009. "The history of ergot of rye (*Claviceps purpurea*) I: From antiquity to 1900." *Journal of the Royal College of Physicians of Edinburgh* 179-84.

Lehman, Leane, Harald L. Esch, J. Wagner, L. Rohnstock, and Manfred Metzler. 2005. "Estrogenic and genotoxic potential of equol and two hydroxylated metabolites of Daidzein in cultured human Ishikawa cells." *Toxicology Letter* 72-86.

Leung, Hau Y., Lai Hang Yung, Ching Ho Poon, Guoli Shi, A.-Lien Lu, and Lai K. Leung. 2009. "Genistein protects against polycyclic aromatic hydrocarbon-induced oxidative DNA damage in non-cancerous breast cells MCF-10A." *British Journal of Nutrition* 257–262.

Li, Yin, Katherine A. Burns, Yukitomo Arao, Colin J. Luh, and Kenneth S. Korach. 2012. "Differential Estrogenic Actions of Endocrine-Disrupting Chemicals Bisphenol A, Bisphenol AF, and Zearalenone through Estrogen Receptor α and β *in Vitro*." *Environmental Health Perspectives.*

Liu, Huaqing, Juan Du, Chunyan Hu, Hong Qui, Xinru Wang, Shoulin Wang, Qizhan Liu, and Zhong Li. 2010. "Delayed activation of extracellular-signal-regulated kinase 1/2 is involved in genistein- and equol-induced cell proliferation and estrogen-receptor-α-mediated transcription in MCF-7 breast cancer cells." *The Journal of Nutritional Biochemistry* 390-396.

Lo, Emily Kwun Kwan, Jetty Chung-Yung Lee, Paul C. Turner, and Hani El-Nezami. 2021. "Low dose of zearalenone elevated colon cancer cell growth through G protein-coupled estrogenic receptor." *Nature Scientific Reports* 7403-7413.

Loewe, S., and H. Muischnek. 1926. "Über Kombinationswirkungen." *Archiv für experimentielle Pathologie und Pharmakologie* 313–326.

Loftis, Amanda D., and Will K. Reeves. 2012. "Principles of Real-Time PCR." In *Veterinary PCR Diagnostics*, by Chengming Wang and Marc D. Freeman Bernhard Kaltenboeck, 3-17. SAIF Zone: Bentham Science Publishers.

Lovett, Richard A. 2010. "A warming Earth could mean stronger toxins." *Nature* 593.

Lu, Qian, Jiao-Yang Luo, Hao-Nan Ruan, Chang-Jian Wang, and Mei-Hua Yang. 2022. "Structure-toxicity relationships, toxicity mechanisms and health risk assessment of food-borne modified deoxynivalenol and zearalenone: A comprehensive review." *Science of The Total Environment* 151192.

Lui, Cheng, and Ine Van der Fels-Klerx. 2021. "Quantitative Modeling of Climate Change Impacts on Mycotoxins in Cereals: A Review." *Toxins*, 276.

Maggiolini, Marcello, Adele Vivacqua, Giovanna Fasanella, Anna Grazia Recchia, Diego Sisci, Vincenzo Pezzi, Daniela Montanaro, Anna Maria Musti, Didier Picard, and Sebastiano Andò. 2004. "The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17β-estradiol and phytoestrogens in breast cancer cells." *Journal of Biological Chemistry* 27008-27016.

Manoj, Kumar, Sarma Devojit Kumar, Shubham Swasti, Kumawat Manoj, Verma Vinod, Prakash Anil, and Tiwari Rajnarayan. 2020. "Environmental Endocrine-Disrupting Chemical Exposure: Role in Non-Communicable Diseases ." *Frontiers in Public Health.*

Mansoury, Morva, Maya Hamed, Rashid Karmustaji, Fatima Al Hannan, and Stephen T.Safrany. 2021. "The edge effect: A global problem. The trouble with culturing cells in 96-well plates." *Biochemistry and Biophysics Reports* 100987.

Marin, Daniela E., Gina C. Pistol, Valeria C. Bulgaru, and Ionelia Taranu. 2019. "Cytotoxic and inflammatory effects of individual and combined exposure of HepG2 cells to zearalenone and its metabolites." *Naunyn-Schmiedeberg´s Archives of Pharmacology* 937-947.

Marques, Maud, Liette Laflamme, and Luc Gaudreau. 2013. "Estrogen receptor α can selectively repress dioxin receptor-mediated gene expression by targeting DNA methylation." *Nucleic Acid Research* 8094–8106.

Massart, Francesco, Valentina Meucci, Giuseppe Saggese G, and Giulio Soldani. 2008. "High growth rate of girls with precocious puberty exposed to estrogenic mycotoxins." *The Journal of Pediatrics* 690-695.

Maurer, Daniela. 2005. *The Significance of pH Stability for Cell Cultures.* Accessed May 30, 2022. https://www.americanlaboratory.com/914-Application-Notes/30798-The-Significance-of-pH-Stability-for-Cell-Cultures/.

Medjakovic, Svjetlana, Monika Mueller, and Alois Jungbauer. 2010. "Potential Health-modulating Effects of Isoflavone and Metabolites via Activation of PPAR and AhR." *Nutrients* 241-279.

Melissa, Poh Su Wei, Yong Voon Chen Phelim, and Visweswaran Navaratna. 2017. "DNA Microarray Analysis of Estrogen Responsive Genes in Ishikawa Cells by Glabridin." *Biochemistry Insights* 1–14.

Minervini, Fiorenza, Alessandra Giannoccaro, Aldo Cavallini, and Angelo Visconti. 2005. "Investigations on cellular proliferation induced by zearalenone and its derivatives in relation to the estrogenic parameters." *Toxicology Letters* 272-283.

Molina-Molina, Jose-Manuel, Macarena Real, Inmaculada Jimenez-Diaz, Hidaya Belhassen, Abderazzak Hedhili, Pablo Torné, Mariana F. Fernández, and Nicolas Olea. 2014. "Assessment of estrogenic and anti-androgenic activities of the mycotoxin zearalenone and its metabolites using in vitro receptor-specific bioassays." *Food and Chemical Toxicology* 233-239.

Morandi, Sheila, Alessandra D'Agostina, Francesca Ferrario, and Anna Arnoldi. 2005. "Isoflavone content of Italian soy food products and daily intakes of some specific classes of consumers." *European Food Research and Technology* 84–91.

Mukherjee, Dwaipayan, Steven G. Royce, Jocelyn A. Alexander, Brian Buckley, Sastry S. Isukapalli, Elisa V. Bandera, Helmut Zarbl, and Panos G. Georgopoulos. 2014. "Physiologically-Based Toxicokinetic Modeling of Zearalenone and Its Metabolites: Application to the Jersey Girl Study." *PloS One* 113632.

Mumtaz, Moiz. 2010. *Principles and Practice of Mixtures Toxicology.* Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Nachvak, Seyed Mostafa, Shima Moradi, Javad Anjom-Shoae, Jamal Rahmani, Morteza Nasiri, Vahid Maleki, and Omid Sadeghi. 2019. "Soy, Soy Isoflavones, and Protein Intake in Relation to Mortality from All Causes, Cancers, and Cardiovascular Diseases: A Systematic Review and Dose-Response Meta-Analysis of Prospective Cohort Studies." *Journal of the Academy of Nutrition and Dietetics* 1483-1500.

Nishida, Masato. 2002. "The Ishikawa Cells from Birth to the Present ." *Human Cell: Official Journal of Human Cell Research Society* 104-117.

O'Lone, R., M. C. Frith, E. K. Karlsson, and U. Hansen. 2004 . "Genomic targets of nuclear estrogen receptors." *Molecular Endocrinology* 1859–1875.

OECD. 2018. "Consideration for Assessing the Risks of Combined Exposure to Multiple Chemicals ."

Pang, Jian, Qingsong Zhou, Xiaofeng Sun, Lan Li, Bo Zhou, Fanchun Zeng, Yong Zhao, Wei Shen, and Zhongyi Sun. 2017. "Effect of low-dose zearalenone exposure on reproductive capacity of male mice." *Toxicology and Applied Pharmacology* 60-67.

Papoutsi, Zoi, Chunyan Zhao, Milica Putnik, Jan-Ake Gustaffson, and Karin Dahlman-Wright. 2009. "Binding of estrogen recepto α/β heterodimers to chromatin in MCF-7 cells." *Journal of Molecular Endocrinology* 65-72.

Parveen, Meher, Yun Zhu, and Ryoiti Kiyama. 2009. "Expression profiling of the genes responding to zearalenone and its analogues using estrogen-responsive genes." *FEBS Letters* 2377-2384.

Penagos-Tabares, Felipe, Ratchaneewan Khiaosa-ard, Veronika Nagl, Johannes Faas, Timothy Jenkins, Michael Sulyok, and Qendrim Zebeli. 2021. "Mycotoxins, Phytoestrogens and Other Secondary Metabolites in Austrian Pastures: Occurrences, Contamination Levels and Implications of Geo-Climatic Factors." T*oxins* 460.

Pitt, John I., and David J. Miller. 2017. "A Concise History of Mycotoxin Research." *Journal of Agriculture and Food Chemistry* 7021−7033.

Preedy, Victor R. 2013. *Isoflavones : chemistry, analysis, function and effects* . Cambridge: Royal Society of Chemistry

Prossnitz, Eric R., and Marcello Maggiolini. 2009. "Mechanisms of estrogen signaling and gene expression via GPR30." *Molecular and Cellular Endocrinology* 32-38.

Prossnitz, Eric R., and Matthias Barton. 2011. *Nature Reviews Endocrinology* 715–726.

Qiagen. 2009. *QuantiTect® Reverse Transcription Handbook.* Protocol, Qiagen.

Qiagen. 2016. QuantiTect® Reverse Transcription Kit. Quick Start Protocol.

Qiagen. 2019. *RNeasy® Mini Handbook.* Protocol , Qiagen.

Ramos, Joe W. 2008. "The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells." *The International Journal of Biochemistry & Cell Biology* 2707-2719.

Ribeiro, Ana Rita, Alexandra Maia, Mariana Santos, Maria Elizabeth Tiritan, and Cláudia Maria Rosa Ribeiro. 2016. "Occurrence of Natural Contaminants of Emerging Concern in the Douro River Estuary, Portugal." *Archives of Environmental Contamination and Toxicology* 361–371.

Ricci, M. Stacey, Diane G. Toscano, Carolyn J. Mattingly, and William A. Toscano. 1999. "Estrogen Receptor Reduces CYP1A1 Induction in Cultured Human Endometrial Cells." *The Journal of Biological Chemistry* 3430–3438.

Ritchie, Hannah, and Max Roser. 2021. *Soy.* Accessed August 31, 2022. https://ourworldindata.org/soy.

Rodríguez-Carrasco, Yelko, Luigi Castaldo, Anna Gaspari, Giulia Graziani, and Alberto Ritieni. 2019. "Development of an UHPLC-Q-Orbitrap HRMS method for simultaneous determination of mycotoxins and isoflavones in soy-based burgers."*Food Science and Technology* 34-42.

Roell, Kyle, David Reif, and Alison Motsinger-Reif. 2017. "An Introduction to Terminology and Methodology of Chemical Synergy—Perspectives from Across Disciplines." *Frontiers in Pharmacology* 158-169.

Sakamoto, Takako, Hyogo Horiguchi, Etsuko Oguma, and Fujio Kayama. 2010. "Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells." *The Journal of Nutritional Biochemistry* 856-864.

Santes-Palacios, Rebeca, Diego Ornelas-Ayala, Noel Cabañas, Ana Marroquín-Pérez, Alexis Hernández-Magaña, Sitlali del Rosario Olguín-Reyes, Rafael Camacho-Carranza, and Jesús Javier Espinosa-Aguirre. 2016. "Regulation of Human Cytochrome P4501A1 (hCYP1A1): A Plausible Target for Chemoprevention?" *BioMed Research International* 5341081.

Satih, Samir, Nassera Chalabi, Nadege Rabiau, Remy Bosviel, Luc Fontana, and Yves-Jean Bignon. 2010. "Gene expression profiling of breast cancer cell lines in response to

soy isoflavones using a pangenomic microarray approach." *A Journal of Integrative Biology* 231-238.

Scientific Committee on Health and Environmental Risks, Scientific Committee on Emerging and Newly Identified Health Risks, and Scientific Committee on Consumer Safety. 2012. *Toxicity and Assessment of Chemical Mixtures.* European Union. https://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf

Secky, Lena, Martin Svoboda, Lukas Klameth, Erika Bajna, Gerhard Hamilton, Robert Zeillinger, Walter Jäger, and Theresia Thalhammer. 2013. "The Sulfatase Pathway for Estrogen Formation: Targets for the Treatment and Diagnosis of Hormone-Associated Tumors." *Journal of Drug Delivery.*

Setchell, Kenneth D. R., Nadine M. Brown, Pankaj Desai, Linda Zimmer-Nechemias, Brian E. Wolfe, Wayne T. Brashear, Abby S. Kirschner, Aedin Cassidy, and James E. Heubi. 2001. "Bioavailability of Nutrients and Other Bioactive Components from Dietary Supplements." *American Society for Nutritional Sciences.* 1362S-1375S.

Shenoy, Niraj, Mary Stenson, Joshua Lawson, Jithma Abeykoon, Mrinal Patnaik, Xiaosheng Wu, and Thomas Witzig. 2017. "Drugs with anti-oxidant properties can interfere with cell viability measurements by assays that rely on the reducing property of viable cells." *Laboratory Investigation* 494–497.

Skrzydlewski, Pawel, Magdalena Twaruzek, and Jan Grajewski. 2022. "Cytotoxicity of Mycotoxins and Their Combinations on Different Cell Lines: A Review." *Toxins* 244.

Srinivasan, Supriya, and Zafar Nawaz. 2009. “Molecular Biology of Estrogen Receptor Action.” *Hormones, Brain and Behavior* 1187-1220.

Statista. 2022. *Leading soybean producing countries worldwide from 2012/13 to 2021/22.* 11 February. Accessed February 17, 2022. https://www.statista.com/statistics/263926/soybean-production-in-selected-countries-since-1980/.

Stork, Philip J. S., and John M. Schmitt. 2002. "Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation." *Trends in Cell Biology* 258-266.

Stowell, Cheri, Kevin K. Barvian, Peter C.M. Young, Robert M.Bigsby, Dawn E. Verdugo, Carolyn R. Bertozzi, and Theodore S. Widlanski. 2006. "A Role for Sulfation-Desulfation in the Uptake of Bisphenol A into Breast Tumor Cells." *Chemistry & Biology* 891–897.

Ström, Anders, Johan Hartman, James S. Foster, Silke Kietz, Jay Wimalasena, and Jan-Åke Gustafsson. 2004. *PNAS.*

Su, Yang, Yuchen Sun, Daxin Ju, Siying Chang, Baoming Shi, and Anshan Shan. 2018. "The detoxification effect of vitamin C on zearalenone toxicity in piglets." *Ecotoxicology and Environmental Safety* 284–292.

Tchoumtchoua, Job, Maria Makropoulou, Sylvain Benjamin Ateba, Athina Boulaka, Maria Halabaki, George Lambrinidis, Aggeliki K. Meligova, et al. 2016. "Estrogenic activity of isoflavonoids from the stem bark of the tropical tree *Amphimas pterocarpoides*, a source of traditional medicine." *Journal of Steroid Biochemistry and Molecular Biology* 138-148.

Teoh, Eng Soon. 2015. "Secondary Metabolites of Plants." *Medicinal Orchids of Asia* 59–73.

Thermo Fischer Scientific. 2022. *Gibco™ Penicillin-Streptomycin.* Accessed Juli 28, 2022. https://www.thermofisher.com/order/catalog/product/15070063.

Thompson, Melanie J. 2010. *Title: Isoflavones: Biosynthesis, Occurrence and Health Effects.* Hauppauge, N.Y: Nova Biomedical Books.

Tsuchiya, Yuki, Miki Nakajima, Satoru Kyo, Taro Kanaya, Masaki Inoue, and Tsuyoshi Yokoi Human CYP1B1 Is Regulated by Estradiol via Estrogen Receptor. 2004. "Human CYP1B1 Is Regulated by Estradiol via Estrogen Receptor." *Cancer Research* 3119–3125.

Uifalean, Alina, Stefanie Schneider, Corina Ionescu, Michael Lalk, and Christina Adela Iuga. 2016. "Soy Isoflavones and Breast Cancer Cell Lines: Molecular Mechanisms and Future Perspectives." *Molecules* 13.

US EPA. 2003. *Framework for Cumulative Risk Assessment.* Washington, D.C. : US EPA.

Vejdovszky, Katharina, Kathrin Hahn, Dominik Braun, Benedikt Warth, and Doris Marko. 2017. "Synergistic estrogenic effects of Fusarium and Alternaria mycotoxins *in vitro*." *Archives of Toxicology* 1447–1460.

Vejdovszky, Katharina, Verena Schmidt, Benedikt Warth, and Doris Marko. 2017. "Combinatory estrogenic effects between the isoflavone genistein and the mycotoxins zearalenone and alternariol *in vitro*." *Molecular Nutrition & Food Research* 1600526.

Vendl, Oliver, Colin Crews, Susan Macdonald, Rudolf Krska, and Franz Berthiller. 2010. "Occurrence of free and conjugated Fusarium mycotoxins in cereal based food." *Food Additives and Contaminants* 1148-1152.

Vichai, Vanicha, and Kanyawim Kirtikara. 2006. "Sulforhodamine B colorimetric assay for cytotoxicity screening." *Nature Protocols* 1112-1116.

Wang, D. F., Niya. Y. Zhang, Y. Z. Peng, and D. S. Qi. 2010. "Interaction of zearalenone and soybean isoflavone on the development of reproductive organs, reproductive hormones and estrogen receptor expression in prepubertal gilts." *Animal Reproduction Science* 317-323.

Wang, Dingfa, Qinshan Ma, Niya Zhang, and Desheng Qi. 2010. "Genistein inhibit the proliferation induced by zearalenone in MCF-7 cells." *Molecular Cell Toxicology* 25-31.

Wang, Thomas T. Y., Neeraja Sathyamoorthy, and James Phang. 1996. "Molecular effects of genistein on estrogen receptor mediated pathways." *Carcinogenesis* 271-275.

Wang, Zhanyun, Glen W. Walker, Derek C. G. Muir, and Kakuko Nagatani-Yoshida. 2020. "Toward a Global Understanding of Chemical Pollution: A First Comprehensive Analysis of National and Regional Chemical Inventories." *Environmental Science & Technology* 2575-2584.

Welshons, Wade V., Michael F. Wolf, Catherine S. Murphy, and V. Craig Jordan. 1988. "Estrogenic activity of phenol red." *Molecular and Cellular Endocrinology* 169-178.

Yasar, Pelin, Gamze Ayaz, Sirma Damla User, Gizem Güpür, and Mesut Muyan. 2016. "Molecular mechanism of estrogen-estrogen receptor signaling." *Reproductive Medicine and Biology* 4-20.

Yu, Zengli, Dongsheng Hua, and Yin Lib. 2004. "Effects of zearalenone on mRNA expression and activity of cytochrome P450 1A1 and 1B1 in MCF-7 cells." *Ecotoxicology and Environmental Safety* 187-193.

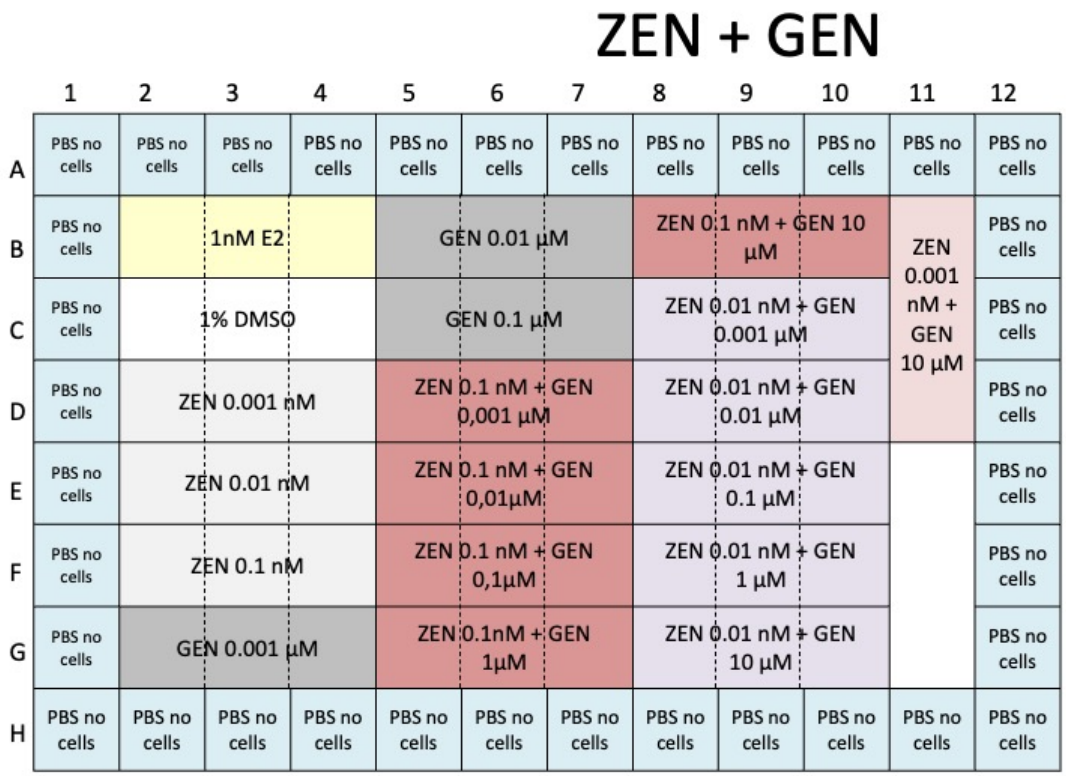
Zentek, Jürgen, and Boroojeni, Farshad Goodarzi. 2020. "(Bio)Technological processing of poultry and pig feed: Impact on the composition, digestibility, anti-nutritional factors and hygiene." *Animal Feed Science and Technology* 114576.

Zhang, Lili, Xiaoping Li, Lijun Zhao, Lifang Zhang, Guo Zhang, Jianliu Wang, and Lihui Wei. 2009. "Nongenomic effect of estrogen on the MAPK signaling pathway and calcium influx in endometrial carcinoma cells." *Cellular Biochemistry* 553-562.

List of Figures

List of Tables

Appendix



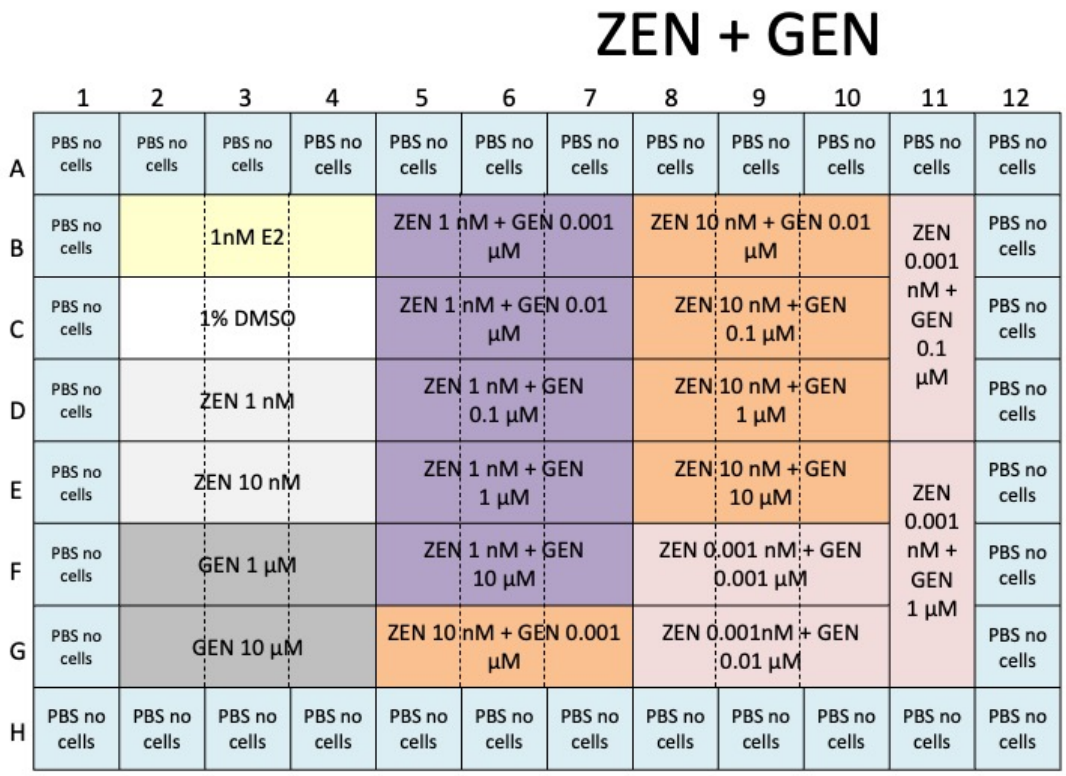


Figure 30: Schematic diagram used for incubations for cytotoxicity assays (example ZEN and GEN)

Table 8: 96-well plates used for cytotoxicity assays

Plate Number	ZEN Metabolite	Phytoestrogen	Plate Number	ZEN Metabolite	Phytoestrogen
3	ZEN	GEN	**43**	ZEN	GLY
4	ZEN	GEN	**44**	ZEN	GLY
5	α-ZEL	GEN	**45**	ZEN	GEN
6	α-ZEL	GEN	**46**	ZEN	GEN
7	ZEN	GEN	**47**	ZEN	GEN
8	ZEN	GEN	**48**	ZEN	GEN
9	ZEN	DAI	**49**	HZEN	GEN
10	ZEN	DAI	**50**	HZEN	GEN
11	ZEN	DAI	**51**	HZEN	GEN
12	ZEN	DAI	**52**	HZEN	GEN
13	ZEN	GEN	**53**	HZEN	DAI
14	ZEN	GEN	**54**	HZEN	DAI
15	ZEN	DAI	**55**	HZEN	DAI
16	ZEN	DAI	**56**	HZEN	DAI
17	ZEN	EQ	**57**	DHZEN	DAI
18	ZEN	EQl	**58**	DHZEN	DAI
19	ZEN	DAI	**59**	DHZEN	DAI
20	ZEN	DAI	**60**	DHZEN	DAI
21	ZEN	EQ	**61**	DHZEN	GEN
22	ZEN	EQ	**62**	DHZEN	GEN
23	ZEN	EQ	**63**	DHZEN	GEN
24	ZEN	EQ	**64**	DHZEN	GEN
25	ZEN	DAI	**65**	DHZEN	GLY
26	ZEN	DAI	**66**	DHZEN	GLY
27	ZEN	GEN	**67**	DHZEN	GLY
28	ZEN	GEN	**68**	DHZEN	GLY
29	ZEN	GLY	**69**	DHZEN	EQ
30	ZEN	GLY	**70**	DHZEN	EQ
31	ZEN	GEN	**71**	HZEN	EQ
32	ZEN	GEN	**72**	HZEN	Equol
33	ZEN	GLY	**73**	α-ZAL	GLY
34	ZEN	GLY	**74**	α-ZAL	GLY
35	ZEN	GLY	**75**	HZEN	EQ
36	ZEN	GLY	**76**	HZEN	EQ
37	ZEN	EQ	**77**	HZEN	EQ
38	ZEN	EQ	**78**	HZEN	EQ
39	ZEN	EQ	**79**	α-ZAL	GLY
40	ZEN	EQ	**80**	α-ZAL	GLY
41	ZEN	GLY	**81**	α-ZAL	GLY
42	ZEN	GLY	**82**	α-ZAL	GLY

Table 8: continued

Plate Number	ZEN Metabolite	Phytoestrogen	Plate Number	ZEN Metabolite	Phytoestrogen
83	DHZEN	EQ	**99**	HZEN	GLY
84	DHZEN	EQ	**100**	HZEN	GLY
85	DHZEN	EQ	**101**	HZEN	GLY
86	DHZEN	EQ	**102**	HZEN	GLY
87	DHZEN	GEN	**103**	ZEN-14-S	DAI
88	DHZEN	GEN	**104**	ZEN-14-S	DAI
89	DHZEN	GLY	**105**	ZEN-14-S	DAI
90	DHZEN	GLY	**106**	ZEN-14-S	DAI
91	HZEN	GEN	**107**	ZEN-14-S	GLY
92	HZEN	GEN	**108**	ZEN-14-S	GLY
93	HZEN	DAI	**109**	ZEN-14-S	GLY
94	HZEN	DAI	**110**	ZEN-14-S	GLY
95	α-ZAL	EQ	**111**	ZEN-14-S	EQ
96	α-ZAL	EQ	**112**	ZEN-14-S	EQ
97	α-ZAL	EQ	**113**	ZEN-14-S	EQ
98	α-ZAL	EQ	**114**	ZEN-14-S	EQ

Table 9: PCR results

	ESR1			*ESR2*		
	48 h		24 h	48 h		24 h
	Average	SD*		Average	SD*	
DMSO [1 %]	1.02606	0.03317	1.06973	1.03886	0.04501	1.06745
E2 [1 nM]	1.56248	0.35706	0.62429	2.02285	0.57243	1.66215
GEN [10 µM]	1.44705	0.17116	2.01698	0.99907	0.22977	4.24142
GEN [1 µM] ZEN [10 nM]	2.41321	1.21646	0.49070	1.21346	0.28160	1.29159
GEN [10 µM] ZEN [10 nM]	1.36133	0.82789	0.57885	2.18690	1.46362	1.19187
ZEN [10 nM]	1.42678	0.81890	0.14606	1.01451	0.20526	0.50053
ZEN [10 nM] E2 [1 nM]	0.69062	0.31028	0.47122	0.41848	0.11176	0.49751

**SD = Standard Deviation*

	CYP1A1		*CYP1B1*		
	48 h		48 h		24 h
	Average	SD*	Average	SD*	
DMSO [1 %]	1.03220	0.03375	1.06659	0.08041	1.02375
E2 [1 nM]	0.85966	0.49790	1.22805	0.39236	0.60916
GEN [10 µM]	1.02908	0.31254	0.94380	0.12510	0.34560
GEN [1 µM] ZEN [10 nM]	1.06719	0.11383	0.22542	0.07611	0.29088
GEN [10 µM] ZEN [10 nM]	1.15743	0.81747	0.94618	0.49611	0.57874
ZEN [10 nM]	0.62399	0.18464	0.69174	0.20813	0.16730
ZEN [10 nM] E2 [1 nM]			0.08104	0.00713	0.08609

**SD = Standard Deviation*

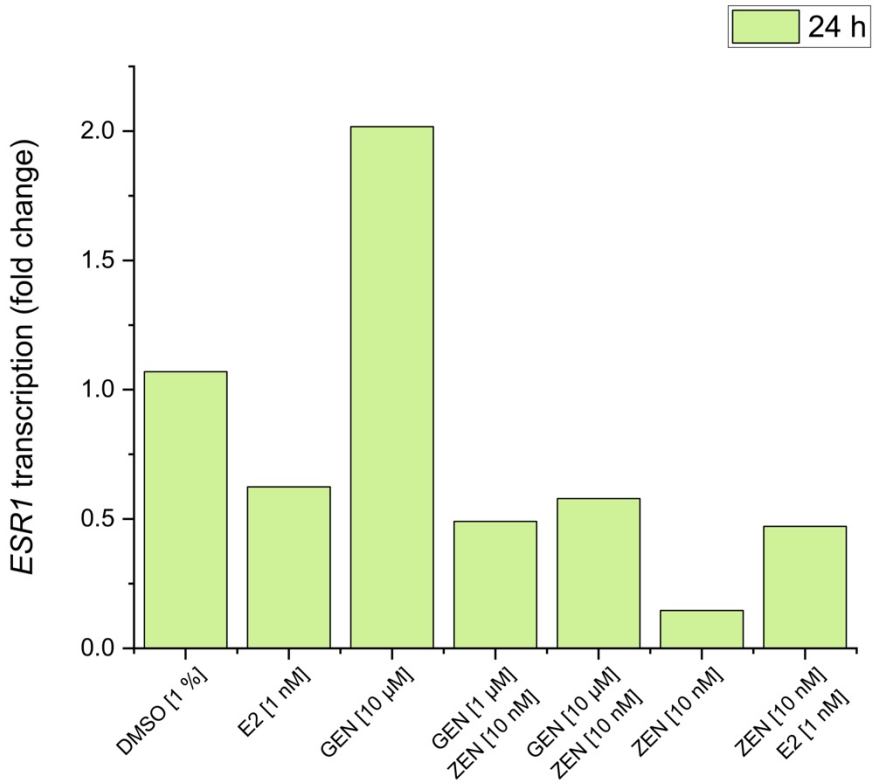


Figure 31: Single replicate of ESR1 transcription fold change upon 24 hours of incubation

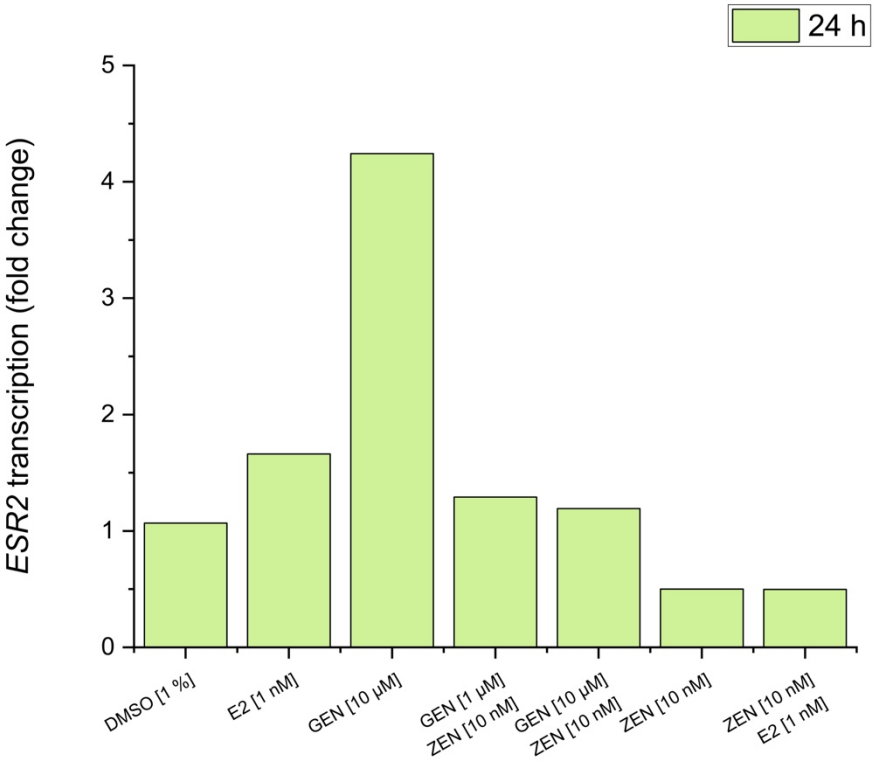


Figure 32: Single replicate of ESR2 transcription fold change upon 24 hours of incubation

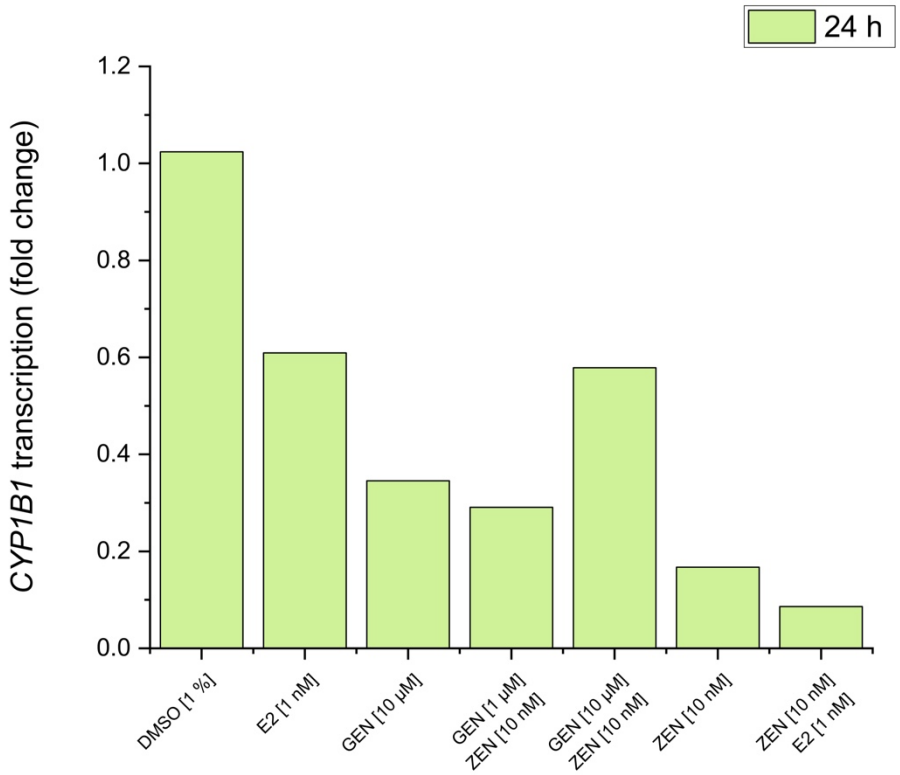


Figure 33: Single replicate of CYP1B1 transcription fold change upon 24 hours of incubation