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„The Impact of Deoxynivalenol (DON) and its Metabolites DON-3-sulfate and DON-15-sulfate on the Human Colorectal Adenocarcinoma Cell Line HT-29“

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

— Marie Curie —

physicist and chemist, first woman to win a Nobel Prize

as quoted in Curie (1937)

Acknowledgements

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Danke!

Declaration of originality

I declare hereby that this diploma thesis and the work reported herein was originated and composed entirely from me.

Furthermore I confirm that the information derived from published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

This work has not been submitted previously or concurrently - insofar as I am aware of – to any examining board.

Vienna, in May 2016 Signature _____

Wölflingseder Lydia

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Index of abbreviations

3-ADON	3-acetyl-deoxynivalenol
3'-UTR	3' untranslated region
15-ADON	15-acetyl-deoxynivalenol
AFB_1	Aflatoxin B_1
ANOVA	Analysis of variance
ATF	Activating Transcription Factor
a_w	Water activity
BAX	Bcl-2-associated X protein
BBB	Blood-brain barrier
bw	Body weight
COX-2	Cyclooxygenase-2
CSF	Cerebral spinal fluid
DAPI	4',6-diamidino-2-phenylindole
DCF	2′,7′-dichlorofluorescein
DCFH	2′,7′-dichlorofluorescin
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOGT1	Deoxynivalenol-glycosyltransferase
DON-3-Glc	Deoxynivalenol-3-glucoside
DOM-1	Deepoxy-deoxynivalenol
EGR	Early growth response
ERK	Extracellular-signal-regulated kinase
EU	European Union

FAO	Food and Agriculture Organization of the United Nations
FB_1	Fumonisin B_1
FCS	Fetal calf serum
FHB	*Fusarium* head blight
GPx	Glutathione peroxidise
GSH	Glutathione
GSSG	Glutathione disulfide
Hck	Hematopoietic cell kinase
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
ICC	Immunocytochemistry
IEA	Intermediate electron acceptor
IEC	Intestinal epithelial cells
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LC-ESI	Liquid chromatography-electrospray ionization
LD_{50}	median lethal dose/ lethal dose, 50%
Log*D*	Distribution coefficient
LPS	Lipopolysaccharide
LSD	Least significant difference
M II	Metaphase II
MAPK	Mitogen-activated protein kinase
MMP	Mitochondrial membrane potential
MPTP	Mitochondial permeability transition pore
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry

NaCl	Sodium chloride
$Na_2HPO_4 \cdot 2\ H_2O$	Disodium hydrogen phosphate dehydrate
NO	Nitric oxide
NOEL	No observed-effect level
O	Oxygen
$O_2^{\cdot -}$	Superoxide radical
OS	Oxidative stress
OTA	Ochratoxin A
PBS	Phosphate-buffered saline
PKR	Protein kinase RNA-activated
PMTDI	Provisional maximum tolerable daily intake
ppm	Parts per million
P/S	Penicillin/streptomycin
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RASFF	Rapid Alert System for Food and Feed
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of the mean
SIM	Structured illumination microscopy
SRB	Sulforhodamine B
ssp	*Species pluralis*
T-2	T-2 toxin
TCA	Trichloroacetic acid
TEER	Transepithelial electrical resistance
TLC	Thin-layer chromatography

TNF-α	Tumor necrosis factor alpha
TRIS	Tris (hydroxymethyl-) aminomethane
tRNA	Transfer ribonucleic acid
WHO	World Health Organization
WST-1	Water Soluble Tetrazolium

1. Introduction

Different moulds, mainly belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera can produce toxic compounds as secondary metabolites (European Food Safety Authority (EFSA), 2016) and accordingly, and as a consequence of their proliferation, mycotoxins can contaminate food and feed both pre-harvest or post-harvest (Whitlow and Hagler, 2002). To date, under the term "mycotoxins" it is possible to identify hundreds of different molecules, that might have been chemically characterised, but so far a comparably small proportion has been comprehensively investigated in (Whitlow and Hagler, 2002). For these reasons, the characterisation of the toxicity of mycotoxins and the underlying mechanisms sustaining their biological effects represent a very actual and vibrant field of investigation.

Although worldwide there is no precise data on the occurrence of mycotoxins in food and feed available, the Food and Agriculture Organization of the United Nations (FAO) has estimated that about 25 % of food world production is contaminated by at least one mycotoxin (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2002). In agreement, a study focusing on the occurrence in food in the European Union (EU) of *Fusarium*-produced trichothecenes, the family of mycotoxins to which belongs also Deoxynivalenol (DON), showed that 57 % out of several thousand samples resulted positive for DON (Schothorst and van Egmond, 2004). Moreover, since metabolites of certain mycotoxins also show toxicologically relevant effects on human and animal health (e.g. Frizzell *et al.* (2011) and Pizzo *et al.* (2016)) it is also important to characterise, apart from the parent compound, its derivatives.

Mycotoxins and their metabolites can enter the food chain through contaminated food and feed, and, as far as DON is concerned, especially cereals can be potentially hazardous to both human and animal health. Many clinical toxicological syndromes associated to the consumption of mycotoxin-contaminated commodities have been well characterized. Acute mortality, slowed growth, reduced reproductive efficiency, gastrointestinal disorders or also altered nutritional efficiency are only a few examples of several acute and chronic diseases identified in association with the ingestion of mycotoxin contaminated food or feed (Pestka and Smolinski, 2005; Rotter *et al.*, 1996).

In order to guarantee food quality without health hazard and environmental contaminants, it is necessary to investigate the toxicity of both parent compounds and metabolites: these information are needed for the introduction of regulatory limits, control measurements, appropriate food processing, and also for the toxicological

evaluation of the potential risk associated to the entrance of mycotoxins into the food chain.

Accordingly, after a brief introduction into the topic of mycotoxins and their production, the theoretical part of this thesis was developed to give an overview about the toxigenic fungal genera *Fusarium.* After the more general introduction, the attention will be focused on the trichothecenes, a subgroup of mycotoxins to which DON and its derivatives belong. The following chapters will progressively go further into the topics concerning DON and its derivatives, discussing their occurrence and biosynthesis and beyond that the metabolic and toxicological mechanisms of action associated with these compounds. This introduction into the theoretical background of DON and its derivatives will lead to the discussion of the aim of this thesis and the presentation of the data, obtained during the experimental work. Furthermore, the conclusion, which can be drawn, will be discussed. Finally, in the chapter “Materials and methods” details concerning the experimental work are explained.

2. Theoretical background

2.1 Mycotoxins

The idiom “myco” derives from the Greek term “mukēs” and can be translated as “fungus” (Collins English Dictionary, 2016). Accordingly, mycotoxins are toxic, low-molecular-weight natural compounds produced as secondary metabolic products by different filamentous fungi (Bennett and Klich, 2003) mainly belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera (European Food Safety Authority (EFSA), 2016).

Fungal metabolites considered as mycotoxins form a toxicologically and chemically heterogenous group, assembled together due to their capacity to cause diseases or even acute death in humans or other species of vertebrates (Bennett, 1987). As a consequence not all toxins produced by fungi are part of this group, because they are classified according to their toxicological target and their concentration. For example fungal metabolites which are toxic to bacteria are defined as antibiotics or those toxic to plants are known as phytotoxins (Bennett and Klich, 2003). Therefore mycotoxins are secondary metabolites of fungal origin that are all, already in low concentrations, toxic to vertebrates and other animal groups (Bennett, 1987).

Due to their amount and variety, mycotoxins are difficult to classify and until today a lot of different classification schemes have been created. For instance, clinicians tend to group toxins according to the target organ and thereby classes have been defined as hepatotoxins, nephrotoxins, neurotoxins or immunotoxins. Furthermore, mycotoxins can be classified into generic groups, according to the main mechanisms of toxicity, such as teratogens, mutagens, carcinogens or allergens. Other types of classification are based on their chemical structures (e.g. coumarins, lactones, etc…), on their biosynthetic origins (i. e. amino acid-derived, polyketides,…) or on the diseases they cause (i. e. stachybotryotoxicosis, St. Anthony’s fire,...). In addition to that, mycotoxins can be also classified by the fungi producing them and as an example *Aspergillus* toxins or *Penicillium* toxins can be given. None of these classification strategies is sufficient and compounds can be placed in completely diverse, cognitive groups. For instance Aflatoxins can be categorized as hepatotoxic, carcinogenic and mutagenic toxins and further as difuran-containing, polyketide-derived mycotoxins produced by the fungal genus *Aspergillus* (Bennett and Klich, 2003).

For the experimental work of this thesis the mycotoxin deoxynivalenol (DON; IUPAC name: 12, 13-epoxy-3α, 7α, 15-trihydroxytrichothec-9-en-8-on), mainly produced by *Fusarium* species and two sulfo-metabolites, namely DON-3-sulfate (DON3S) and DON-15-sulfate (DON15S) were investigated (Figure 1).

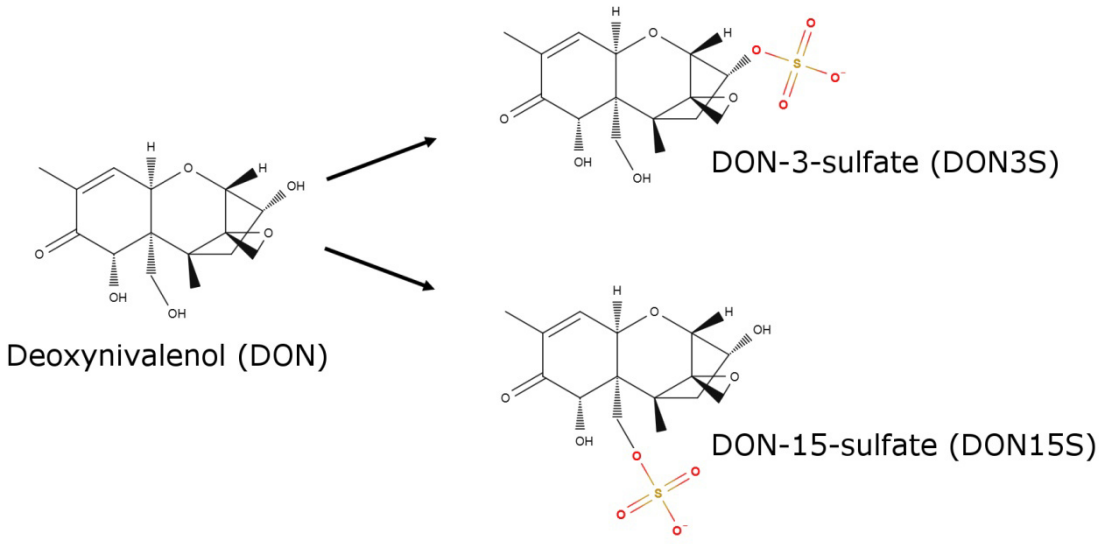

Figure 1: Structure of deoxynivalenol (DON) and its metabolites DON-3-sulfate and DON-15-sulfate

2.1.1 Mycotoxins as food contaminants

As already mentioned above, mycotoxins have a lot of different ways of action and "carcinogenic", "teratogenic", "nephrotoxic" and "mutagenic" are only a few terms describing their biological activities and give an idea why their presence in the food chain is a potential hazard to food safety and further to human and animal health (Oueslati *et al.*, 2014). Even though mycotoxins can be produced by various fungal genera, the most relevant groups of mycotoxins and the respective producing organism are shown in Table 1.

Due to the fungal contamination of crops, mycotoxins can easily enter the food chain and be ingested by human beings either directly and/or through products like milk, meat or eggs. In fact, if contaminated feed is used, mycotoxins can be accumulated in the organs and tissues of livestock (Capcarova *et al.*, 2016). Furthermore, mycotoxin-contaminated feed is reducing its conversion to animal protein and can have negative effects on animal health and fertility, also related to conspicuous economic losses (Bhat and Miller, 1991; Chowdhury and Smith, 2005). Some important factors

influencing fungal growth, mycotoxin production and toxin contamination of food and feed and in addition, regulatory and different control strategies concerning these toxic colonisations will be presented in the following paragraphs.

Table 1: Overview of most relevant mycotoxin groups found in food and feed (Marin et al., 2013)

Mycotoxins	Producing fungal genera
Aflatoxins	*Aspergillus*
Ochratoxins	*Aspergillus* and *Penicillium*
Trichothecenes, **zearalenone,** **fumonisins,** **emerging mycotoxins** (fusaproliferin, moniliformin, beauvericin, ennatins)	mainly by *Fusarium* species
Ergot alkaloids	*Claviceps*
Altenuene, **alternariol,** **alternariol methyl ether,** **altertoxin,** **tenuazonic acid**	*Alternaria*

2.1.1.1 Mycotoxins in food and feed - regulatory and control strategies

Containment of mycotoxin levels in food and feed would not only lead to lower costs in healthcare, but also reduce economic losses caused by contaminated livestock, forage crops and feeds and also regulatory costs and research grants (Zain, 2011). Inadequate agricultural practice, improper storage conditions and adverse environmental conditions are all known to have an impact on mould and mycotoxin contamination of crops. Regarding the environment, extreme conditions like drought, but also an increase in temperature or in relative humidity can affect and extend the fungal colonisation of crops and their toxigenic metabolism (Russell *et al.*, 1991).

Beyond that, as already indicated above, good agricultural practices can help to decrease mycotoxin levels in food and feed (Figure 2). Different strategies can be adopted in this respect, according to the organism and/or toxin involved. For instance, in some cases early harvesting can reduce pre-harvest the fungal colonization of crops and therefore also the mycotoxin levels of the deriving products (Zain, 2011). In agreement, Rachaputi *et al.* (2002) investigated aflatoxin levels in peanuts and found

up to 27 % lower toxin concentrations after early harvest compared to delayed harvesting treatments.

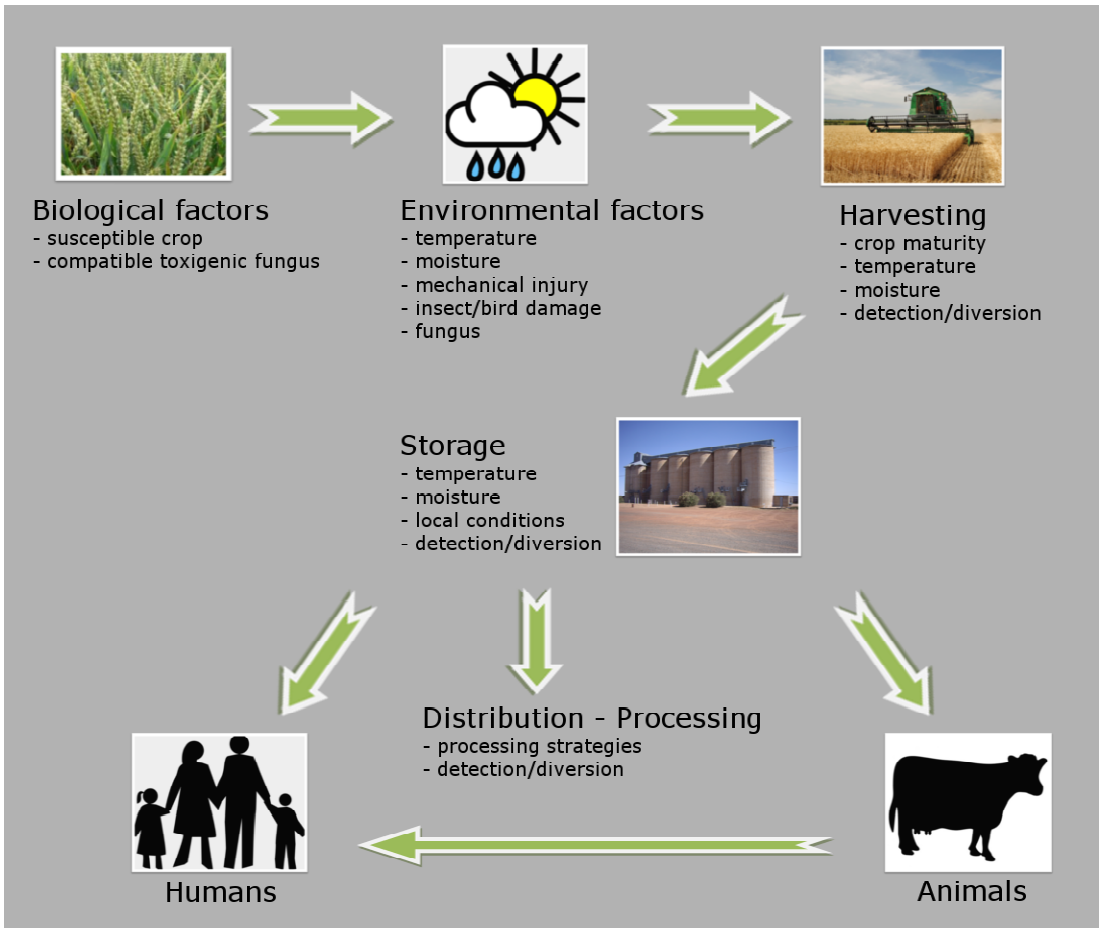

Figure 2: Factors related to the mycotoxin occurrence in the food and feed chain - taken and modified from Bryden (2012)

Regarding the drying process, in order to create less adequate conditions for mould growth and insect infestation, a quick reduction of moisture levels in agricultural products should be preferred (Awuah and Ellis, 2002). Harvested maize dried within 24 to 48 h can contain up to 15 % or even lower moisture content and this reduces the risk of *Aspergillus* growth and mycotoxin production (Lanyasunya *et al.*, 2005).

Physical, post-harvest treatment strategies aimed to remove significantly mycotoxin contaminations were reported by Park (2002). It was shown that physical treatments as sorting, winnowing, washing and crushing combined with de-hulling can be used to remove effectively mycotoxins to acceptable contamination levels. These decontamination techniques are based on the separation of affected seeds from the rest (Zain, 2011) and are conducted to inactivate, destroy, or remove mycotoxins.

Furthermore, during and after these physical treatments it needs to be guaranteed that toxic residues are not produced or left in the food and feed and that the nutritive values of the agricultural products are maintained (Park, 2002).

Insect infestation is a further component related to the extent of toxin levels in crops. Avantaggio *et al.* (2002) reported that it can be used as an early indicator of *Fusarium* mycotoxin contamination. Insect activity leads to the formation of infection wounds and enables thereby spores of mycotoxin-producing fungi to enter interior parts of the crops. So, proper management and control of insect pests, both pre- and post-harvest, could reduce development of toxigenic fungi and their following mycotoxin contaminations (Munkvold, 2003).

During the storage of dried agricultural products it's mandatory to prevent an increase of their moisture content caused by leaks or condensations due to temperature differences between the centre and the periphery of the stored crops. Furthermore, microbial and insect activity during the storage phase leads to moisture condensation and creates in localised areas possibilities for potential mould growth (Wicklow, 1995). Growing conditions for mycotoxin-producing fungi are given at 70-150 g/kg moisture, 80-85 % relative humidity and at temperatures between 0 °C to 35 °C, depending on the different fungal genera (Wicklow, 1995).

In order to reduce mycotoxin levels further processing strategies have been developed. For instance a technique using dispersive liquid-liquid microextraction to decrease quantitatively ochratoxin A (OTA) levels from raisin samples (Karami-Osboo *et al.*, 2015) or, beyond that, various bio-control strategies. For example Cleveland *et al.* (2003) established a strategy where atoxigenic bio-control fungi are grown in order to outcompete other closely related toxigenic mould species and reduce as a consequence also mycotoxin contamination in crops.

In order to show the relevance of these strategies, the following paragraphs focus on the effects on human health caused by mycotoxin exposure.

2.1.1.2 Negative effects of mycotoxins on humans – mycotoxicosis

Since not even good agricultural and processing practice can completely avoid mycotoxin contamination of food products, the human body is continuously confronted with the toxic fungal metabolites. Due to such exposure to mycotoxins, acute or chronic poisonings, known as mycotoxicosis, can occur (Peraica *et al.*, 2014). Most mycotoxicosis can be related to the consumption of contaminated food and feed, but

also direct skin contact with mould or the inhalation of spore-borne toxins are possible sources of mycotoxin poisoning (Bennett and Klich, 2003).

The different mycotoxicosis, as all toxicological syndromes, can be categorised in two groups: acute and chronic (Capcarova *et al.*, 2016). Acute poisonings are characterised by a fast inclination and a marked occurrence of the toxic effects. In comparison, chronic toxicity describes adverse health effects due to exposure, often at lower doses, over a longer time period, which can lead to irreversible effects or cancer (James, 1985). Most human and animal diseases related to mycotoxin exposure can be classified as chronic effects, for instance, kidney toxicity, carcinogenic effects or immune suppression. Nevertheless, among the acute cases of intoxications there are well-known, historical incidences, which were connected directly to the toxicity of mycotoxins, for example an outbreak of gastrointestinal disorders in 1987 in India affecting several thousands of people (Bhat *et al.*, 1989) or in 1935 in the United States of leukoencephalomalacia due to *Fusarium*-contaminated feed causing the deaths of around 5 000 horses (Sheldon, 1904).

Theoretically, a disease can be defined as a mycotoxicoses only if a dose-response relationship between the mycotoxins and the caused disease is given. Therefore, for human population epidemiological data needs to be evaluated, but also animal models can generate supportive data for the identification of potential human mycotoxicoses, especially if reproducible symptoms are evoked by similar mycotoxin exposures (Hsieh, 1988).

The most relevant mycotoxicoses and the commodities in which the toxins frequently occur are shown in Table 2. Aflatoxins, for example, depending on the dose and duration of exposure, can cause a variety of toxic effects including hepatotoxicity and carcinogenicity (Lewis *et al.*, 2005). In severe cases, acute aflatoxicosis can induce a toxic hepatitis resulting in jaundice and leading ultimately to death (Zain, 2011). Lewis *et al.* (2005) reported one of the largest aflatoxicosis outbreaks ever documented, which took place in rural Kenya in April 2004 and lead to 317 cases and 125 deaths. Chronic dietary exposure to aflatoxins has been associated with the occurrence of human hepatocellular carcinomas, especially in areas where hepatitis B infections can be found (Lewis *et al.*, 2005). Among the mycotoxins included in Table 2, aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1) and ochratoxin A are considered the more toxic compounds to mammalian organisms. In addition, the International Agency for Research on Cancer (IARC) classified AFB_1 as a Class I human carcinogen and FB_1 and OTA as Class 2B,

thus probable human carcinogens (International Agency for Research on Cancer, 1993).

Table 2: The most relevant food contaminating mycotoxins, the major fungal species which produce them, the products frequently colonized and the human diseases they may cause – taken and modified from Reddy *et al.* (2010)

Mycotoxin	Producing fungal species	Food commodity	Mycotoxicosis
Aflatoxins	*A. flavus,* *A. parasiticus*	– Maize, – wheat, – rice, – sorghum, – ground nuts, – tree nuts, – figs,...	– Liver lesions, – cirrhosis, – primary hepatocellular carcinoma, – Kwashiorkor, – Reye's syndrome...
Fumonisins	*F. verticillioides,* *F. proliferatum*	– Maize, – maize products, – sorghum,...	– Esophageal carcinoma...
Ochratoxin A	*A. ochraceus,* *P. verrucosum,* *A. carbonarius*	– Cereals, – dried fruit, – wine – coffee,...	– Endemic nephropathy, – urothelial tumours,...
Deoxynivalenol	*F. graminearum,* *F. culmorum*	– Cereals, – cereal products,...	– Nausea, – vomiting, – abdominal pain, – diarrhea, – dizziness, – headache,...
Zearalenone	*F. graminearum,* *F. culmorum*	– Cereals, – cereal products,...	– Premature puberty in girls, – cervical cancer,...
Patulin	*P. expansum*	– Apples, – apple juice,...	Damage of – gastrointestinal, – respiratory systems, – DNA, – many enzymes,...

High and recurrent mycotoxin exposure is of particular concern in rural communities of developing countries, where less attention is given to regulatory limits and also good agricultural practice and proper storage could be problematic. Additionally, mycotoxin contaminations are a problem in developed countries as well (Bennett and Klich, 2003). This can be deducted for example from the annual report of the Rapid Alert System for Food and Feed (RASFF), since e. g. in 2014 mycotoxins were the main hazard in border rejection notifications in the European Union (European Commission, 2014).

Accordingly, to effectively protect humans and animals against various mycotoxicosis, detailed toxicological characterisation of the compounds and the development of appropriate analysis methods is inevitable and essential.

Since, during this thesis, it was tried to investigate different toxic effects of DON, a mycotoxin primarily produced by *Fusarium* genera, and its sulfated derivatives, the following chapter should highlight details concerning primarily mycotoxins formed by various *Fusarium* species.

2.2 *Fusarium* mycotoxins

2.2.1 Introduction

Species belonging to the fungal genus *Fusarium* are mycotoxin producing fungi, which can be classified as so called "field fungi". In contrast to storage fungi, such as *Aspergillus* and *Penicillium* species, which are frequently contaminating crops post-harvest during storage, field fungi are commonly related to pre-harvest contaminations of crops (Geraldo *et al.*, 2006).

2.2.2 Occurrence and growth conditions

In general, *Fusarium* colonisations occur worldwide in diverse habitats and to date more than 70 species of the genus *Fusarium* are identified (Bhatnagar *et al.*, 2006). For proliferation and toxin production *Fusarium* fungi require lower temperatures in comparison to several other species, such as *Aspergillus* species (Placinta *et al.*, 1999). In this respect, Hope and co-workers studied and monitored for 40 days the optimum growth conditions of two toxigenic *Fusarium* species (*F. culmorum, F. graminearum*). They analysed the contamination of layers of wheat and identified at 25 °C for *F. culmorum* a maximum growth at a water activity (a_w) of 0,98 and for *F. graminearum* at 0,99 a_w. Below an a_w level of 0,90 no mycelia growth could be observed. Furthermore, DON production of these fungal species was monitored and the toxin production occurred between of 0,995 and 0,96 a_w at 15-25 °C (Hope *et al.*, 2005). Due to these differences their geographical distribution is influenced by climate conditions. *Fusarium culmorum* is commonly found in Europe, whereas *Fusarium graminearum* occurs in Europe as well as North America (Ovando-Martínez *et al.*, 2013; Wegulo, 2012).

2.2.3 Fusarium toxins and their mycotoxicosis

Fusarium fungi can produce a variety of mycotoxins, but Trichothecenes (e. g. DON and T-2 toxin (T-2)), Zearalenone and Fumonisin B_1 are the ones considered as most

toxicologically relevant (Tima *et al.*, 2016). Mycotoxins produced by *Fusarium* species can cause both acute and chronic toxic effects in humans and in farm animals. A case of acute intoxication was described for instance in pigs after exposure to high concentrations of T-2 and it was characterised by multiple haemorrhages on the serous membrane of the liver and along the intestinal tract (Weaver *et al.*, 1978). Various other symptoms caused by acute intoxication of broiler chickens included internal haemorrhage, mouth and skin lesions, neural disorders and negative effects on the feather quality (Sokolovic *et al.*, 2008). Nowadays, due to modern agricultural practice, these high contamination levels are not common and research focus mainly on the investigation of chronic toxic effects.

In case of regular consumption of low to moderate quantities of *Fusarium* mycotoxins through contaminated food, the gastro-intestinal tract and its epithelial cell layer serves as the first barrier against the toxin. In this respect, any effect of the mycotoxins on the "barrier function" of the intestine can be particularly dangerous. Bouhet *et al.* (2004) for instance reported, by measuring the transepithelial electrical resistance (TEER), that FB_1 decreases the TEER of a porcine intestinal epithelial cell line (IPEC-1) in a time- and dose-dependent manner.

Furthermore *Fusarium* mycotoxins can influence the amount of goblet cells and their mucus production in different ways (Obremski *et al.*, 2005; Obremski *et al.*, 2008) and in general the viability and proliferation activity of intestinal epithelial cells (IECs) (Bouhet *et al.*, 2004). The effects of *Fusarium* mycotoxins at intestinal level are summarized in Figure 3.

Moreover, the protective function of the gastrointestinal tract is ensured by components of both innate and adaptive immunity (Bouhet and Oswald, 2005) and can as well be affected by *Fusarium* mycotoxins (Figure 3).If *Fusarium* mycotoxins, crossed already the IEC layer, influence the innate and adaptive immunity different toxic effects are reported. It can be seen, for example, in an altered function of macrophages and neutrophils or also in a decreased T- and B-lymphocyte activity and antibody production (Bondy and Pestka, 2000; Gerberick and Sorenson, 1983; Osweiler *et al.*, 1993; Zain, 2011).

On the whole, it is clear how various negative effects on human and animal health and especially on the intestinal function and immune system can be caused by the regular intake of *Fusarium* toxin contaminated food and feed. Moreover, in addition to the above mentioned direct effects of *Fusarium* mycotoxins at intestinal level, the toxins

can favour the onset of other pathologic conditions: these changes of the intestinal tract, triggered by *Fusarium* mycotoxins may result in an increased human and animal susceptibility to pathogens and further in a decrease of host resistance to various infections or other food-borne contaminants (Antonissen *et al.*, 2014).

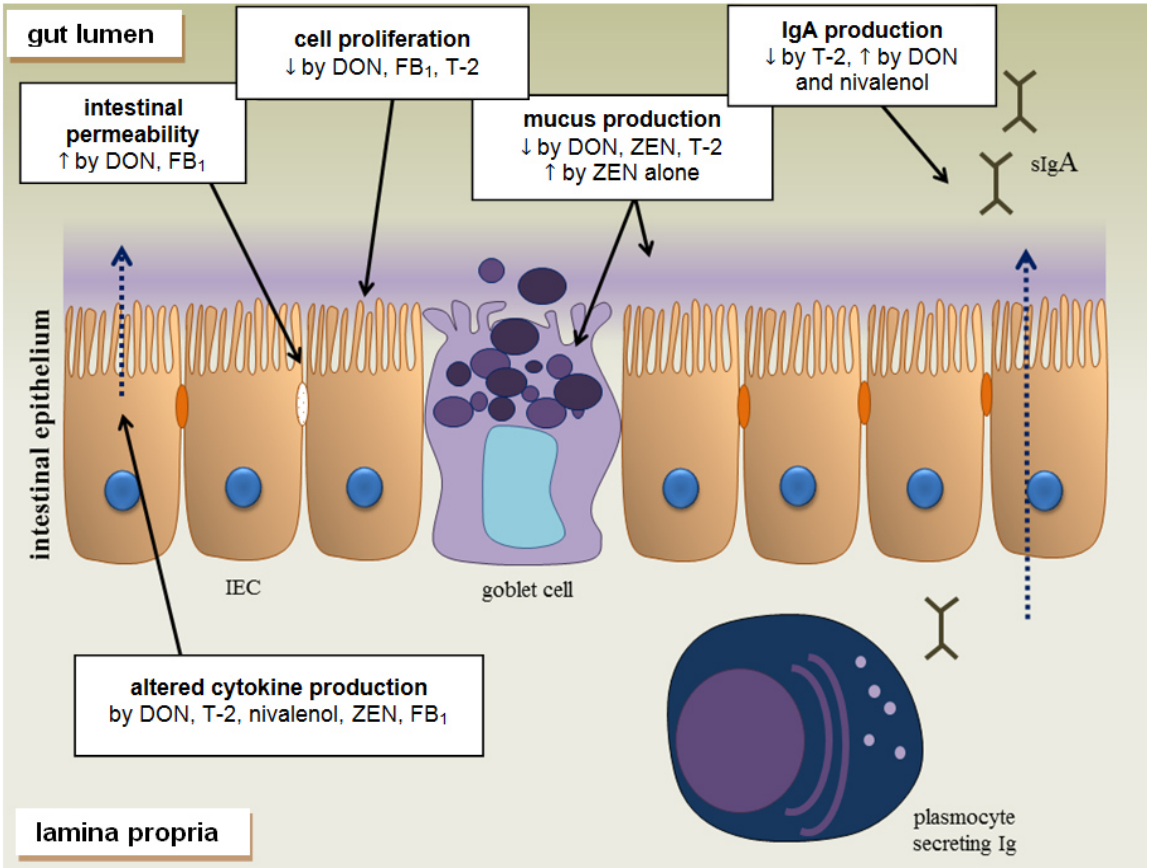

Figure 3: Schematic oveview of the toxic effects on the intestinal epithelium caused by *Fusarium* mycotoxins

– taken and modified from Antonissen et al. (2014)

Since to date hundreds of different mycotoxins, representing numerous mechanisms of action, have already been identified, for the toxicological characterisation of a new compound, it is necessary to know in detail chemically related molecules. Therefore in the following chapter a class of mycotoxins, the so called trichothecenes, including DON and its derivatives, will be described.

2.2.4 Fusarium metabolites: Trichothecene mycotoxins

2.2.4.1 Introduction

The trichothecenes mycotoxins form a subgroup of the secondary metabolites produced by *Fusarium* spp, which includes over 200 compounds (Grovey, 2007). Structurally, these molecules share the same 12, 13-epoxytrichothec-9-ene cyclic structure (Figure 4) and the term “trichothecene” derives from the name of one of the first identified compounds of this class, the so called “trichothecin” (Bennett and Klich, 2003). The conditions for the formation of trichothecene mycotoxins include temperatures between 6-24 °C and high humidity, and for these reasons they are found in North and South America, Europe, Asia, Australia and Africa (Quiroga *et al.*, 1995).

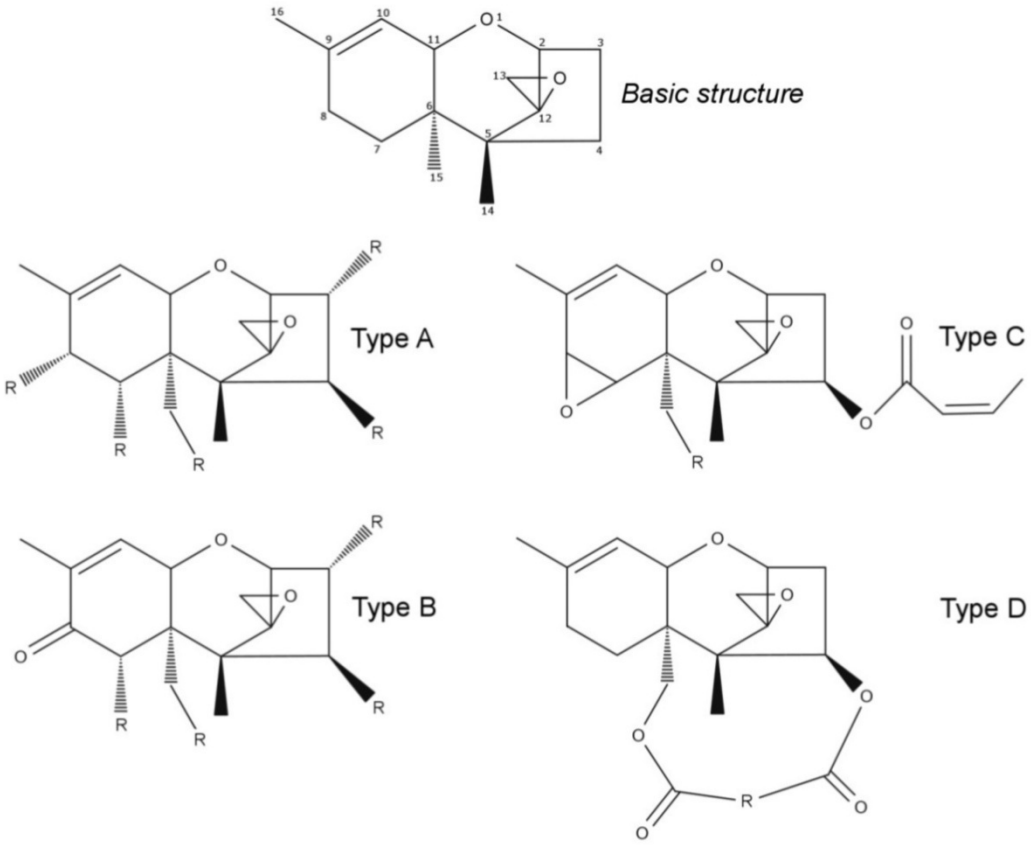

Figure 4: Basic classification of trichothecenes mycotoxins according to their chemical structure

Trichothecenes can be divided into four different categories (Type A, B, C and D) according to various substitution patterns. The only structural differences between Type A, B and C can be found in the substituent at C-8. Compounds belonging to the

A-type are characterised by a hydroxyl, an ester function (e.g. neosolaniol or T-2 toxin, respectively) or the absence of an oxygen substitution (e.g. trichodermin, harzianum A). DON, nivalenol or trichotecin, for instance, are classified as Type B trichothecenes and possess in C-8 position a carbonyl function. Type C compounds are identified by an epoxide group in positions C-7 and C-8 (e.g. crotocin), whereas in Type D trichothecenes, such as roridin A, an additional cyclic system, linking the C-4 to the C-15, can be found (Marasas *et al.*, 1984; McCormick *et al.*, 2011).

Chemically, the trichothecene mycotoxins are relatively stable against heat treatment and cannot be eliminated by usual food processing techniques. Furthermore, trichothecenes are stable at neutral and acidic pH conditions (Eriksen, 2003) and are able, due to their small, amphiphilic structure to pass cell membranes. At the cellular level ingested compounds affect highly proliferating tissues (Wannemacher and Winer, 1977), for instance those lining the gastrointestinal system, lymphoid and erythroid cells or also the skin (Zain, 2011).

2.2.4.2 Occurrence and production conditions of trichothecene mycotoxins

Different genera of fungi are able to produce trichothecene mycotoxins, including *Myrothecium*, *Spicellum*, *Stachybotrys*, *Trichoderma*, *Trichothecium* and others (Cole and Cox, 1981). However, most trichothecenes have been identified as metabolites of *Fusarium* species (Zain, 2011) and are considered important plant pathogens (Desjardins, 2006). For optimal growth of these fungi substrates with a high disposability of moisture and nutrients are needed and these are among the reasons why, for instance, *Myrothecium* species colonise muskmelon or tomato (Kuti *et al.*, 1989) and *Trichothecium* fungi can be found in the soil or decomposing organic material (Pandey *et al.*, 1990).

Two species of trichothecene producing *Fusarium* fungi, namely *Fusarium graminearum* and *Fusarium culmorum* can lead in small grain cereals to one of the most common damaging diseases, the so called "*Fusarium* head blight" (FHB). FHB causes accumulations of trichothecene mycotoxins, for instance of DON, in the colonised grain. This event, but even less severe contaminations, may lead to the entrance of toxins into the food chain, and consequently to a potential food safety risk for human and animal health (Bin-Umer *et al.*, 2014; McCormick *et al.*, 2013).

2.2.4.3 Mechanisms of action and toxicity of trichothecene mycotoxins

Regarding the mechanisms of action of trichothecenes several studies showed that these mycotoxins, inhibit the formation of peptide bonds by interfering with the peptidyl transferase center of the ribosomal subunit 60S (Wei *et al.*, 1974), In fact, thrichothecene mycotoxins are strong inhibitors of eukaryotic protein synthesis. For this mechanism of toxicity the moiety of the molecules that contains the 12,13-epoxide group is crucial; in addition, different compounds can affect the different stages of initiation, elongation and termination of the polypeptide chain (Cundliffe and Davies, 1977). A reduction of the double bond at the C-9 and C-10 weakens the reactivity of the toxins (Ehrlich and Daigle, 1987).

Further ways of action of trichothecene mycotoxins include the inhibition of the mitochondrial protein synthesis (Pace *et al.*, 1988) and their interaction with protein sulfhydryl groups (Ueno and Matsumoto, 1975). In addition the toxicity of trichothecenes is coupled to cellular oxidative stress since these mycotoxins are able to increase the levels of free radicals, including reactive oxygen species (ROS). An increased ROS level is an initiator of the process of lipid peroxidation, which leads to changes in phospholipids, membrane fluidity and the cellular antioxidant status (Krishnaswamy *et al.*, 2010) and causes furthermore DNA damage by propagating chain reactions (Yang *et al.*, 2014).

Exposure to low concentrations of trichothecene mycotoxins can result in disorders of the gastrointestinal tract such as vomiting, diarrhea or gastroenteritis. In addition, severe damage of the intestinal epithelial cells and the gastric mucosa have been reported as consequences of trichothecene intake at higher concentrations. In the most severe cases these events can lead to hemorrhages, endotoxemia or even shock-like death (Pestka, 2010b). Further toxic effects of these fungal metabolites include growth retardation, reduced bone marrow and immune dysfunction and their toxic action was also associated with the Kashin-Beck disease, which is a chronic, endemic, age-related osteochondropathy (Li *et al.*, 2014) and the alimentary toxic aleukia (Lutsky and Mor, 1981).

As trichothecenes are one of the major contaminants of cereals and grains and their occurrence causes on a global scale not only significant economic losses, but presents also a potential health hazard to humans and animals, investigation of their pathogenicity, their toxicological activity and their metabolism in the organism is of crucial importance. DON is one of the most prevalent and, as such, one of the

trichothecenes that potentially has the highest economic repercussions. Since DON can be considered the parent compound of the sulfated metabolites DON3S and DON15S investigated in this thesis, comprehensive knowledge about its mechanisms of action and toxicology is crucial. Accordingly, the following chapter will try to summarise the most relevant facts about DON's toxicological profile.

2.2.5 Important trichothecene mycotoxins: DON and its derivatives

2.2.5.1 Introduction

DON, a trichothecene of the Type B group, is mainly produced, as already mentioned above, by *Fusarium* species, such as *Fusarium graminearum* or *Fusarium culmorum* (Mirocha *et al.*, 1994) and was first isolated as the so called "Rd-toxin" by a Japanese group (Morooka *et al.*, 1972) . Due to its strong toxic effects on the gastrointestinal tracts, resulting in nausea, vomiting and diarrhea (Forsyth *et al.*, 1977), DON is also known as vomitoxin (Vesonder *et al.*, 1973).

In 2001 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg body weight (bw) based on a no observed-effect level (NOEL) of 100 µg/kg bw per day for decreased body weight gain observed in a feeding study conducted for two years in mice (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2002).

As explained in more details in 2.2.5.2, DON is synthesised together with its acetyl derivatives (Figure 5), namely the 3-acetyl-deoxynivalenol (3-ADON) and the 15-acetly-deoxynivalenol (15-ADON) (Garvey *et al.*, 2009).

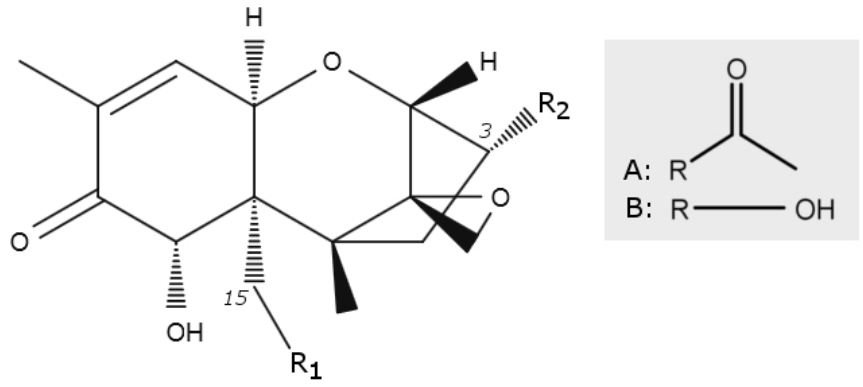

Figure 5: Chemical structure of 3-acetyl-deoxynivalenol (3-ADON; R_1: B and R_2: A) and 15-acetyl deoxynivalenol (15-ADON; R_1: A and R_2: B)

These acetylated DON metabolites co-occurred with their parent compound in concentrations up to 20 % of the identified DON concentrations. The highest contamination levels among wheat, barley, rye or corn were found in the latter ones (European Commission, 2003).

Beyond that, another DON derivative, deoxynivalenol-3-glucoside (DON-3-Glc, Figure 6), was found in several cereal crops. The latter seems to be a metabolic product of a detoxification mechanism, in which the plant glucosylates the parent compound DON. Since one or more glucose molecules coupled to DON reduce the compounds toxicity, substances like the DON-3-Glc are also known as so called modified mycotoxins. According to this it was shown that the inhibition of protein biosynthesis caused by DON-3-Glc was less compared to the inhibition activity of the DON (Poppenberger *et al.*, 2003).

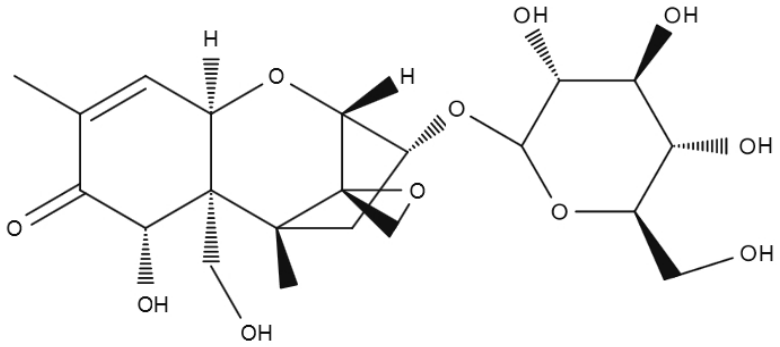

Figure 6: Chemical structure of DON-3-β-D-glucopyranoside (DON-3-Glc)

Ten years after the preceding report, in 2011, the JECFA evaluated several recent studies about DON and beyond that also about its acetylated derivatives comparing their metabolism, their toxicity profile, in terms of acute toxicity, genotoxicity and in general their toxicokinetics. Conclusion of these evaluations, concerning the risk assessment of these compounds, was that the toxicity of the acetylated derivatives should be considered equal to that of DON. JECFA drew its conclusion on the basis of: *i)* studies on the acute lethality of 3-ADON and 15-ADON in mice; *ii) in vivo* data which showed conversion of the acetylated derivatives to DON. As a consequence, the Committee extended the PMTDI of 1 µg/kg bw and included the acetyl congeners of DON into the regulations. Furthermore, an acute reference dose (ARfD) of 8 µg/kg bw was established by the JECFA for both DON and its acetyl metabolites (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2011).

DON is a structurally polar organic molecule with three free hydroxyl groups at the C-3, C-7 and the C-15 position. Further physical and chemical properties of this compound are summarised in Table 3. Among these, the thermal stability of DON severely affects their occurrence in food and feed. Several studies have reported that DON does barely degrade at high temperatures and that it is stable under normal food processing conditions as for example during the sterilization process at 120 °C (Hughes *et al.*, 1999; Wolf and Bullerman, 1998). Wolf and Bullerman (1998) correlated the dependence of DON's stability in aqueous environment on the temperature, the pH and time. DON did not show any degradation at 100 or 120 °C at a pH 4 and was only partially destructed at higher temperatures (170 °C) after one hour. Higher pH values (pH 7) affected the heat stability of DON only at 170 °C, where more destruction of the compound was found after 15 minutes and a further increase of pH conditions (pH 10) lead to partial degradation of DON at 100 °C and total destruction at 120 °C after half an hour.

Table 3: Physical and chemical properties of DON (National Center for Biotechnology Information. PubChem Compound Database, 2016)

Property	Data
IUPAC name	12, 13-epoxy-3α, 7α, 15-trihydroxytrichothec-9-en-8on
Molecular formula	$C_{15}H_{20}O_6$
Molecular weight	296, 32 g/mol
Colour and physical state	Colourless fine needles
Melting point	151-153 °C
Solubility	in water: 5,5 x 10^4 mg/L at 25 °C and soluble in polar organic solvents (e.g., aqueous methanol, ethanol, chloroform, acetonitrile, and ethyl acetate)

2.2.5.2 Biosynthesis of DON and its derivatives in Fusarium genera

The biosynthesis of *Fusarium* produced DON, schematically illustrated in Figure 7, starts with a cyclisation of farnesyl pyrophosphate molecules forming the compound trichodiene. This step is catalysed by the cyclase trichodiene synthase (Tri5) and is encoded by the gene *TRI5* (Cane *et al.*, 1985; Hohn and Beremand, 1989). Trichodiene is then oxygenised several times and so, catalysed by a cytochrome P450 monooxygenase, four O-atoms are added, one of them creating the epoxide function at the C-12 and C-13 position (McCormick *et al.*, 2006a). Isotrichotriol is the resulting compound of these preceding reaction steps, which is forming, due to a non-enzymatic isomerisation followed by another cyclisation, isotrichodermol (McCormick *et al.*, 2011).

The following step leads to a strong reduction in toxicity of the *Fusarium* produced trichothecenes and that is why it can be interpreted as a self-protecting strategy of the toxigenic organism. In this reaction isotrichodermol undergoes a conversion to isotrichodermin catalysed enzymatically by an acetyltransferase (Kimura *et al.*, 1998).

Afterwards, at the C-15 position a further hydroxyl-function is added (Alexander *et al.*, 1998), which undergoes then an acetylating reaction encoded by the *TRI3* gene (Garvey *et al.*, 2009). Two further hydroxyl groups are added at the C-7 and the C-8 position (McCormick *et al.*, 2006b), of which the latter is then transformed into a carbonyl function. As the final reactions of the DON biosynthesis an esterase removes the acetyl groups at the C-3 and C-15 position producing either the intermediate compound 3-acetyl-deoxynivalenol or 15-acetyldeoxynivalenol. Differences in the activity of this enzyme, due to corresponding variations in the DNA, determine which of the intermediate compounds is synthesised (Alexander *et al.*, 2011; Garvey *et al.*, 2009).

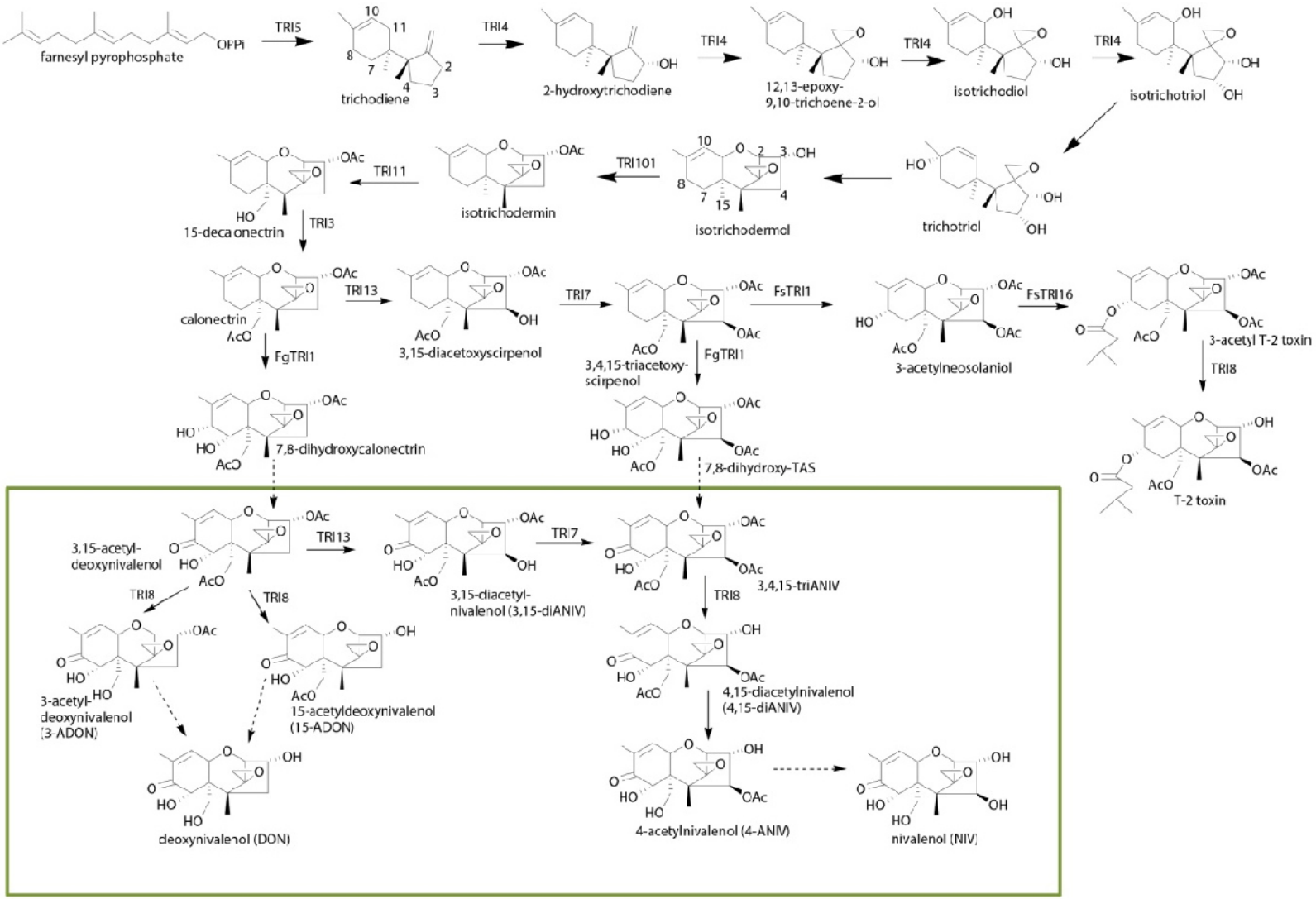

Figure 7: **Schematic overview of the proposed biosynthetic pathway in *Fusarium* species.**

(McCormick *et al.*, 2011)

2.2.5.3 Occurrence of DON and its derivatives

The trichothecene mycotoxin DON has been studied over decades and therefore it is probably not only one of the best known but also most frequent contaminants of grains and their depending products. Since the extent of *Fusarium* infections on crops depends on the weather and the fungal growth is favoured by high moisture availability at the time of flowering, DON contamination is primarily found in temperate areas (European Food Safety Authority (EFSA), 2013). The occurrence data of DON gathered by the European Commission (2003) showed that a high incidence of the toxic compound can be found in wheat and wheat flour, but also in barley, oats or rye and rye flour.

The European Food Safety Authority reviewed 2013 in a new report about the occurrence and exposure of DON, its acetylated derivatives (3-ADON and 15-ADON) and of its conjugate deoxynivalenol-3-glucoside (DON-3-Glc) in food and feed in total data of 26 613 analysis. These analytical results correspond to 18 844 sample, which were collected between 2007 and 2012 by 21 member states of the EU and Norway. DON contaminations across samples of food, feed and unprocessed grains of undefined end-use were reported as 43,5 %, 75,2 % and 44,6 %, of the analysed samples, respectively. The contamination of feed was much severer in comparison to the results of foods and unprocessed grains of undefined end-use. In general, the highest toxin contamination levels, compared to the other analysed cereals, were found among the maize, wheat and oat grain samples and beyond that in the analysed derived food and feed products. Furthermore, differences between various processed cereal products could be found. For instance, wheat bran was significantly higher contaminated by DON than the other what milling products, while significantly lower toxin levels were found in processed cereals, such as bread, pasta, fine bakery products or breakfast cereals, compared to unprocessed grains and their milling products (European Food Safety Authority (EFSA), 2013).

Concerning the occurrence of 3-ADON and 15-ADON, the data of around 4 000 analysed samples showed far less frequent contaminations by these compounds than by DON and beyond that the concentrations of the contaminants were lower than those of the parent compound. In nearly all the samples analysed during the survey, the acetylated derivatives co-occurred with DON. Furthermore it was reported that the identified 3-ADON contaminations contribute to the sum of DON on average at the upper bound estimate around 13-20 % and less than 2 % at the lower bound estimate.

However, the contribution of 15-ADON was reported around 10-15 % at both the lower and upper bound estimates (European Food Safety Authority (EFSA), 2013).

A positive correlation between an increasing production of DON and its glycosylated metabolite, DON-3-Glc, was reported by Rasmussen *et al.* (2012). Due to few available data about DON-3-Glc the European Food Safety Authority (EFSA) (2013) was not able to consider this compound in the food safety assessment. Nevertheless of 177 analysed samples of unprocessed wheat and rye grain 5 % (all wheat grains) contained the modified mycotoxin DON-3-Glc and in only one of these positive samples DON did not co-occur (European Food Safety Authority (EFSA), 2013). Furthermore, DON-3-Glc was identified in both naturally contaminated barley and in the malt and beer produced from it (Lancova *et al.*, 2008).

2.2.5.4 Metabolism

As part of the evolution, plants and animals have both developed several strategies for the metabolism of xenobiotics. In general, plants have a high capacity to metabolise and detoxify exogenous compounds, such as mycotoxins, forming, for instance, glucose conjugates. Mammals are also able to release, at least partially, the ingested and metabolised compounds via the digestive tract, a route that can potentially reactivate the mycotoxins (Berthiller *et al.*, 2007).

- *Metabolism of DON and its derivatives in plants*

  In general the metabolism of xenobiotics in plants is described in three phases. During phase I, also known “transformation phase”, the compound undergoes, in order to introduce reactive groups, various reactions as for example oxidations or dealkylations. Often this metabolic phase is required to activate the molecule and enable the following step of the metabolic process such as the conjugation (Komives and Gullner, 2005). So in phase II, the so called “conjugation phase”, polar compounds, (i. e. glucose molecules) are conjugated to the products of phase I reaction. This step often serves concretely to detoxify or inactivate the metabolised toxic compound. (Schröder and Collins, 2011). During the last metabolic phase, phase III or “compartmentation phase”, the resulting metabolites are transported to vacuoles and stored or undergo further modifications and are deposited in the cellular membrane (Sandermann, 1992).

  Detoxification strategies of plants seem to be effective for DON, in fact Miller *et al.* (1983) studied the oscillation of the concentrations of different mycotoxins

including DON and 15-ADON in wheat contaminated with *Fusarium graminearum* and reported a sharp decrease of the DON levels six weeks after inoculation of the spores. Similarly, the 15-ADON levels decreased two weeks after inoculation (Miller *et al.*, 1983).

In line with the above mentioned mechanisms of detoxification, after the incubation *in vitro* of maize cell suspension cultures with DON the metabolite deoxynivalenol-3-β-D-glucoside (DON-3-Glc) was identified using NMR analysis (Sewald *et al.*, 1992). Afterwards, DON-3-Glc was also reported as natural contaminant in maize and wheat samples co-occurring with DON (Berthiller *et al.*, 2005).

Using the model plant *Arabidopsis thaliana* the enzyme responsible for the metabolism of DON was identified. In fact, the UDP-glycosyltransferase is able to produce the masked mycotoxin DON-3-Glc by catalysing the addition of glucose from UDP-glucose to the hydroxyl group at the C-3 position of DON. Due to this mechanism of action this deoxynivalenol-glycosyltransferase (DOGT1) detoxifies both DON and also its acetyl derivative 15-ADON (Poppenberger *et al.*, 2003).

Even before the identification of the sulfation as a detoxification reaction in plants, a sulfate conjugate of zearalenone was characterised in the model plant *A. thaliana* (Berthiller *et al.*, 2006). The interpretation of the sulfation as a detoxification pathway for the mycotoxin is gaining more and more strength, in fact treating the plant with DON and T-2 toxin seems to lead to an upregulation of a probable sulfotransferase gene (Warth *et al.*, 2015). This hypothesis originated after the discovery of a potential sulfotransferase gene in wheat (Schweiger *et al.*, 2013). Using a liquid chromatography-electrospray ionization (LC-ESI)-MS/MS-based “dilute and shoot” method to analyse both samples directly treated with DON and samples artificially exposed to *Fusarium graminearum* for 96 hours DON-3-sulfate and DON-15-sulfate were identified as two novel metabolites of the trichothecene mycotoxin DON in wheat (Warth *et al.*, 2015). The two compounds have been biologically characterised for the first time in the present thesis and, concomitantly, the DON-3-sulfate was also identified as a urinary metabolite in human samples (Warth *et al.*, unpublished).

- *Metabolism and metabolic products of DON and its derivatives in human and animals*

  The metabolism of DON in animals has been studied quite intensively during the last decades and recently more studies also try to focus the research on the metabolic reactions in humans. Different *in vitro* systems and methods, such as liver microsomes or faecal fermentations are used, to determine the metabolic fate of DON in the human organism (Wu *et al.*, 2014). Recently Warth *et al.* (2013) conducted also *in vivo* experiments, analysing urine samples after a naturally contaminated diet for four days of a volunteer. Generally, it can be said that the major human metabolites identified yet are conjugated products (Wu *et al.*, 2014).

  Regardless the metabolic pathways that leads to the formation of DON derivatives, these events generally result in an alteration in the polar properties of the molecule. This can be illustrated well by the different log*D* values of DON and its derivatives at various pH levels (Figure 8). DON, for instance has a log*D* of - 0,97 at a pH of 7, and for this reason it can be interpreted as a polar compound. The metabolic addition of an acetyl function, like for instance in the 3-ADON or 15-ADON, leads to a decrease in polarity (log*D* values at pH 7: - 0,35 and – 0,53, respectively), whereas a glucoside or glucuronide conjugation results in an increased polarity (log*D* value of DON-3-Glc at pH 7: - 2,74) (Maresca, 2013).

  To date, the mechanisms mediating the entrance of the trichothecene mycotoxin DON in cells has not been identified. In spite of that, it was shown in Caco-2 cells that an increase of the extracellular toxin concentration does not lead to a saturation of the entrance of the toxin into the intestinal cells, suggesting that a passive diffusion mechanism is possible (Sergent *et al.*, 2006). Taking into account that DON is a polar molecule, a property that affects strongly its ability to cross the cell membrane through lipid diffusion, a bulk phase endocytosis/pinocytosis mechanism or an unknown membrane transporter are the most probable hypothesis to describe the cellular uptake of DON (Maresca, 2013).

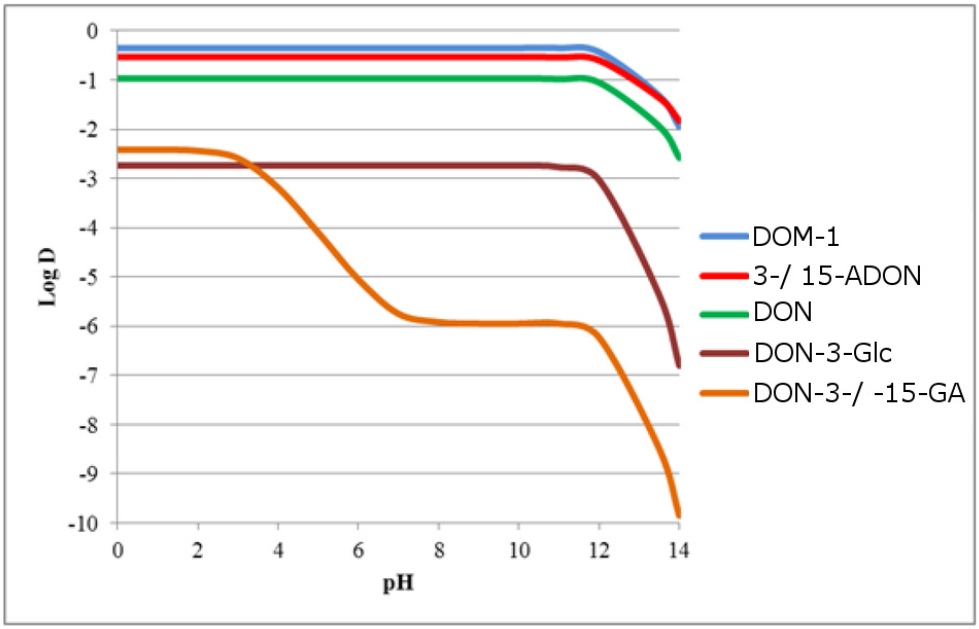

Figure 8: Log*D* values of DON and its metabolites at different pH values - taken and modified from Maresca (2013)

Moreover, when considering the intestinal absorption of DON and its derivatives, also the contribution of gut microbiota needs to be taken into account. In fact, resident microorganisms in the gastrointestinal tract affect strongly not only the maturation of the intestinal and immune system of the host, but plays also an important role in nutrition, metabolism and excretion of mycotoxins (Karlovsky, 2011; Walter and Ley, 2011). According to the localisation of the microbiota in the digestive tract, the hosts can be classified into those having high bacterial content only in their colon, thus after their small intestine (e. g. monogastric species such as humans or pigs) and into species having bacteria before and after the small intestine, e. g. polygastric animals or birds (Maresca, 2013). These differences in the localisation of the microorganisms influences the sensitivity of the hosts to oral administration of DON, since gut microbiota are able to detoxify DON through the formation of a less toxic de-epoxide metabolite (Figure 9), also known as 9,12-diene DON or DOM-1 (Eriksen *et al.*, 2004; Worrell *et al.*, 1989). High bacterial content before the small intestine guarantees the transformation of the toxic trichothecene DON before it reaches the small intestine and that is why, for instance, in cows only 1 % of the ingested compound can be found in the blood (Prelusky *et al.*, 1984) and why those animals are almost insensitive to oral DON intoxication (Prelusky *et al.*, 1986; Rotter *et al.*, 1996). Monogastric animals

instead, since high amounts of the ingested DON is absorbed in the small intestine and does not even reach the colon, are not able to detoxify as much DON to DOM-1 as polygastric animals. As a consequence, in their faeces less DOM-1, as a metabolic product of ingested DON, is found in comparison to faeces of polygastric animals (Eriksen *et al.*, 2002). Beyond that, as already indicated above, in monogastric animals high percentages of the ingested DON are able to enter the blood stream. For instance, an *in* vivo study in pigs showed that 89 % of the ingested mycotoxin crossed the intestinal epithelium after chronic exposure to naturally contaminated wheat and 54 % absorption was identified after acute oral exposure (Goyarts and Danicke, 2006).

Since also DON metabolites, such as 3-ADON, 15-ADON or DON-3-Glc occur in food (described in 2.2.5.3), they can be substrates of the intestinal metabolism as well. A study conducted by Eriksen *et al.* (2003) in pigs showed that after two and a half days of feeding with 3-ADON contaminated feed at a concentration of 2.5 mg/kg, 3-ADON could not be detected in the blood, but 58 % was metabolised to DON and 42 % to its glucuronide derivative and DOM-1.

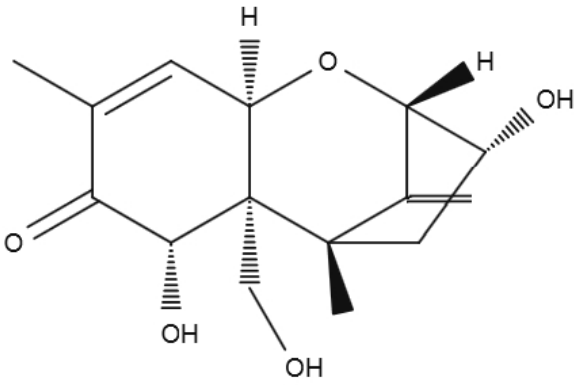

Figure 9: Chemical structure of deepoxy-deoxynivalenol (DOM-1)

To date, little data are available for the description of the more polar metabolites of DON (i. e. DON-3-Glc, DON-3-GA and DON-15-GA). In spite of that, several *in vitro* studies reported that DON-3-Glc, although it is stable in the upper human gut, can be transformed by some intestinal bacteria through the hydrolysis of its glucoside function into DON (Gratz *et al.*, 2013). Since DON-3-Glc is poorly bioavailable and most of the DON released during its hydrolysis after the small intestine remains in the faeces, thus not absorbed, it can be concluded that the toxicological relevance of DON-3-Glc is limited compared to that of the parent compound (Nagl *et al.*, 2012).

As already described for the plants, also mammalian cells metabolise DON in different ways and are e. g. able to glucuronidate DON and produce DON-3-GA or DON-15-GA (Figure 10). This is an important metabolic reaction catalysed by UDP-glucuronosyltransferases and it is used by the organism to form a less toxic molecule compared to the parent compound DON (Nagl *et al.*, 2012). In fact, the membranes and to interact with ribosomes (Maresca, 2013).

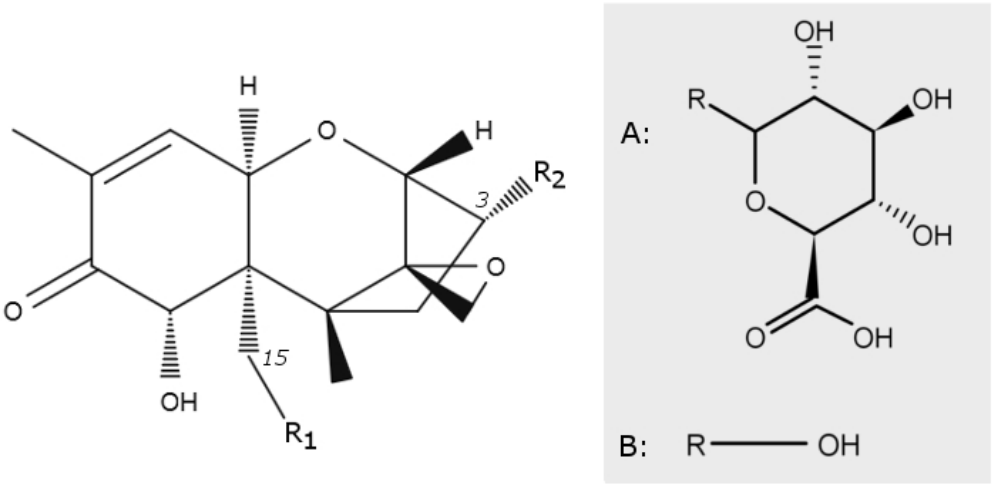

Figure 10: Chemical structure of deoxynivalenol-3-glucuronide (DON-3-GA; R_1: A and R_2: B) and of deoxynivalenol-15-glucuronide (DON-15-GA; R_1: B and R_2: A)

Studies focused on the excretion of DON showed that DON is partially metabolised into DOM-1 and glucuronidated DON and DOM-1 derivatives and excreted in the urine (Turner *et al.*, 2011). Recently, Warth *et al.* (2012) showed, analysing human urine samples, that 79-95 % of the total ingested DON could be found as either DON-3-GA or DON-15-GA and furthermore, that 75 % of total glucuronides present in the samples were detected as DON-15-GA (Warth *et al.*, 2012).
In a recent study, also conducted by Warth *et al.*, unpublished, a sulfated DON metabolite, namely DON-3-sulfate was detected as well in human urine samples.

2.3 Intoxications and epidemiological studies regarding DON and its derivatives

2.3.1 Toxic effects after acute exposure to DON and its derivatives

Acute DON intoxication, as already described above, is characterised by a severe involvement of the gastrointestinal tract. In fact, it can lead in pigs, to various emetic effects, such as abdominal distress, diarrhoea, malaise, induced salivation and emesis (Forsyth *et al.*, 1977; Friend *et al.*, 1982; Young *et al.*, 1983). Young *et al.* (1983) reported in pigs that on average a dietary level of 19,7 ppm caused a rapid onset of vomiting and that already toxin levels of 12 ppm lead to almost complete feed refusal.

Exposure to high DON concentrations was reported in mice to result even in shock-like death and beyond that several studies described effects such as heart lesions, necrosis in lymphoid tissue, of the intestinal tract and in bone marrow (Pestka, 2007). According to acute toxicity studies it was possible to calculate LD_{50} values for mice between 46 to 78 mg/kg bw reported for oral exposure (Yoshizawa and Morooka, 1973).

Regarding the acute toxicity of acetylated DON derivatives LD_{50} values were also determined in mice. One study for 3-ADON and DON reported LD_{50} values after oral administration of 34 and 46 mg/kg bw, respectively (Yoshizawa and Morooka, 1974). For 15-ADON LD_{50} values for oral ingestion were 34 and for DON 49 mg/kg bw, which lead to conclude that the acetylated derivative seems to be slightly more toxic than DON, whereas observed LD_{50} values after intraperitoneal injection were 113 for 15-ADON and 78 mg/kg bw for DON, suggesting that DON shows higher toxicity (Forsell *et al.*, 1987).

2.3.2 Short-term exposure to DON and sub-chronic effects

In order to mimic repetitive exposure to DON in a limited timeframe, short term and sub-chronic have been performed. In this respect, rats, after short term and sub-chronic exposure to DON showed effects such as feed refusal, reduced weight gain and body weight (Sprando *et al.*, 2005). Concerning organ to body weight ratios no significant changes after short-term feeding were reported in rats (Arnold *et al.*, 1986; Sprando *et al.*, 2005). However, Sprando *et al.* (2005) described further treatment related effects such as thymic lymphoid depletions, increased hematopoiesis of spleens and lesions in the non-glandular stomach and reported in male rats a dose-related increase of sperm tail abnormalities.

2.3.3 Toxic effects after chronic exposure to DON and its derivatives

Animal studies performed shortly after the discovery of DON, revealed a variety of chronic effects after long-term exposure to the toxin. For instance decreased growth rates, induced weight loss right up to anorexia and severe nutritional changes (Hughes *et al.*, 1999; Rotter *et al.*, 1994; Trenholm *et al.*, 1984). Since the contamination with DON, represent a serious problem for feed, a lot of studies were performed using farm animals as models. In this respect, pigs showed already at a dietary level of 1,3 ppm DON a significant decrease in feed intake and, as already mentioned above, at 12 ppm complete feed refusal (Young *et al.*, 1983). The reduction in weight gain during the experimental diet with DON contaminated feed (eight weeks; 2 and 4 ppm) a concentration dependency was identified in pigs. Beyond that in this study a NOAEL was set at 0,04 mg/kg bw/day (Bergsjo *et al.*, 1992). Feeding studies conducted with contamination levels at 3,5 ppm showed again a decrease in body weight gain, but also reduced nutritional efficiency, slaughter weight, serum protein and albumin concentrations. Furthermore, chronic toxic effects such as an increased liver weight and reduced packed blood cell volume were observed and lead to an estimated NOAEL of 0,03 mg/kg bw/day (Bergsjo *et al.*, 1993).

Rotter *et al.* (1994) conducted a study in swine focused on low-level exposure using DON concentrations between 0.75, 1.50, and 3.00 mg DON/kg diet. Toxic effects occurred in the whole concentration range. For instance, concentration-dependent reductions in absolute and relative thyroid sizes, increased levels of serum T4 and a thicker and more folded oesophageal region of the stomach were reported (Rotter *et al.*, 1994).

Studies conducted in poultry did not show serious adverse effects on health and productivity after experimental diet containing DON concentrations up to 8 ppm (Hamilton *et al.*, 1985a; Hamilton *et al.*, 1985b). Awad *et al.* (2004) reported that nutritional efficiency and uptake by broilers exposed to DON levels of 10 ppm was affected. DON exposed to young broiler chickens in combination with aflatoxin showed reduced growth rates and an increased relative organ weight of liver, kidney, spleen, gizzard and proventriculus. Beyond that anaemia, decreased enzymatic activity and reduced protein and albumin levels were identified. Overall the combinatory effects were not considered as a synergism but rather as additive toxicity (Huff *et al.*, 1986).

As also dairy cows did not show unusual symptoms of illness attributed to the mycotoxin-contaminated feed (Trenholm *et al.*, 1985). In accordance with the above

mentioned considerations (2.2.5.4) it can be concluded that monogastric species show higher sensitivity to chronic DON exposure compared to poultry and ruminants. Former showed primarily alterations of the gastrointestinal tract and lymphoid tissues (Pestka, 2007).

2.3.3.1 Carcinogenetic effects of DON

Concerning carcinogenetic effects of DON IARC classified DON in Group 3 which means “not classifiable as to its carcinogenicity to humans” (International Agency for Research on Cancer, 1993). A feeding study conducted over two years in mice focused on dietary levels between 1 and 10 ppm DON did not show an increasing number of spontaneous hepatic neoplasms. In male animals even a decreased incidence was found, which could result from the known positive correlation between body weight and neoplasmic growth (Iverson *et al.*, 1995).

2.3.4 Human intoxications and epidemiological studies regarding DON and its derivatives

During the last decades different outbreaks of acute human illness associated with the consumption of contaminated food, including DON, were reported. In China, for instance, between 1961 and 1985 around 35 outbreaks with around 8000 victims were described. The affected persons suffered, after the consumption of contaminated maize and wheat, for instance from abdominal pain, vomiting, nausea, diarrhoea, fever, dizziness and headache (Joint FAO/WHO Expert Committee on Food Additives, 2001). After an outbreak in 1984 in Xingtai County thin-layer chromatography (TLC) analysis were conducted of five samples, which determined DON at concentrations between 3,8 and 93 mg/kg (Luo, 1988).

A further outbreak in 1987 with about 50 000 victims in India was caused by the consumption of bread produced from contaminated wheat, containing various trichothecene mycotoxins, such as DON (0,34-8,4 mg/kg in 11 of 24 samples) and ADON (0,6-2,4 mg/kg in 4 of 24 samples). The affected persons suffered from moderate digestive disorders and gastrointestinal tract symptoms (Bhat *et al.*, 1989).

The regular intake of DON was also associated with a higher incidence of oesophageal cancer. In fact, in 1989 maize samples from Linxian, a Chinese high-risk area for this type of cancer with a mortality rate of 132 per 100 000, were analysed and their DON concentration compared with samples from Shagqiu (mortality rate of oesophageal cancer: 16 per 100 000). The mean concentration of DON was 0,57 mg/kg in maize samples from Linxian and 0,099 mg/kg in samples taken from Shangqiu (Luo *et al.*,

1990). In a further study conducted by Gao and Yoshizawa (1997) these two high- and low-incidence areas of oesophageal cancer were compared. In this case, significantly higher mean concentrations of DON and 15-ADON were found in the maize samples of Linxian in comparison to the toxin levels in samples taken from Shangqui. Thus, leading to conclude that the higher incidence of oesophageal cancer could be correlated with the mycotoxin concentration in the food (Gao and Yoshizawa, 1997).

Furthermore in 1989 maize and wheat samples of Chinese high- and low risk areas for Kashin-Beck disease, an endemic osteochondropathy, were analysed for mycotoxin contaminations. In high-incidence areas DON was found to occur in significantly higher concentrations in comparison to the low-incidence regions and in line also 3-ADON and 15-ADON levels were significantly increased in the high-risk areas (Luo, 1992).

2.4 Mechanisms of action and toxicology of DON and its derivatives

2.4.1 Cellular effects of DON and its derivatives

2.4.1.1 Different cellular response to DON

In vitro studies about mechanisms of action of toxins are routinely conducted in cultured cells systems. The different cell types used in cytotoxicity studies of DON comprise a wide variety of models from cloned to primary cells originating from the liver, the gut epithelium or the kidney to lymphoid, lung or bone marrow cells (Pestka *et al.*, 2004). Obviously, the sensitivity to DON varies according to the model. In this respect, several research groups reported that the mononuclear phagocyte system represents one of the most susceptible lineages to DON. *In vitro* and *in vivo* treatment with low to moderate concentrations of DON, which results in partial translation inhibition, promote both the transcriptional and post-transcriptional expression of different inflammatory associated genes, including different cytokines (Chung *et al.*, 2003; Zhou *et al.*, 1997), cyclooxygenase-2 (COX-2) (Moon and Pestka, 2002) and chemokines (Islam *et al.*, 2006). Higher DON concentrations, on the other hand, induced apoptosis of leukocytes (Pestka *et al.*, 1994; Yang *et al.*, 2000), which, since cells of the immune system protect the organism against infections and foreign material, resulted in immune suppression (Pestka *et al.*, 2004).

2.4.1.2 Effects of DON and its derivatives on cell signaling pathways

- *Effects of DON on cell signalling pathways*

  At the cellular level the toxicity of most trichothecene mycotoxins is sustained by the inhibition of the protein synthesis of proliferating eukaryotic cells (Ueno *et al.*, 1973). For this effect, the presence of the epoxide moiety of the molecule is crucial (Carter and Cannon, 1977) and was reported for DON in both *in vitro* and *in vivo* studies (Azcona-Olivera *et al.*, 1995; De Walle *et al.*, 2010).

  Several studies describing the mechanism of action of DON and related compounds have been already conducted over the last 40 years and it is known that the molecular target involved in the inhibition of eukaryotic protein biosynthesis is the ribosomal subunit 60S. Ribosomes are composed of RNA molecules and proteins, where in detail DON and beyond that also other mycotoxins such as T-2 toxin or Verrucarin A target a part of the so called peptidyl transferase centre, also known as the catalytic centre of the ribosome. In order to form peptide bonds during the translation process two substrates, aminoacyl-tRNA and peptidyl-tRNA, need to interact in the right way with the A- and P-site of the peptidyl transferase center. Various inhibitors of protein synthesis align similarly in the binding pockets, and so do the indicated trichothecenes mycotoxins at the A-site (Garreau de Loubresse *et al.*, 2014).

  Since nucleic acids and proteins possess nucleophile structural elements, nucleotides and amino acids, respectively, they have the ability to react with the C-12/C-13 epoxide function of DON (probable reactions shown in Figure 11) (Maresca, 2013). Such reactions of DON have not been reported yet, but in regard to aflatoxins interactions between amine and the toxins were identified by Sabbioni *et al.* (1987).

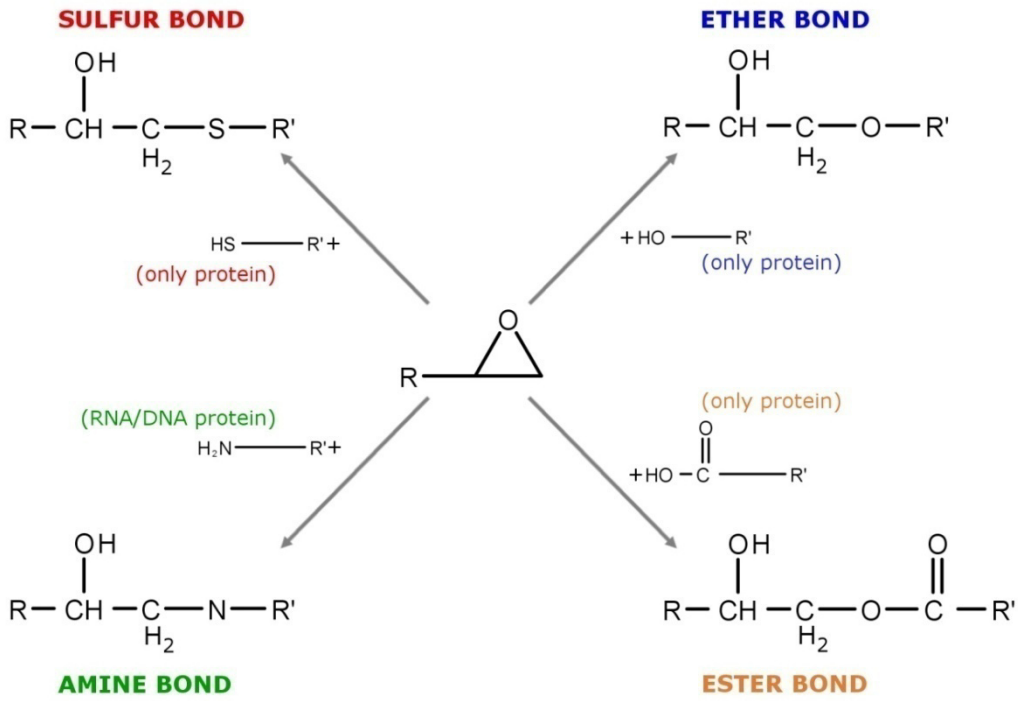

Figure 11: Possible reactions of DON's epoxide function with nucleophile functions of nucleic acids or proteins.

- taken and modified from Maresca (2013)

In agreement with the above mentioned mechanism of action, cellular signaling pathways affected by ribotoxic stress and ribotoxins mirrors those activated by DON. In particular the double-stranded RNA- (dsRNA)-associated protein kinase (PKR) (Zhou *et al.*, 2003) and the hematopoietic cell kinase (Hck) are influenced (Zhou *et al.*, 2005) and beyond that mitogen-activated protein kinases (MAPKs) are activated by DON. As a consequence, physiological processes such as cell proliferation and differentiation but also protein expressions, regulating innate immunity (e. g. activation of NF-κB) and apoptosis (e. g. via p53) are affected (Baltriukiene *et al.*, 2007; Pestka, 2010a; Yang *et al.*, 2000). Even though it was first proposed the opposite way, it was recently reported that rRNA cleavage is a result of apoptosis activation through caspase and RNases stimulation and not their cause. It was shown an exposure of low or high DON concentrations activated the signaling pathway already after some minutes, whereas rRNA cleavage occurred only after far longer exposure times at high concentrations (He *et al.*, 2012a; He *et al.*, 2012b).

Overall, the most actual hypothesis concerning molecular mechanisms of action of DON can be summarised the following way (Figure 12):

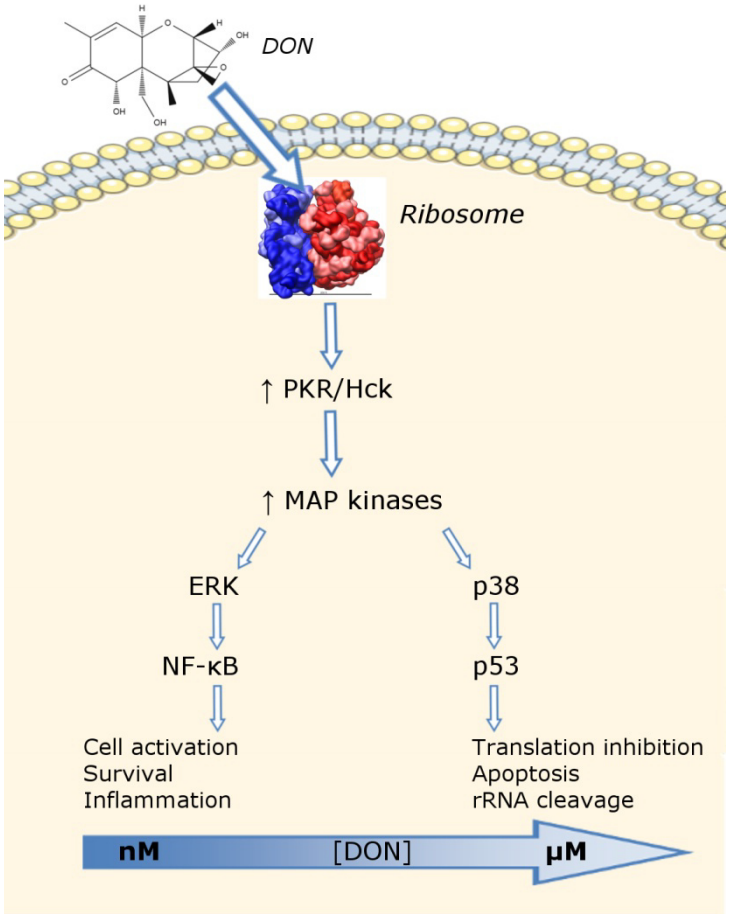

Figure 12: Cellular effects of DON on cell signaling pathways in macrophages.

- taken and modified from Maresca (2013)

Following the most quoted mechanism of action, after the passage through the cellular membrane, the epoxide group of DON is supposed to interact with the functional groups of nucleotides or rRNA-bound proteins (e. g. PKR and Hck) resulting in DON binding to rRNA. Rapidly PKR and Hck, rRNA-activated protein kinases are activated, which further leads to an activation of different MAPKs. Which MAPK types are induced in this step depends on the affecting DON concentration. In macrophages, for instance, it was reported that low mycotoxin concentrations (nM) lead primarily to activation of ERK and as a consequence to gene expression and a survival of the cell, whereas higher DON

concentrations (µM) activated p38 MAPKs causing apoptosis, rRNA cleavage and inhibition of protein biosynthesis (Maresca, 2013).

Intriguingly, it seems that the cellular mechanisms sustaining the biological effects of DON at low and high concentrations are different. In fact, if at high concentrations the effect of DON on protein synthesis prevails, at low concentrations DON alters transcriptionally and post-transcriptionally the expression of genes which primarily influence inflammatory reactions (Chung *et al.*, 2003) and the innate immune response (Moon and Pestka, 2002) via regulations of gene transcription, translation and stabilisation of messenger RNA (mRNA) (Pestka, 2010b). For instance, the toxin leads to an upregulation of mRNAs for TNF-α and IL-6 in macrophages (Chung *et al.*, 2003; Jia *et al.*, 2006) and was further reported to induce mRNA expression of IL-2 in EL-4 T cells (Li *et al.*, 1997) and IL-8 in monocyctes (Gray *et al.*, 2008; Gray and Pestka, 2007). As already mentioned above, DON exposure also results in a stabilisation of COX-2, TNF-α and IL-6 mRNAs in macrophages (Chung *et al.*, 2003; Jia *et al.*, 2006; Moon and Pestka, 2003) and also mRNAs from IL-2 in T cells (Li *et al.*, 1997). In this respect, the stabilisation is resulting of multiple copies of AUUUA motifs which lead to an alteration in the 3'-untranslated region (3'-UTR), regulating mRNA degradation (Moon *et al.*, 2003; Pestka, 2010b).

Beyond PKR, Hck, MAPKs and NF-κB, the effects of DON influence numerous other cellular proteins. Among these, regulations by DON of the early growth response gene 1 (EGR-1) (Moon *et al.*, 2007) or the activating transcription factor 2 (ATF 3) (Yang *et al.*, 2009) were already described and in addition Pan *et al.* (2013) described recently the involvement of DON in the phosphorylation of 188 proteins associated with regulations of transcription, translation, RNA processing, ribosome biogenesis, cell cycle, cytoskeleton organisation and cell differentiation.

- *Effects of DON derivatives, including DON3S and DON15S on cell signaling pathways*

  The description of the effects of DON derivatives on cellular signaling pathways is relatively limited in comparison to DON. In spite of that, for years it has been known, as already indicated above, that DON derivatives without the epoxide group are less toxic than their parent compound. DOM-1, for example, is one of

these derivatives and it is, due to the missing epoxide moiety, not able to bind to ribosomal units (Eriksen *et al.*, 2004).

Furthermore, derivatives as DON-3-Glc, DON-3-GA or DON-15-GA, which are characterised by a more polar behaviour, have not been reported as toxic. To date it is not clarified if they are not able to enter the cell due to their polar structure and/or to bind to ribosomal units (Poppenberger *et al.*, 2003; Wu *et al.*, 2007).

Toxic effects caused by 3-ADON and 15-ADON instead depend on the affected animal species and cell types. Additionally, there are also specie-specific differences in their ability to cross the cellular membrane and or to bind to ribosomes potentially influence their toxicity. Studies conducted in lymphocytes showed less toxicity compared to the effects caused by DON (Bondy *et al.*, 1991; Forsell and Pestka, 1985). On the contrary, Pinton *et al.* (2012) identified *in vitro,* in porcine IECs, and *ex vivo,* using intestinal explants, due to its ability to activate MAPKs 15-AODN as the most toxic compound. 3-ADON showed in comparison to DON less toxicity (Pinton *et al.*, 2012). Further studies, for instance, *in vivo* in mice showed as well higher toxicity of 15-ADON to intestinal tissue than DON but only in case of ingestion. Analysis after intraperitoneal injection did not confirm those data and lead to the conclusion that IECs react particularly sensitive to 15-ADON (Forsell *et al.*, 1987). As in theory, in all cell types ribosomes are known to be the same, these different toxic effects may be given due to different amounts of acetylated derivatives entering the cells. Consequently IECs seem to have, in comparison to lymphocytes, the higher ability to transport 15-ADON across the cellular membrane (Maresca, 2013). In addition, the differences could be explained as follows: Only DON is able to interact with ribosomes, although it has the same ability to enter the cells as its acetylated derivatives. As a consequence the different toxic effects suggest that IECs are more efficient removing the acetyl moiety and converting 3- or 15-ADON into DON (Maresca, 2013).

In regard to the sulfated derivatives DON3S and DON15S, since they have been described quite recently for the first time as plant and human metabolites (Warth *et al.*, 2015; Warth *et al.*, unpublished), toxicity data is only available *in vitro* concerning translation inhibition of wheat ribosomes. Warth *et al.* (2015) reported that a 50 % reduced translation was found after a DON-treatment of 1,5 µM, whereas DON3S did not cause translation inhibition at concentrations up to 50 µM

and DON15S moderately inhibited the ribosomes with an estimated IC_{50} value of 66 µM.

These data led to conclude that sulfation could be considered a strategy to detoxify the parent compound DON (Warth *et al.*, 2015). A more detailed biological characterisation of the effects of the sulfated DON metabolites DON3S and DON15S at intestinal level is the primary objective of the present thesis.

2.4.1.3 Impact of DON and its derivatives on cellular proliferation

High proliferating cells, such for example IECs, show high susceptibility to trichothecene treatment and in fact, several studies showed alterations in cell proliferations and resulting barrier functions (Diesing *et al.*, 2011a; Harhaj and Antonetti, 2004; Pinton and Oswald, 2014).

Regarding the impact of DON on cell proliferation after 48 hours of treatment was reported, for instance, in bovine large follicle granulose cells, where a biphasic cellular response was induced by the toxin. At 0,33 µM cell numbers increased by 14% whereas the highest DON concentration of 3,3 µM resulted in a 22 % decrease in cell proliferation by 22%. An intermediate toxin concentration (0.31 µM) stimulated in comparison to an untreated control cell growth by 20% (Pizzo *et al.*, 2015). In agreement, Diesing *et al.* (2011a) reported in intestinal porcine epithelial cells a significant relative increase in cell proliferation after 48 and 72 hours of DON-treatment (200 ng/ml).

Concerning the effects of the DON derivatives 3-ADON and 15-ADON Pinton *et al.* (2012) observed a strong inhibitory effect between 5 to 30 µM DON, 3-ADON and 15-ADON.

2.4.1.4 Cytotoxic effects of DON and its derivatives

Cytotoxic effects of DON have been investigated in numerous studies and different cell lines. Kolf-Clauw *et al.* (2009) analysed jejuna explants of weanling pigs treated for four hour with DON concentrations between 0,2 and 30 µM DON. The higher doses (10 and 30 µM) resulted in severe autolysis of the tissue. Treatment with DON (between 0.2 and 5 µM) lead to concentration dependent histological lesions and necrosis in the lamina propria, where numerous apoptotic cells were found at samples exposed to 5 µM DON.

In vitro experiments conducted in human intestinal cells, such as Caco-2 cells, using DON concentrations ranging from 1 to 150 µM showed dose dependent cytotoxic effects already at 1 µM, which became significant at 10 µM (Kouadio *et al.*, 2005).

Furthermore, cytotoxic effects of acetylated DON derivatives were investigated in porcine intestinal epithelial cells, known to be highly sensitive to trichothecenes. After a treatment for 24 hours with 30 µM DON, 3-ADON or 15-ADON a decrease of cell proliferation was reported, respectively of 60 %, 13 % and 69 %. According to these results 15-ADON can be considered as more toxic to proliferating intestinal epithelial cells in comparison to DON and 3-ADON (Pinton *et al.*, 2012).

2.4.1.5 Impact of DON on the oxidative stress response of cells

To date several *in vitro* studies have investigated the ability of DON to induce oxidative stress which is further strongly connected to cytotoxic effects and apoptosis induction caused by exposure to the mycotoxin (Braicu *et al.*, 2009; Costa *et al.*, 2009; Zhang *et al.*, 2009). Oxidative stress occurs in cells as the result of an intracellular imbalance (Figure 13) in which the level of reactive oxygen species (ROS) exceeds antioxidant capacities (Sies, 1991). ROS and cellular oxidative stress are able to induce lipid peroxidation which further leads not only to modifications and damage in the cell membrane but results also in a rise of DNA fragmentations (Chaudhari *et al.*, 2009). Subsequently, different signaling pathways, such as the one concerning mitogen-activated protein kinases and "ribotoxic stress response" described in 2.4.1.2, are activated resulting, for instance, in cell apoptosis or cytotoxicity (Sehata *et al.*, 2005).

Sahu *et al.* (2008) observed after incubation with µg/ml DON in rat liver cells an increase of ROS levels associated with hepatotoxic effects. A further study conducted in U937 cells (human leukemic monocyte lymphoma cell line) showed that treatment with quite high concentrations of DON (80 and 160 µM) caused increased ROS generation and severe cell damage. Even though glutathione peroxidase (GPx) was stimulated during this process the increased activity of the detoxifying enzyme could not prevent cell death (Costa *et al.*, 2009).

Furthermore, it was demonstrated in human colon cancer cells HCT116 that mitochondrial ROS seems to be strongly involved in DON induced apoptosis, since DON (100 µM) can open the mitochondrial permeability transition pore (MPTP) and trigger a loss of the mitochondrial transmembrane potential (MMP) (Bensassi *et al.*, 2012; Orrenius *et al.*, 2007; Ralph *et al.*, 2010). Furthermore, Bensassi *et al.* (2012) described the activation of caspase-9 and caspase-3 and modifications in

cytochrome c and BAX expression, which suggest that the mitochondrial intrinsic pathway plays a key role in DON-induced apoptotic processes. In HT-29 similar results have been observed as well, since, also in this model, the incubation with DON (500 ng/ml) induced a significant loss of MMP (between 30 and 100 %) (Kalaiselvi *et al.*, 2013).

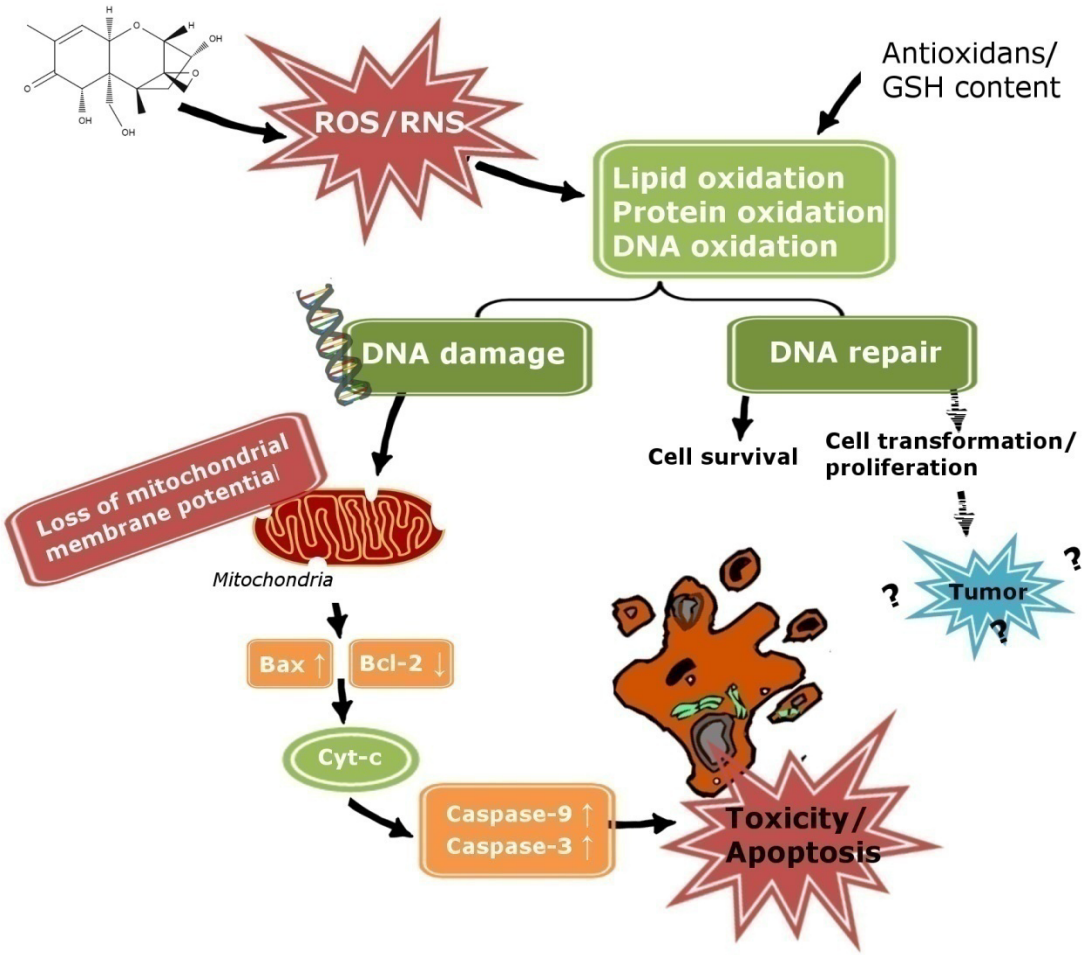

Figure 13: Mode of action mediated by cellular oxidative stress.

- taken and modified from (Mishra *et al.*, 2014b)

In Jurkat cells, beside an induction of oxidative stress, also the activation of the anti-oxidative Nrf2/Keap1 signaling pathway was reported. After DON exposure of 3 hours at a concentration of 0,5 µM a translocation of Nrf2 and Keap1 from the cytoplasm into the nucleus was observed (Katika *et al.*, 2015).

Overall, stimulation of ROS generation due to DON exposure should not be interpreted in light of the toxicity of the compound. In fact, ROS production and the resulting

oxidative stress are involved in several cellular pathways and mechanisms and can lead as a consequence to severe damage and alteration in the cells through to apoptosis (Mishra *et al.*, 2014b).

2.4.1.6 Impacts of DON on intestinal functions

Considering the occurrence of the DON and its metabolites in food and feed, the comprehension of the effects of the toxins on the gastrointestinal tract is of crucial importance. In accordance, after exposure to DON-contaminated food, IECs are directly affected by the trichothecene toxin and modified in their function.

- *Impact of DON on intestinal cell viability and proliferation*

  Numerous studies investigated alterations in cell proliferation and viability as a consequence of DON exposure in models that mimic the gastrointestinal tract. In fact, for a food contaminant this often represents one of the primary sites of action. For instance, an *in vitro* treatment of Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, for 24 hours showed cytotoxic effects at DON concentrations between 30 and 40 µM and an inhibition of cell proliferation at lower concentrations. In agreement, an IC_{50} value was determined between 1 and 5 µM (Instanes and Hetland, 2004). Further studies conducted in IECs originated from rats and pigs reported that DON caused cytotoxicity and apoptosis and stimated in this model IC_{50} values between 10 and 50 µM (Bianco *et al.*, 2012; Diesing *et al.*, 2011a; Diesing *et al.*, 2011b). Furthermore, Diesing and co-workers described differences in toxicity between undifferentiated and differentiated porcine epithelial cells and also a difference between apical and basolateral exposure. Undifferentiated cells showed a 10-fold higher sensitivity to DON treatment than the differentiated ones. Moreover, DON seemed to be more toxic when applied basolaterally rather than in apically (Diesing *et al.*, 2011a). In order to explain the first effect mentioned above, the authors suggested differences in cell cycle stage between transformed and non-transformed cells. The differential sensitivity depending on the route of application has not been explained yet (Diesing *et al.*, 2011a) and further experiments need to be performed, for instance to evaluate if different amounts of toxin are absorbed by IECs treated basolaterally or apically.

- *Impact of DON on nutrient absorption in intestinal cells*

  Two major ways exist to transfer dietary nutrients, electrolytes and water from the intestinal lumen into the blood: *i)* transcellular permeability: this is primarily followed by compounds transferred trough the IECs by selective transporters and *ii)* the paracellular permeability where the transport takes place in the gap between the cells in association with intercellular membrane junctional complexes (Groschwitz and Hogan, 2009). DON and also other trichothecenes are able to influence and inhibit the intestinal nutrient absorption. Maresca *et al.* (2002) described, for instance, that incubation of DON (10 µM) *in vitro* with human IECs, so called HT-29-clone D4, inhibited activity of the D-glucose/D-galactose sodium-dependent transporter (SGLT1), the glucose transporter-5 (GLUT5), the D-fructose transporter and of different amino acid transporters. These alterations of the nutrient absorption via IECs caused by the trichothecene mycotoxin DON could further be associated with toxic effects such as growth retardation and inhibition (Maresca *et al.*, 2002).

- *Impact of DON and its derivatives on tight junctions and barrier functions in intestinal cells*

  As already mentioned in section 2.2.3, various mycotoxins, including DON, have the ability to modify the organisation of the tight junctions within the epithelial membrane. These changes in the epithelial integrity can be investigated by measuring the TEER (Harhaj and Antonetti, 2004) and several studies in different human intestinal epithelial cell lines, such as Caco-2, HT-29 and T84 have shown a concentration-dependent reduction of the TEER (Kasuga *et al.*, 1998; Maresca *et al.*, 2002; Pinton *et al.*, 2009; Sergent *et al.*, 2006). Similar effects were obtained in porcine IECs, which, however, showed higher susceptibility than the human cancer cells (Diesing *et al.*, 2011a; Pinton *et al.*, 2009).

  In the porcine cell line IPEC-1 DON was compared to its acetylated derivatives 3-ADON and 15-ADON. After 24 hours of incubation (10 µM) 15-ADON was more potent in altering the cellular permeability (TEER) than DON, whereas 3-ADON was the less toxic compound of those three (Pinton *et al.*, 2012). In Caco-2 cells similar results in the toxic responses to the mycotoxin and its acetylated derivatives were reported (Kadota *et al.*, 2013).

Furthermore, a concentration- and time-dependent increase in the paracellular permeability determined by using a tracer was found after DON-treatment in human carcinoma cells such as Caco-2 and T84 (Kasuga *et al.*, 1998; Pinton *et al.*, 2009).

Similarly increasing effects were shown in a long-term study during which rats were exposed for 28 days to DON-contaminated feed (2 mg/kg feed). After analysis of pieces of jejunum a decreased TEER combined with an increase of paracellular permeability were found.

Beside DON, also its acetylated derivatives decreased paracellular permeability and again 15-ADON identified as the most toxic compound, followed by DON and 3-ADON (Pinton *et al.*, 2009).

Overall, the intestinal tract and especially its epithelial layer and the IECs represent the first biological barrier to protect the organism against a passage of ingested toxins from the lumen into the circulation. In agreement, after exposure to contaminated food, intestinal cells can be exposed to relatively high doses of foreign compounds such as mycotoxins which can result in different types of intoxications and their related effects on human and animal health. Since in several studies during the last decades an innumerable amount of toxic effects caused by DON exposure to the gastrointestinal tract were reported, the preceded paragraphs try to summarise the major topics concerning the toxicological effects on the intestinal functions, but represents for sure only a fraction of the actual knowledge. Nevertheless, it should be considered that in addition to DON, other related compounds, such as the sulfated derivatives DON3S and DON15S or also DON-3-Glc represent an emerging problem due to their accumulation in food an feed (Berthiller *et al.*, 2013). Not only their occurrence and toxicity, but also appropriated methods to analyse and regulate such novel modified mycotoxins are still missing and in order to evaluate their toxicological relevance, need to be investigated in detail.

3. Aim of the thesis

Beside acetylates, glycosides and glucuronides, recent studies have identified, as already mentioned above, two novel sulfo-metabolites of DON, namely deoxynivalenol-3-sulfate (DON3S) and deoxynivalenol-15-sulfate (DON15S) in wheat and the former in human urine as well (Warth *et al.*, 2015; Warth *et al.*, unpublished).

In regard to such derivatives, and especially to the novel sulfated compounds, neither regulatory limits nor regular analysis are established yet. Nevertheless, since such metabolites can be considered so called modified mycotoxins and several studies have revealed that they show the ability to be reactivated through metabolic processes in the digestive tract (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2011; Gratz *et al.*, 2013). Thus, also the novel metabolites DON3S and DON15S might pose a potential risk to human and animal health entering the food and feed chain.

Accordingly, this thesis aims to contribute to the toxicological characterisation of DON3S and DON15S in comparison to the parent compound deoxynivalenol (DON). In this respect, a preliminary toxicological profiling concerning their cytotoxic effects, their ability to cause cellular oxidative stress and further mechanisms of action has been conducted and since the gastrointestinal tract is the first barrier against feed contaminants as well as the first target for mycotoxins the human colorectal adenocarcinoma cell line HT-29 was chosen for the experiments. To estimate cytotoxic effects of DON and its sulfo-metabolites and also their effects on cell proliferation different assays such as the Sulforhodamine B (SRB) assay or the WST-1 assay were performed. Furthermore, a microscopic confluence analysis and an immunocytochemical evaluation of a cell proliferation marker, namely of KI-67, were conducted for this thesis. In order to investigate the cellular oxidative stress caused by DON and the sulfated derivatives DON3S and DON15S, the dichlorofluorescein (DCF) assay, a fluorometric assay was performed.

As already indicated above, the two modified mycotoxins, DON3S and DON15S could probably be related in their toxicity to the parent compound DON. Consequently in both, the experimental and also in the theoretical part of this thesis it was attempted to highlight the similarities and the differences between the three compounds.

The experiments presented in this thesis represent the starting point for the toxicological evaluation of DON3S and DON15S and for estimation of their importance as food contaminants. Even though, further work is currently in progress, the

toxicological characterisation of the sulfated DON derivatives presented in the following pages opens new perspectives in the risk assessment of trichothecene mycotoxins.

4. Results of the experimental work

In the following chapter the results of the experimental work conducted during this thesis will be presented. Details concerning the exact practical workflow and test protocols can be found in the paragraphs of chapter 7, “Materials and methods”. In addition, in the same chapter, the different procedures applied for the analysis of data and statistical methods are explained in detail.

4.1 Dichlorofluorescein assay - Impact of DON and its sulfo-derivatives DON3S and DON15S on the cellular reactive oxygen species levels

Since several studies (see section 2.4.1.5 for details) have reported the strong ability of DON to induce oxidative stress and ROS production in various cell lines (Braicu *et al.*, 2009; Costa *et al.*, 2009; Zhang *et al.*, 2009), the DCF assay was chosen as a first step for the investigation of the behaviour of the novel metabolites DON3S and DON15S. This assay is based on the principle that a non-fluorescent precursor (DCFH-DA) enters the cell, and once there, it is oxidised by ROS in a fast reaction that leads to the formation of a fluorescent molecule (DCF). As a consequence, fluorescence intensity detected during the assay can be considered directly proportional to the intracellular ROS levels (LeBel *et al.*, 1992).

Considering that this is the first biological characterization of the DON3S and DON15S in a cellular system, every experiment was conducted in comparison to the parent compound DON, plus positive controls (50 µM and 500 µM H_2O_2 for the DCF) and negative controls (solvent). During the first experiments, for the definition of the more appropriate protocol, fluorescence intensity was measured every ten minutes, in order to identify the time points of major interest. On the basis of this optimisation, the following results summarise data acquirecd after 3, 10, 30, 60, 120 and 180 minutes of incubation.

Figure 14 shows the concentration-dependent results of the measurements of the fluorescence intensity in the DCF assay after 3, 10, 30, 60, 120 and 180 minutes of incubation with DON, DON3S and DON15S at the concentrations of 0,1, 1 and 10 µM.

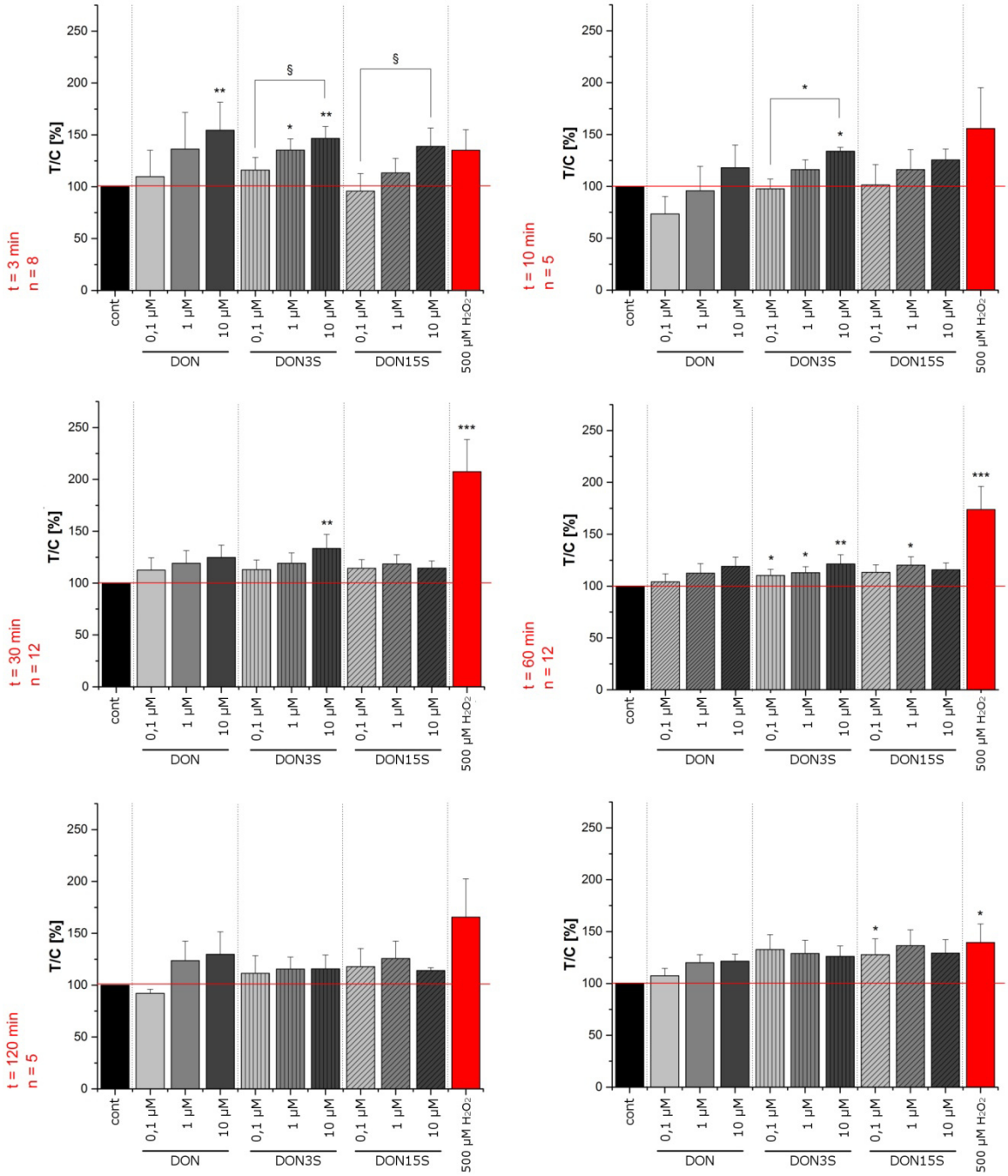

Figure 14: Concentration-dependent results of the measurements of the fluorescence intensity in the DCF assay after 3, 10, 30, 60, 120 and 180 minutes of incubation with DON, DON3S and DON15S at the concentrations 0,1, 1 and 10 µM.

** indicates significant differences compared to the negative control (* p<0,05; ** p<0,01; *** p <0,001).*

§ indicates significant differences compared to different concentrations of the same compound (§ p<0,05).

Values are expressed as mean ± SEM of at least five independent experiments performed in hexuplicates. Statistical significance to the negative control was evaluated by one-way ANOVA and Fisher post-hoc test.

After short-time incubation (3 min) 10 µM DON triggered a significant increase of the intracellular ROS (fluorescence increase as % of the solvent control 154, ± 27,0 %; p<0,01). However, this response appeared transient, since it could not be observed after longer incubation times.

DON3S showed in comparison to the other compounds increased in a different way intracellular ROS levels. In fact, a significant increase in ROS production was detected already after 3 minutes and was still evident after 60 minutes of incubation with 10 µM DON3S (p<0,05 and p<0,01). In addition, also 1 µM DON3S significantly induced intracellular ROS increase after 3 and 60 minutes of incubation (p<0,05).

The 15-sulfate of DON showed a more limited effect in sustaining intracellular oxidative stress in comparison to the DON3S. In fact, except for a transient increase after 60 minutes (120,3 ± 8,1 % increase; p<0,05) and after 180 minutes (136,4 ± 15,4 % increase; p<0,05) of incubation with 1 µM, no other effects were detectable.

Moreover, comparing these graphs between the different concentrations, their step-like appearances indicate quite well the concentration dependence of the reaction.

For clarity, all the statistical significances and the respective T/C-values in percentage terms are summarised in Table 4 to Table 8 as well.

Table 4: Significant differences in fluorescence intensity after 3 minutes of incubation evaluated by one-way ANOVA and Fisher's LSD post-hoc test.

3 min	**10 µM DON** 154,6 ± 27,0 %	**1 µM DON3S** 135,5 ± 10,8 %	**10 µM DON3S** 146,6 ± 11,5 %	**10 µM DON15S** 139,0 ± 17,6 %
neg. control	p<0,01	p<0,05	p<0,01	
0,1 µM DON3S 116,1 ± 12,2 %			p<0,05	
0,1 µM DON15S 95,8 ± 16,9 %				p<0,05

Table 5: Significant differences in fluorescence intensity after 10 minutes of incubation evaluated by one-way ANOVA and Fisher's LSD post-hoc test.

10 min	**10 µM DON3S** 134,0 ± 3,6 %
neg. control	p<0,05
0,1 µM DON3S 97,8 ± 9,2 %	p<0,05

Table 6: Significant difference in fluorescence intensity after 30 minutes of incubation evaluated by one-way ANOVA and Fisher's LSD post-hoc test.

30 min	**10 µM DON3S** 133,5 ± 13,5 %
neg. control	p<0,01

Table 7: Significant differences in fluorescence intensity after 60 minutes of incubation evaluated by one-way ANOVA and Fisher's LSD post-hoc test.

60 min	**0,1 µM DON3S** 110,2 ± 5,9 %	**1 µM DON3S** 113,1 ± 5,7 %	**10 µM DON3S** 121,4 ± 8,9 %	**1 µM DON15S** 120,3 ± 8,1 %
neg. control	p<0,05	p<0,05	p<0,01	p<0,05

Table 8: Significant difference in fluorescence intensity after 180 minutes of incubation evaluated by one-way ANOVA and Fisher's LSD post-hoc test.

180 min	**1 µM DON15S** 136,4 ± 15,4 %
neg. control	p<0,05

In order to appreciate the whole information included in these experiments, the same data were also plotted to show the time dependency of the effect of single concentrations (0,1 µM: Figure 15; 1 µM: Figure 16; 10 µM: Figure 17).

When analysing the cellular response from this angle, all three graphs show that the induction of intracellular ROS productions from the trichothecene toxins seem to be characterised by a fast reaction. In fact, fluorescence values are decreasing already after ten minutes of incubation and they can be considered relatively stable after 30 up to 90 minutes of incubation. Interestingly, after longer incubation times the concentrations of ROS inside the cells showed again a tendency toward increase.

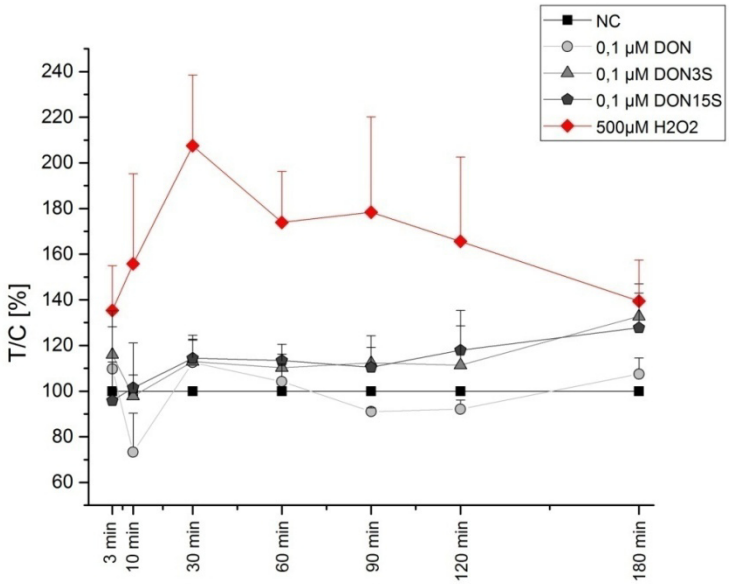

Figure 15: Time-dependent results of the measurements of the fluorescence intensity in the DCF assay after from 3 to 180 minutes of incubation with DON, DON3S and DON15S at the concentrations 0,1 µM.

Values are expressed as mean ± SEM of at least five independent experiments performed in hexuplicates.

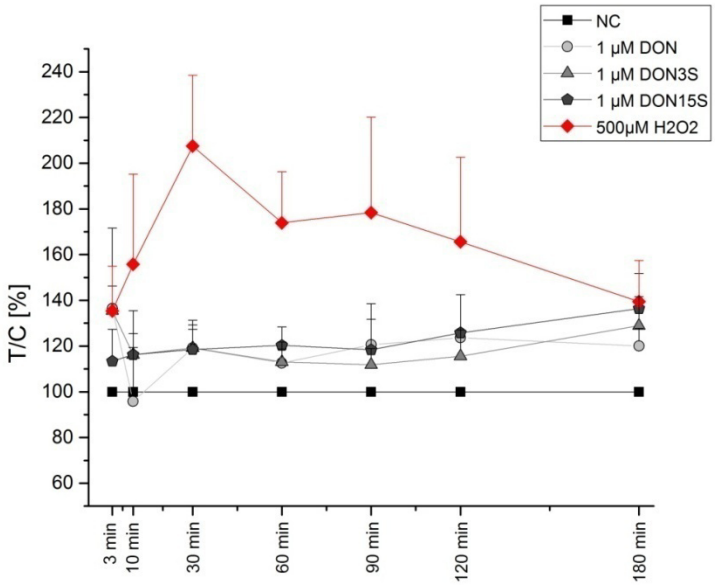

Figure 16: Time-dependent results of the measurements of the fluorescence intensity in the DCF assay after from 3 to 180 minutes of incubation with DON, DON3S and DON15S at the concentrations 1 µM.

Values are expressed as mean ± SEM of at least five independent experiments performed in hexuplicates.

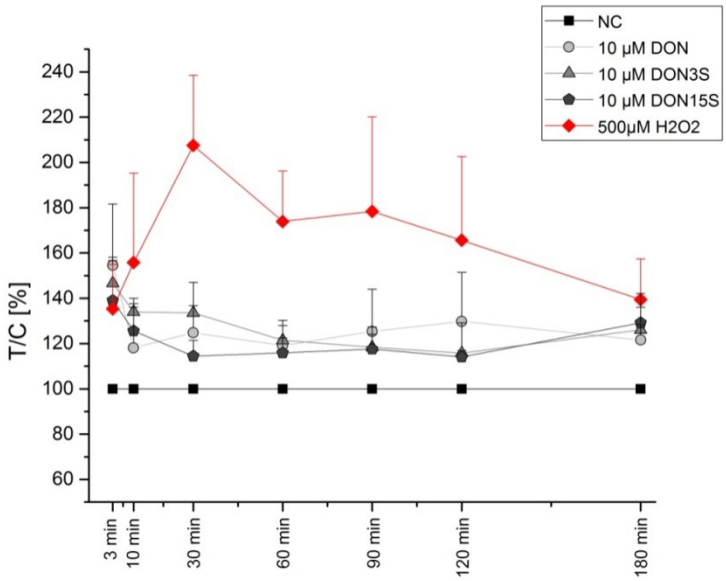

Figure 17: Time-dependent results of the measurements of the fluorescence intensity in the DCF assay after from 3 to 180 minutes of incubation with DON, DON3S and DON15S at the concentrations 10 µM.

Values are expressed as mean ± SEM of at least five independent experiments performed in hexuplicates.

4.2 Sulforhodamine B – Impact of DON and its sulfo-derivatives DON3S and DON15S on cytotoxicity and cell proliferation

Since increased intracellular ROS can be associated, according to the concentration, to both cytotoxic effects (Kouadio *et al.*, 2005) or to cellular proliferation (Mishra *et al.*, 2014a; Mishra *et al.*, 2014b), experiments were performed to evaluate the impact of the three molecules on the viability of HT-29 intestinal cells. As a first step, the SRB assay was performed.

In principle, the assay is based on the ability of the dye sulforhodamine B to stain the protein content of trichloroacetic acid-fixed cells. While the staining step requires acidic conditions, the SRB dye can be solubilised again under mild basic conditions and the absorbance of the solution measured. The absorbance values are directly proportional to the total protein content and thus provide an information about the cells present in the well and, indirectly, information about the cell viability (Skehan *et al.*, 1990).

The experiments were conducted in HT-29 cells at incubation times of 24 and 48 hours. In order to get an idea about the required concentration range preliminary experiments were conducted using the same incubation concentrations as in the DCF

assay (0,1, 1 and 10 µM). Subsequently, on the basis of preliminary results, the range of concentrations was further expanded (between 0,1 nM and 100 µM) and experiments were performed accordingly.

The results of the SRB assay after 24 hours of incubation with DON, DON3S and DON15S are summarised in Figure 18 as percentage of control.

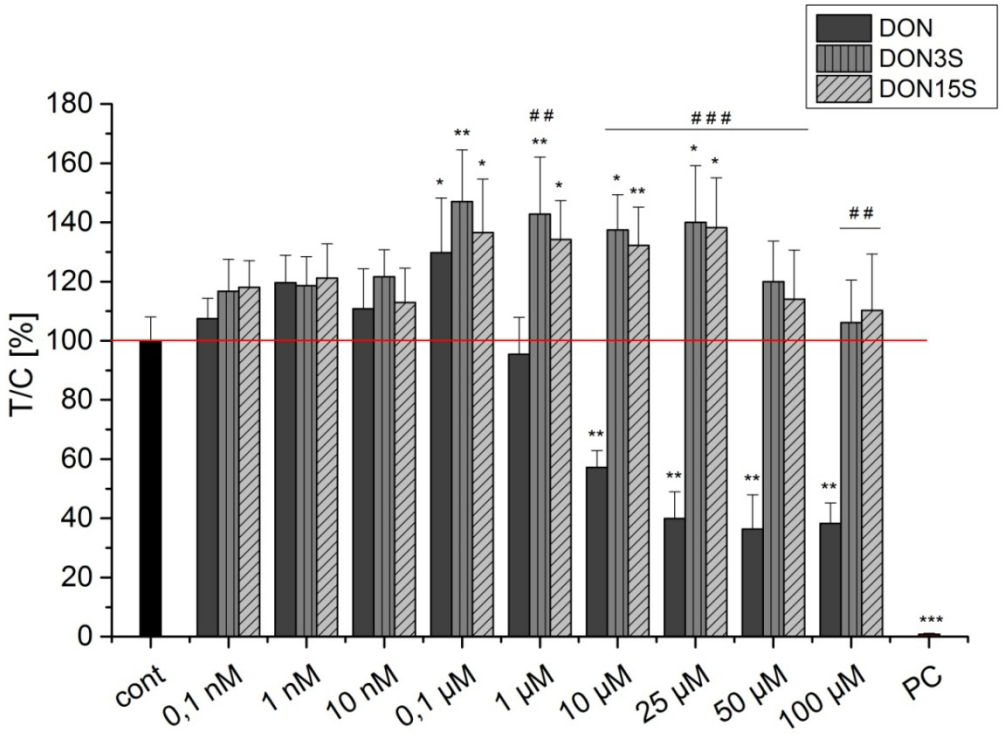

Figure 18: Results of the measured absorbance values in the sulforhodamine B assay after an incubation of 24 hours with DON, DON3S and DON15S at concentrations between 0,1 nM and 100 µM.

** indicates significant differences compared to the negative control (* p<0,05; ** p<0,01; *** p<0,001).*

 # indicates significant differences compared to the values of DON at the same concentration (## p<0,01; ### p<0,001).

Values are expressed as mean ± SEM of at least three independent experiments performed in quadruplicates. Statistical significance was evaluated by Kruskal-Wallis ANOVA.

In these experiments, clear differences between the parent compound DON and its sulfated derivatives were found. DON showed a biphasic behaviour: at the concentration of 0,1 µM it significantly induced cell proliferation (129,7 ± 18,5 % of increase; p<0,05), whereas at concentrations higher than 1 µM cytotoxic effects started to appear and became significant at concentrations higher than 10 µM (p<0,01). At

10 µM only 57,2 ± 5,7 % protein content of the negative control was still detectable and progressively decreased to the highest concentration tested (100 µM; 38,3 ± 6,9 %).

On the contrary, no cytotoxic effects have been observed in cells incubated with the sulfated DON metabolites DON3S and DON15S. A significant increase in cell proliferation was identified at concentrations between 0,1 and 25 µM ($p<0,05$ and $p<0,01$) in comparison to the negative control with a maximum value of 147,1 ± 17,4 % at 0,1 µM. Accordingly, the cellular viability of cells incubated with DON3S and DON15S was significantly higher in comparison to those incubated with the parent compound DON (indicated in the graph with #-symbols).

For clarity, the respective T/C-values in percentage terms and the statistical significances calculated by Kruskal-Wallis ANOVA are in addition indicated in the tables below, where "+" symbolises proliferating stimuli and "–" represents significant cytotoxic effects (DON: Table 9; DON3S: Table 10; DON15S: Table 11)

Table 9: Significant differences in protein content after 24 hours of incubation with DON evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative protein content.

24 hours	0,1 µM DON 129,8 ± 18,5 %	10 µM DON 57,2 ± 5,7 %	25 µM DON 39,9 ± 9,0 %	50 µM DON 36,3 ± 11,6 %	100 µM DON 38,3 ± 6,9 %
negative control	+ p<0,05	- p<0,01	- p<0,01	- p<0,01	- p<0,01

Table 10: Significant differences in protein content after 24 hours of incubation with DON3S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control and to the respective DON concentration. + indicates an increase and - a decrease in relative protein content.

24 hours	0,1 µM DON3S 147,1 ± 17,4 %	1 µM DON3S 142,8 ± 19,2 %	10 µM DON3S 137,4 ± 11,9 %	25 µM DON3S 140,1 ± 19,1 %	50 µM DON3S 119,9 ± 13,7 %	100 µM DON3S 106,2 ± 14,3 %
negative control	+ p<0,01	+ p<0,01	+ p<0,05	+ p<0,05		
DON at the same conc. as compared		p<0,01	p<0,001	p<0,001	p<0,001	p<0,01

Table 11: Significant differences in protein content after 24 hours of incubation with DON15S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control and to the respective DON concentration. + indicates an increase and - a decrease in relative protein content.

24 hours	0,1 µM DON15S 136,5 ± 18,1 %	1 µM DON15S 134,2 ± 13,2 %	10 µM DON15S 132,2 ± 12,9 %	25 µM DON15S 138,2 ± 16,8 %	50 µM DON15S 114,1 ± 16,6 %	100 µM DON15S 110,3 ± 19,0 %
negative control	+ p<0,05	+ p<0,05	+ p<0,01	+ p<0,05		
DON at the same conc. as compared			p<0,001	p<0,001	p<0,001	p<0,01

In order to evaluate the effect of the same concentration and substances after longer incubation times, the experiments were conducted beside 24 hours also with 48 hours of incubation. The results for these evaluations are illustrated in Figure 19.

After longer incubation times (48 hours), it can be clearly seen that the effects on cell proliferation was less pronounced in comparison to that detectable after 24 hours. Accordingly, the cytotoxic effect was stronger.

After 48 hours of incubation with DON any effect on cellular proliferation was lost, whereas the cytotoxic effects were even more marked. Therefore the total protein content of cells incubated 48 hours with 10 µM DON was determined as 39,1 ± 7,7 % of the negative control and further significant decreased at the higher concentrations ($p<0,001$).

A significantly increased cell proliferation could only be found after treatment with DON3S at concentrations between 0,1 and 25 µM, which even if significant, was weaker than the effects found after 24 hours ($p<0,05$).

DON15S increased the total protein content significantly only at the concentration of 0,1 µM ($p<0,05$), where an increase of 117,3 ± 7,5 % was determined. Furthermore, the effects at higher concentrations were less strong than the ones after shorter incubation times. In comparison to the respective effects caused by DON, again DON3S and DON15S showed a significant difference between 1 and 100 µM (1 µM: $p<0,05$; ≥10 µM: $p<0,001$).

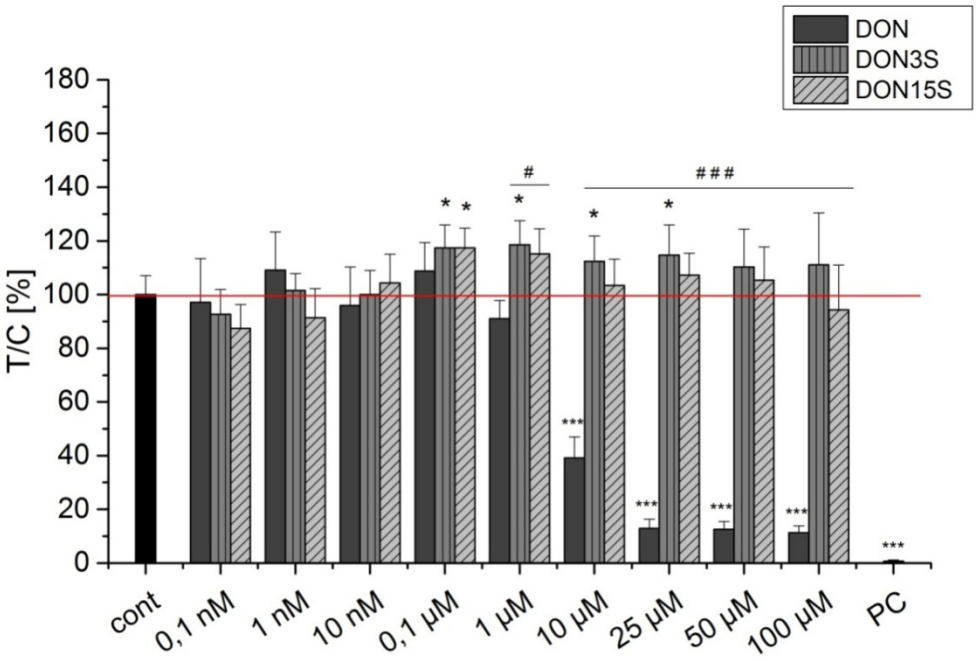

Figure 19: Results of the measured absorbance values in the sulforhodamine B assay after an incubation of 48 hours with DON, DON3S and DON15S at concentrations between 0,1 nM and 100 µM.

** indicates significant differences compared to the negative control (* p<0,05; *** p<0,001).*

 # indicates significant differences compared to the values of DON at the same concentration (# p<0,05; ### p<0,001).

Values are expressed as mean ± SEM of at least three independent experiments performed in quadruplicates. Statistical significance was evaluated by Kruskal-Wallis ANOVA.

For clarity, all the statistical significances evaluated by Kruskal-Wallis ANOVA and also the respective T/C-values in percentage terms are further indicated in the tables below, where consistently with the previous summary "+" represents proliferating stimuli and "–"significant cytotoxic effects (DON: Table 12; DON3S:

Table 13; DON15S: Table 14).

Table 12: Significant differences in protein content after 48 hours of incubation with DON evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative protein content.

48 hours	**10 µM DON** 39,2 ± 7,7 %	**25 µM DON** 12,9 ± 3,4 %	**50 µM DON** 12,5 ± 2,9 %	**100 µM DON** 11,2 ± 2,6 %
negative control	- p<0,001	- p<0,001	- p<0,001	- p<0,001

Table 13: Significant differences in protein content after 48 hours of incubation with DON3S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control and to the respective DON concentration. + indicates an increase and - a decrease in relative protein content.

48 hours	0,1 µM DON3S 117,4 ± 8,6 %	1 µM DON3S 118,6 ± 8,9 %	10 µM DON3S 112,4 ± 9,4 %	25 µM DON3S 114,6 ± 11,2 %	50 µM DON3S 110,2 ± 14,1 %	100 µM DON3S 111,1 ± 19,3 %
negative control	+ p<0,05	+ p<0,05	+ p<0,05	+ p<0,05		
DON at the same conc. as compared		p<0,05	p<0,001	p<0,001	p<0,001	p<0,01

Table 14: Significant differences in protein content after 48 hours of incubation with DON15S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control and to the respective DON concentration. + indicates an increase and - a decrease in relative protein content.

48 hours	0,1 µM DON15S 117,3 ± 7,4 %	1 µM DON15S 115,2 ± 9,3 %	10 µM DON15S 103,4 ± 9,7 %	25 µM DON15S 107,2 ± 8,2 %	50 µM DON15S 105,4 ± 12,4 %	100 µM DON15S 94,3 ± 16,8 %
negative control	+ p<0,05					
DON at the same conc. as compared		p<0,05	p<0,001	p<0,001	p<0,001	p<0,001

4.3 WST-1 - further evaluation of the impact of DON and its sulfo-derivatives DON3S and DON15S on cytotoxicity and cell proliferation

Since the SRB assay evaluates the potential for cytotoxicity only on the basis of one single endpoint, thus the protein content, a second cytotoxicity experiment was performed, namely the WST-1 assay. This assay is conducted on living cells and it is used to detect the mitochondrial activity (Berridge *et al.*, 2005). For the WST-1, the more interesting incubation conditions of DON, DON3S and DON15S have been further investigated. In line, since the SRB assay showed both effects on cytotoxicity and cell proliferation more pronounced after 24 hours, the WST-1 assay was performed only after this incubation time.

As it can be seen in Figure 20, DON showed again a tendency toward a biphasic behaviour depending on its concentration of incubation. In fact, even if its stimulating effect at 0,1 µM was not pronounced as it was in the SRB assay, it is possible to see a progressive and concentration dependent increase in the WST-1 signal between 1 nM an 0,1 µM. However, in line with SRB experiments, a significant ($p<0,01$) decrease in the mitochondrial activity was observed starting from the concentrations of 1 µM DON (83,8 ± 4,2 % mitochondrial activity) and more marked at 10 µM (72,3 ± 3,5 % mitochondrial activity).

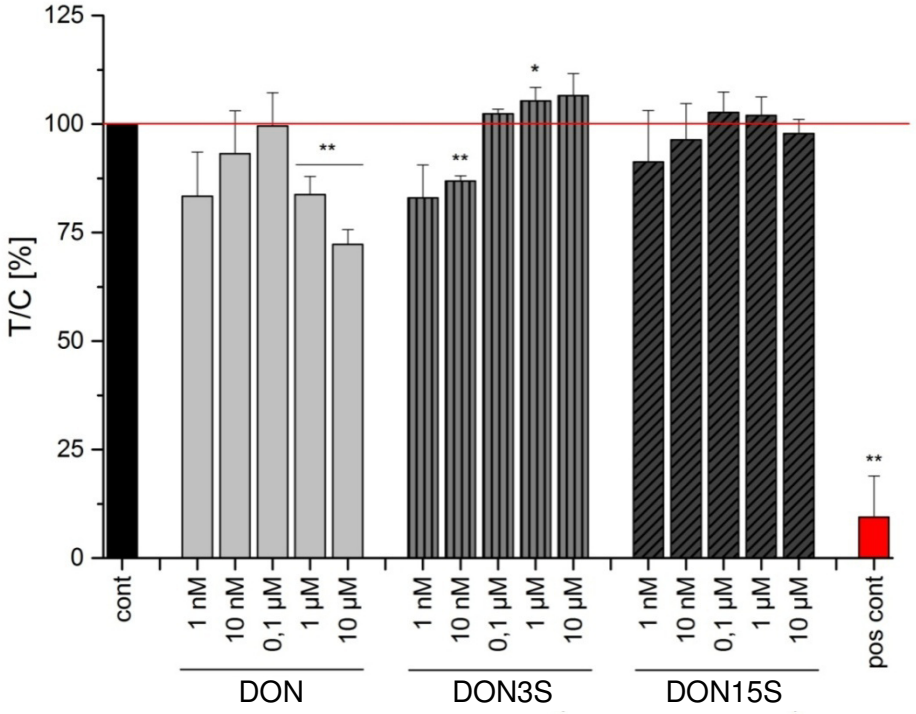

Figure 20: Results of the measured absorbance values in the WST-1 assay after an incubation of 24 hours with DON, DON3S and DON15S at concentrations between 1 nM and 10 µM.

** indicates significant differences compared to the negative control (* p<0,05; ** p<0,01).*

Values are expressed as mean ± SEM of at least five independent experiments performed in quadruplicates. Statistical significance was evaluated by one-way ANOVA and Fisher post-hoc test.

Interestingly, DON3S decreased the mitochondrial activity of the cells incubated with 1 and 10 nM, the latter significantly ($p<0,01$). At 1 and 10 µM instead an increased

mitochondrial activity could be observed, which was evaluated significant at 1 µM with a value of 105,3 ± 3,1 %.

Cells treated with the sulfated derivative DON15S between 1 nM and 10 µM did not show any significant increase or decrease in comparison to the negative control.

For clarity, the statistical significances evaluated by one-way ANOVA and Fisher's LSD post-hoc test and additionally the respective T/C-values in percentage terms are indicated in the tables below, where "+" represents an increase and "–" a decrease in mitochondrial activity (DON: Table 15; DON3S: Table 16)

Table 15: Significant differences in mitochondrial activity after 24 hours of incubation with DON evaluated by one-way ANOVA and Fisher's LSD post-hoc test in comparison to the negative control. + indicates an increase and - a decrease in relative mitochondrial activity.

24 hours	**1 µM DON** 83,8 ± 4,2 %	**10 µM DON** 72,3 ± 3,5 %
negative control	- p<0,01	- p<0,01

Table 16: Significant differences in mitochondrial activity after 24 hours of incubation with DON3S evaluated by one-way ANOVA and Fisher's LSD post-hoc test in comparison to the negative control. + indicates an increase and - a decrease in relative mitochondrial activity.

24 hours	**10 nM DON3S** 86,9 ± 1,2 %	**1 µM DON3S** 105,3 ± 3,1 %
negative control	- p<0,01	+ p<0,05

4.4 Confluence analysis – non photometric evaluation of the impact of DON and its sulfo-derivatives DON3S and DON15S on cytotoxicity and cell proliferation

Both cytotoxicity/cell proliferation assays presented before are photometric assays. In order to confirm the above mentioned effects also in a totally different experimental setup, a microscopy-based assay was optimised. In fact, a confluence analysis was performed using the Cytation™ 3 Cell Imaging Multi-Mode Reader. The whole procedure, which is based on a digital phase contrast image analysis of brightfield images, is described in detail in 7.3.3 and the results are shown in Figure 21. This approach is particularly useful to discriminate if the increase of the SBR signal could be

related to an effective increase of the cell number or the description of the variation of the content of proteins.

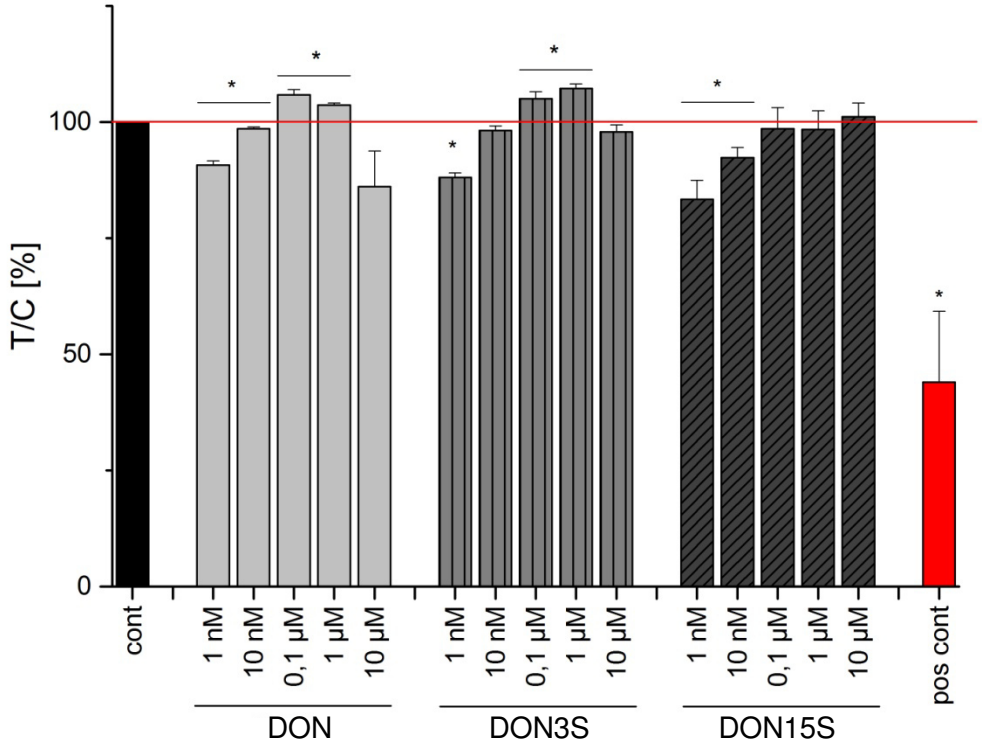

Figure 21: Results of the confluence analysis after an incubation of 24 hours with DON, DON3S and DON15S at concentrations between 1 nM and 10 µM.

** indicates significant differences compared to the negative control (* p<0,05).*

Values are expressed as mean ± SEM of at least three independent experiments performed in quadruplicates. Statistical significance was evaluated by Kruskal-Wallis ANOVA.

The data of the confluence analysis is in accordance with the results already shown above.

DON, for instance showed a significant decrease in the confluence at the low concentrations 1 nM and 10 nM with a confluence of 90,7 ± 0,9 % and 98,6 ± 0,4 % of the negative control respectively (p<0,05). At 0,1 µM it was again identified a stimulating effect sustained also at 1 µM. Thus, a significant higher confluence of 105,9 ± 1,1 % and 103,7 ± 0,5 % compared to the negative control was found (p<0,05). This data are in agreement with results already presented in literature (Pizzo *et al.*, 2015).

In the confluence assay DON3S showed similar effects as already observed in the SRB and WST-1 assay: growth stimulus and thus a higher confluence was determined at the concentrations 0,1 and 1 µM. In line with the data of the WST-1 assay, in cells incubated with 1 and 10 nM DON15S, and 1 nM DON3S it was possible to observe a significant decrease in the confluence down to 88,1 ± 0,9 % of the negative control ($p<0,05$). DON15S significantly reduced the confluence to 83,4 ± 4,1 % and 92,3 ± 2,2 % of the control values after 24 hours of incubation at 1 and 10 nM.

For clarity, all the statistical significances evaluated by Kruskal-Wallis ANOVA and the respective T/C-values in percentage terms are indicated in the tables below, where "+" represents an increase and "–" a decrease in confluence (DON: Table 17; DON3S: Table 18; DON15S: Table 19).

Table 17: Significant differences in confluence after 24 hours of incubation with DON evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative confluence.

24 hours	**1 nM DON** 90,7 ± 0,9 %	**10 nM DON** 98,6 ± 0,4 %	**0,1 µM DON** 105,9 ± 1,1 %	**1 µM DON** 103,7 ± 0,5 %
negative control	- $p<0,05$	- $p<0,05$	+ $p<0,05$	+ $p<0,05$

Table 18: Significant differences in confluence after 24 hours of incubation with DON3S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative confluence.

24 hours	**1 nM DON3S** 88,1 ± 0,9 %	**0,1 µM DON3S** 105,1 ± 1,5 %	**1 µM DON3S** 107,3 ± 0,9 %
negative control	- $p<0,05$	+ $p<0,05$	+ $p<0,05$

Table 19: Significant differences in confluence after 24 hours of incubation with DON15S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative confluence.

24 hours	**1 nM DON15S** 83,4 ± 4,1 %	**10 nM DON15S** 92,3 ± 2,2 %
negative control	- $p<0,05$	- $p<0,05$

4.5 Immunocytochemical KI-67 quantification - further evaluation of the stimulating effect on cell proliferation caused by DON and its sulfo-derivatives DON3S and DON15S

Preceding experiments and their results showed clearly a growth stimulating effect caused by treatment with DON3S and DON15S, more pronounced after 24 hours of incubation than after 48 hours.

In this respect, it was decided to evaluate the effects of the three molecules on one of the most important biomarkers of cellular proliferation, thus KI-67. Several immunocytochemistry studies (Aaltomaa *et al.*, 2006; Abdel-Aziz and Amin, 2012) previously showed that KI-67 can be used in this respect, since it is a nuclear protein which is present in proliferating cells in all phases of the active cell cycle but is not expressed in resting (G0) cells (Abdel-Aziz and Amin, 2012).

Considering the complexity of the preparation of the microscopy samples, as well as the time necessary for the data acquisition and analysis, the most relevant concentrations of the three substances were chosen. Moreover, the structure of the microscopy slides allows the acquisition of a maximum of 8 different experimental conditions each time. Taking these limitations into account, the concentration of 0,1 µM was selected, as it showed the highest stimulating effect on cell proliferation for all the tested conditions. Moreover, the concentration of 10 µM was also selected, since it showed a clear difference between the behaviour of the sulfates and that of DON.

Qualitative analysis of the images already showed a substantial difference in the localisation of KI-67 between the different experimental conditions. Accordingly, representative images are included in Figure 22 (incubations with 0,1 µM DON, DON3S and DON15S) and in Figure 23 (incubations with 10 µM DON, DON3S and DON15S). In the cells treated with DON3S and DON15S the fluorescence signal of KI-67 seems to be in both figures much stronger compared to the negative control and especially compared to the fluorescence intensity of cells incubated with DON. Furthermore, in wells incubated with the sulfated derivatives a high incidence of mitotic structures could be observed, whereas in cells treated with DON or the solvent control less of this ongoing mitosis could be identified. The latter were later quantified in a further assay, in detail described in 7.3.4.1 and the respective results are presented in 4.7.

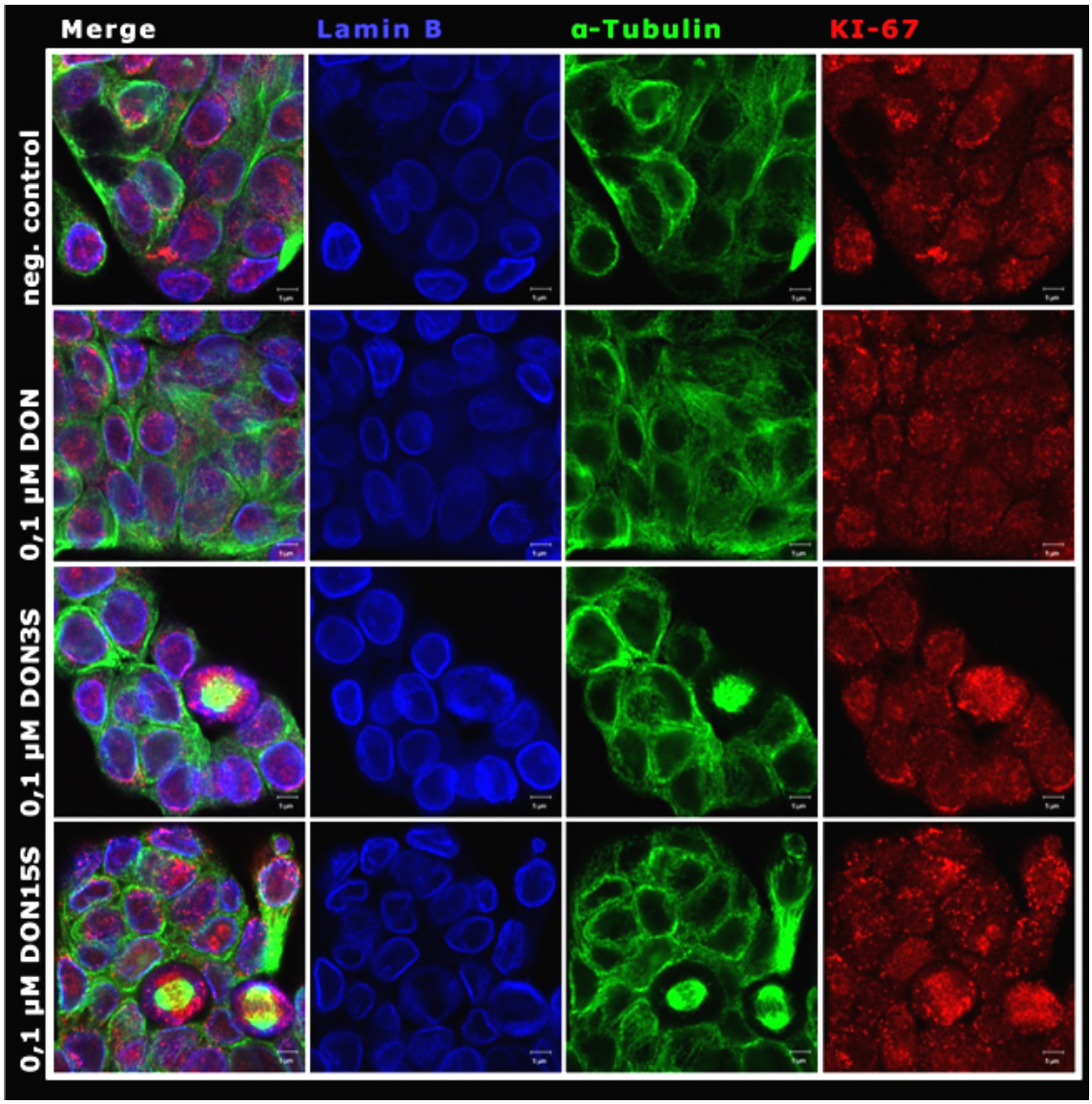

Figure 22: Immunocytochemistry and confocal images of HT-29 cells demonstrating the expression of the cellular proliferation marker KI-67 (right column), as the nuclear structure lamin B and as a component of the cytoskeleton, α-tubulin after 24 hours of incubation with DON, DON3S and DON15S at 0,1 µM and with the solvent control. In the left column merge of the three channels is shown. (scale bar: 5 µm)

Images are acquired using an oil immersion objective of a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system at the following configurations: 63x magnification, zoom 2, average 16, speed 7.

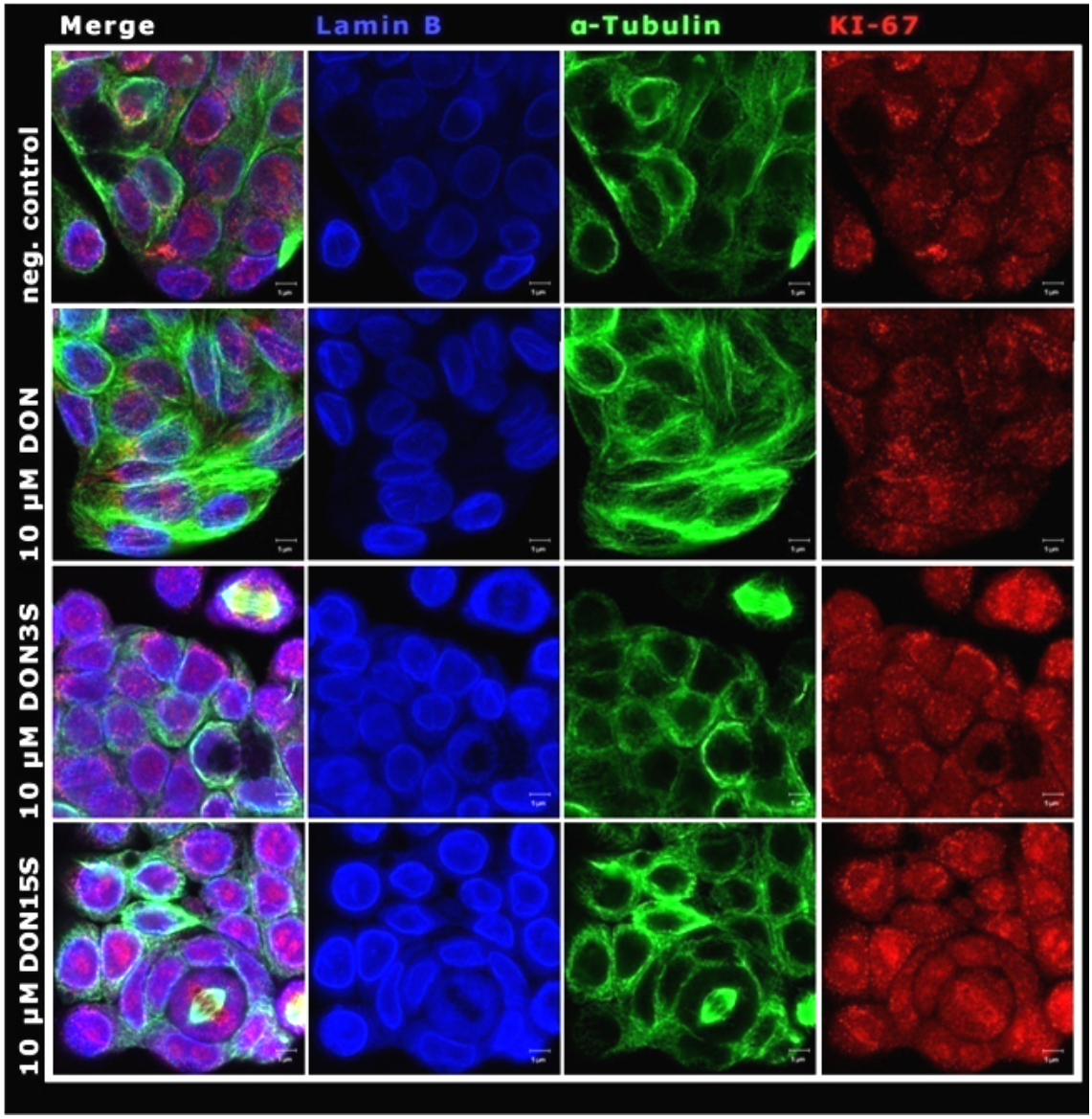

Figure 23: Immunocytochemistry and confocal images of HT-29 cells demonstrating the expression of the cellular proliferation marker KI-67 (right column), as the nuclear structure lamin B and as a component of the cytoskeleton, α-tubulin after 24 hours of incubation with DON, DON3S and DON15S at 10 µM and with the solvent control. In the left column merge of the three channels is shown. (scale bar: 5 µm)

Images are acquired using an oil immersion objective of a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system at the following configurations: 63x magnification, zoom 2, average 16, speed 7.

The quantification of the proliferation marker KI-67 was conducted through analysis of 3D reconstructions taken from the immunocytochemical stainings of KI-67, lamin B and α-tubulin where details regarding the image acquisition can be found in 7.3.4.1.

Further image analysis was performed then with the image processing and analysis software ImageJ and the results of the quantified fluorescence signals are shown in Figure 24.

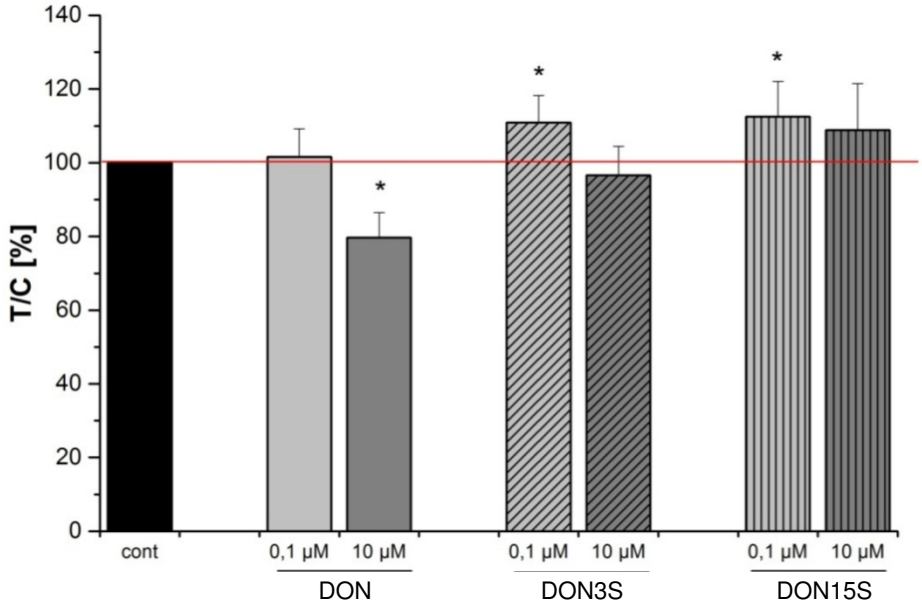

Figure 24: Results of the quantified fluorescence signal of the nuclear protein KI-67 after an incubation of 24 hours with DON, DON3S and DON15S at concentrations at 0,1 µM and 10 µM.

** indicates significant differences compared to the negative control (* p<0,05)*

Values are expressed as mean ± SEM of four independent experiments performed at least in triplicates. Statistical significance was evaluated by Kruskal-Wallis ANOVA.

Image analysis revealed that the incubation with 0,1 µM DON has no significant effect on the expression of KI-67. Nevertheless, 10 µM DON caused a significant reduction of KI-67 (79,7 ± 6,7 % of the fluorescence signal of the negative control), a concentration which was identified in the SRB and WST-1 as cytotoxic.

In line with the previous results, cells treated with both sulfated DON derivatives DON3S and DON15S at 0,1 µM showed a significant higher fluorescence intensity of KI-67 compared to the negative control. The higher concentration of 10 µM DON3S

and DON15S did not result in a significant effect, even though a tendency toward the increase was observed for the DON15S. For clarity, all the statistical significances evaluated by Kruskal-Wallis ANOVA and the respective T/C-values in percentage terms are indicated in Table 20 below, where "+" represents an increase and "–" a decrease in the fluorescence signal of KI-67.

Table 20: Significant differences in the fluorescence signal of KI-67 after 24 hours of incubation with DON, DON3S and DON15S evaluated by Kruskal-Wallis ANOVA in comparison to the negative control. + indicates an increase and - a decrease in relative KI-67 signal.

24 hours	**10 µM DON** 79,7 ± 6,7 %	**0,1 µM DON3S** 110,9 ± 7,3 %	**0,1 µM DON15S** 112,5 ± 9,6 %
negative control	- p<0,05	+ p<0,05	+ p<0,05

4.6 Structure illumination Microscopy (SIM) images of the cell proliferation marker KI-67 and lamin B

Beside the confocal images taken with the 63x oil immersion objective, SIM images , were acquired with a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system using a 100x oil objective (zoom 2), in order to illustrate and support the quantification data outlined above. Thanks to the Structure Illumination Microscopy it is possible to double the resolution of the confocal images for a more detailed localization of the structures of interest. The images, demonstrating in blue lamin B as the nuclear structure and in white the proliferation marker KI-67, are shown in Figure 25.

As already observed in Figure 22 and Figure 23 at a magnification of 63x (zoom 2), the KI-67 signal appears in some areas of the cells treated with the sulfated derivatives much stronger than in comparison to the cells incubated with 0,1 µM of the parent compound DON.

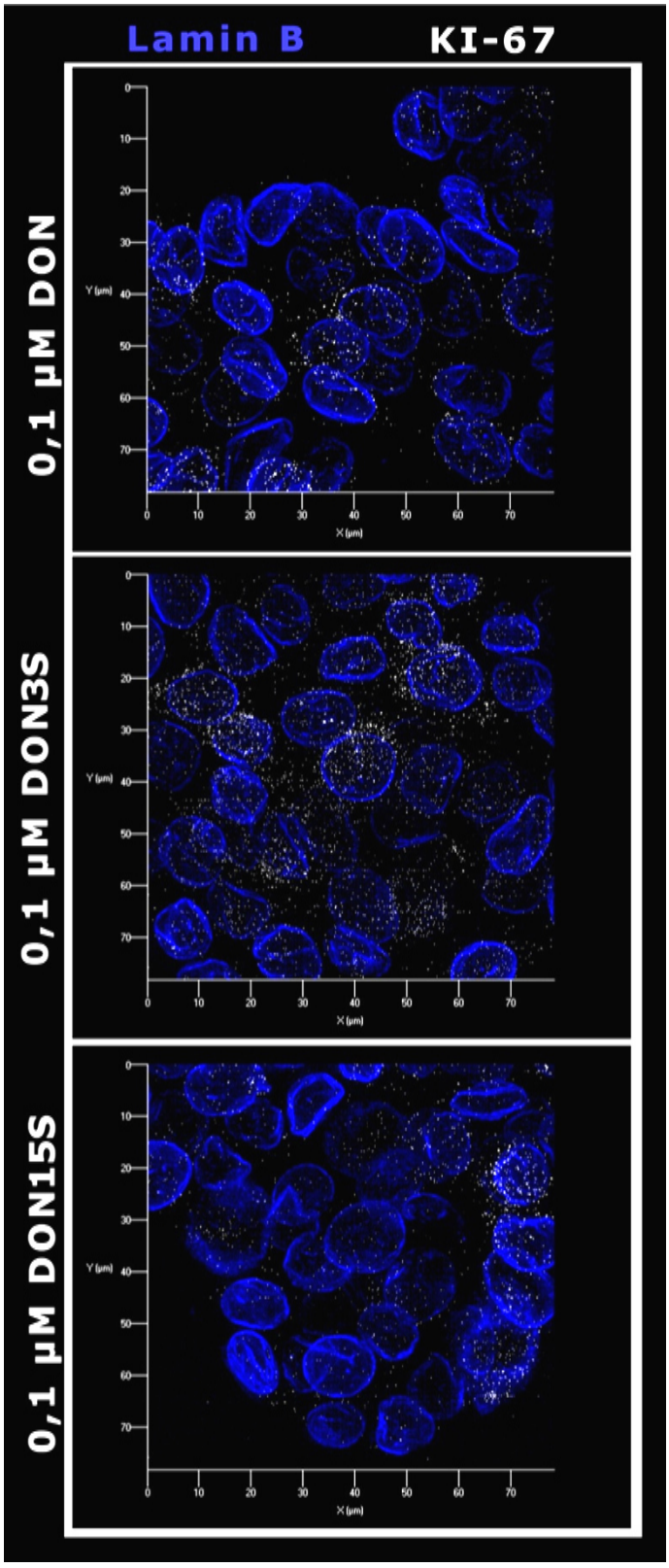

Figure 25: SIM images of HT-29 cells demonstrating the proliferation marker KI-67 (white) and as the nuclear structure lamin B (blue) after 24 hours of incubation with 0,1 µM DON, DON3S and DON15S.

Images are acquired using an oil immersion objective of a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system at the following configurations: 100x magnification, rotations 5, phases 5.

4.7 Analysis of mitotic structures - further evaluation of the stimulating effect on the formation of mitotic structures caused by DON and its sulfo-derivatives DON3S and DON15S

During the performance of the confocal experiments and the image acquisition for the KI-67 quantification an exceeding occurrence of cells in mitosis was visible in intestinal cells exposed to the sulfated DON derivatives. In order to quantify the presence of cells in active mitosis, optical fields were imaged at low magnification (10x magnification, zoom 3) and the percentage of mitotic structures (mitotic spindles) was calculated, as described in detail below in 7.3.4.1. The results regarding this analysis are demonstrated in Figure 26.

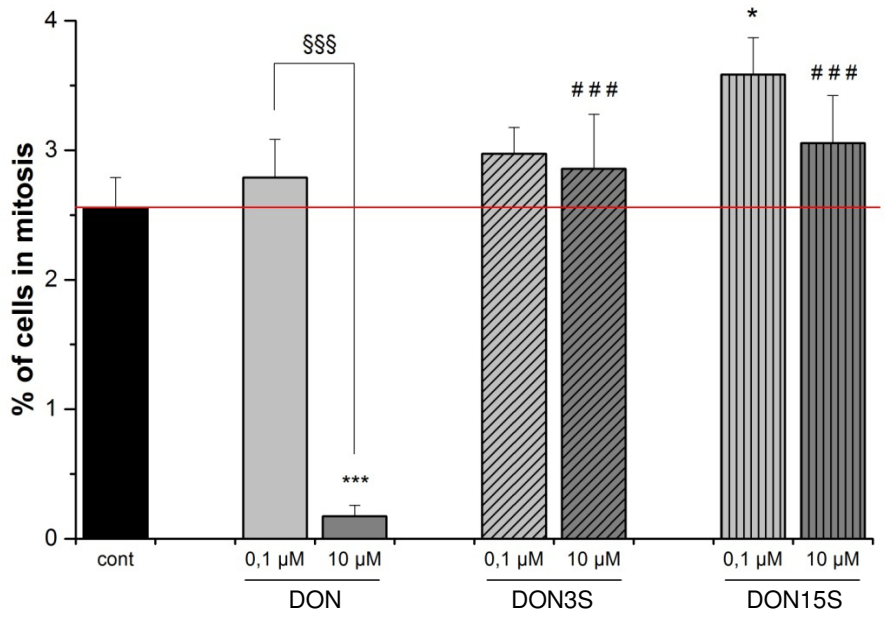

Figure 26: Results of the quantified amount of cells in mitosis after an incubation of 24 hours with DON, DON3S and DON15S at concentrations at 0,1 µM and 10 µM.

** indicates significant differences compared to the negative control (* p<0,05, *** p<0,001)*

 # indicates significant differences compared to the values of DON at the same concentration (### p<0,001).

Values are expressed as mean ± SEM of four independent experiments performed at least in duplicates. Statistical significance was evaluated by one-way ANOVA and Fisher post-hoc test.

Mitotic structures were barely found in the cells incubated with 10 µM DON (0,17 ± 0,09 % of mitotic cells were identified) which mirrored a significant decrease of

intestinal cells actively replicating in comparison to the negative control. On the contrary, a significant increase of mitotic cells was evaluated in cells incubated 24 hours with 0,1 µM DON15 ($p<0,05$) and also in regard to 0,1 µM DON3S a trend to increase the amount of cells in mitosis could be observed, which, however, did not result in a significant difference to the control.

Compared to the low percentage value obtained after 24 hours of incubation with DON 10 µM, a significant difference could be identified in cells incubated with 10 µM DON3S and DON15S ($p<0,001$). These significances are indicated in the graph in Figure 26 with #-symbols.

For clarity, all the statistical significances evaluated by one-way ANOVA and Fisher's LSD post-hoc test and also the respective values in percentage terms are indicated in Table 21 below, where "+" represents an increase and "–" a decrease in the percentage of cells in mitosis.

Table 21: Significant differences in the percentage of cells in mitosis after 24 hours of incubation with DON, DON3S and DON15S evaluated by one-way ANOVA and Fisher's LSD post-hoc test in comparison to the negative control and to the respective DON concentration. + indicates an increase and - a decrease in the percentage of cells in mitosis.

24 hours	**10 µM DON** 0,17 ± 0,09 %	**10 µM DON3S** 2,86 ± 0,42 %	**0,1 µM DON15S** 3,59 ± 0,29 %	**10 µM DON15S** 3,05 ± 0,37 %
negative control 2,56 ± 0,23 %	- $p<0,001$		+ $p<0,05$	
0,1 µM DON 2,79 ± 0,29 %	$p<0,001$			
10 µM DON 0,17 ± 0,09 %		$p<0,001$		$p<0,001$

Furthermore, in addition to bipolar spindle structures, a high incidence of multipolar spindles, such as shown in Figure 27, was observed especially in cells treated with DON at a concentration of 0,1 µM and also with the sulfated DON derivatives DON3S and DON15S. However, a complete characterization of this effect is beyond the scope of the present thesis and is currently under investigation at the Department of Food Chemistry and Toxicology.

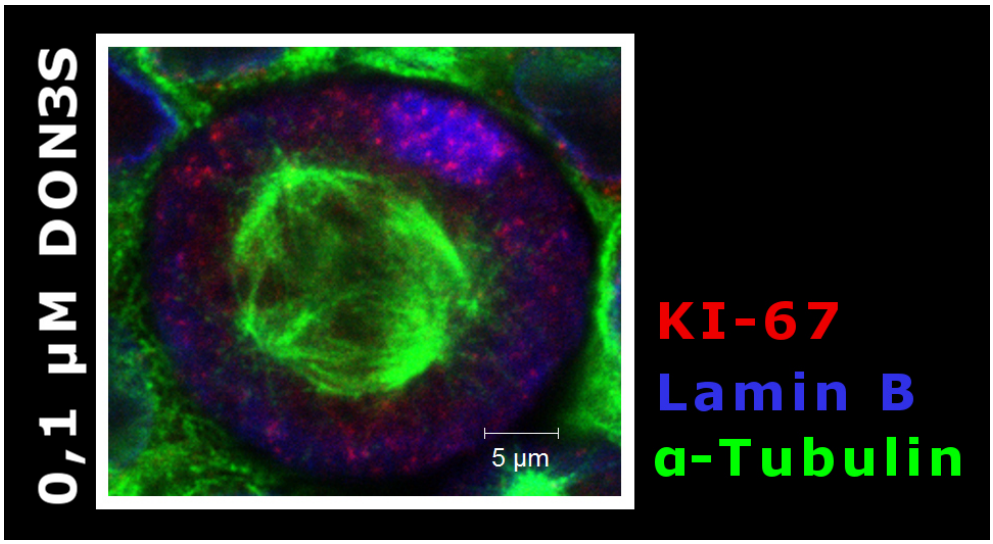

Figure 27: An example of a multipolar spindle as a confocal image of HT-29 cells demonstrating the expression of the cellular proliferation marker KI-67, as the nuclear structure lamin B and as a component of the cytoskeleton, α-tubulin after 24 hours of incubation with DON3S 0,1 µM. (scale bar: 5 µm)

Images are acquired using an oil immersion objective of a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system at the following configurations: 63x magnification, zoom 2, average 16, speed 7.

5. Discussion

During the development of the present thesis, two novel metabolites of the mycotoxin DON were characterised for the first time in human intestinal cells (HT-29). In order to obtain this result a panel of assays was used and this included both routinely used techniques (DCF assay, SRB assay, WST-1 assay), as well as the optimisation of new microscopy-based assays (confluence analysis and advanced confocal imaging).

Several studies previously reported that DON is able to trigger an increase in intracellular ROS in different cell lines, such as animal cells like rat liver clone-9 cells (Sahu *et al.*, 2008) or also human leukemic monocyte lymphoma cells, U937. In the latter example, DON was effective at concentrations of 80 and 160 µM after 8 hours of incubation (Costa *et al.*, 2009). Previous studies characterised the effect of DON on ROS production in HT-29 cells (Krishnaswamy *et al.*, 2010). In this study significantly increased ROS levels were reported at concentrations between 0,8 and 1,6 µM after 30 minutes of incubation (Krishnaswamy *et al.*, 2010). In addition, Katika *et al.* (2015) investigated the impact of 0,5 µM DON on the cellular ROS levels of human Jurkat T-lymphocytes and showed a significant increase of ROS after 6 hours of incubation, whereas after 3 hours no statistical significant differences could be observed. In our experimental system the cellular ROS levels of HT-29 cells after DON-treatment showed a significant effect only at the highest incubation concentration of 10 µM ($p<0,01$). The toxin seemed to react relatively fast, since these significant increases could be identified only after three minutes of incubation, followed even by a slight decrease of ROS levels after ten minutes, before it increased again. Literature data suggest a wide variability in the cellular response to DON in terms of ROS production, and the data presented in this thesis can be considered on average, in line with those presented in literature.

In the cells treated with the metabolites a significant induction of ROS production was mainly observed in cells treated with DON3S at 10 µM between three minutes and one hour of incubation. Nevertheless a slight increase of ROS (5-15 %) was also identified after one hour of incubation with the lower DON3S concentrations 0,1 and 1 µM and with 1 µM of DON15S as well. These results are particularly intriguing since it was previously reported that induction of low intracellular oxidative stress can serve as a stimulus for cell proliferation in cancerogenic cells (Liou and Storz, 2010). In BHK-21 hamster fibroblasts, for instance, a treatment with 1 µM H_2O_2 stimulated cell proliferation (Burdon *et al.*, 1996) and similar results have also been found in human cell lines. For example, Murrell *et al.* (1990) investigated different types of fibroblasts

which showed after a release of the superoxide radical $O_2^{\bullet -}$ an increased cell proliferation. Furthermore, using a free radical scavenger, a concentration-dependent inhibition of proliferation could be identified (Murrell *et al.*, 1990). Accordingly, low inductions of oxidative stress seem to induce a growth stimulus and to activate cell proliferation (Liou and Storz, 2010).

The cytotoxicity and cell proliferation assays conducted during this thesis in HT-29 cells treated with DON3S and DON15S (concentration range between 0,1 nM and 100 µM) pointed towards a strong stimulating effect of the compounds on the cell growth. After 24 hours of incubation, significant increase of protein content was identified in cells incubated with DON3S, DON15S and to a minor extent with DON. The increase reached the maximal value (147,1 ± 17,4 % of controls) after incubation with 0,1 µM DON3S, but also incubations with 1 to 25 µM of DON3S and DON15S caused significant increases of the cellular protein content around 130 %. These results on cell proliferation combined with the low intracellular concentrations of ROS, found after the incubation with the sulfated derivatives in the DCF assay experiments, seems to suggest a link between these two events. The occurrence of the compounds DON-3-sulfate and DON-15-sulfate as wheat metabolites has been lately discovered in 2015 (Warth *et al.*, 2015) and their identification as human metabolites in the urine is even more recent (Warth *et al.*, unpublished), thus further studies are needed to evaluate if these effects can be translated also to other cell types.

In agreement with literature, DON showed a clear cytotoxic response at concentrations higher than or equal to 1 µM, whereas after 24 hours of incubation with 0,1 µM DON a significant increase has been identified in the SRB assay. After 48 hours of DON-incubation the level of total cellular protein was still around 8 % higher than the one of the negative control, but no statistically significant increase could be detected. These data are in agreement with literature, since it was recently demonstrated by Pizzo *et al.* (2015) a stimulating effects of DON in bovine large follicle granulose cells. At DON concentrations of 0,33 µM (48 hours of incubation) a significant increase of the cell number was identified using a coulter counter (+ 14 %). In addition, a different study performed in intestinal porcine epithelial cells after 48 and 72 hours of incubation with DON (concentrations around 6 µM; basolateral exposure) demonstrated a significant relative increase in cell proliferation determined by BrdU incorporation (Diesing *et al.*, 2011a).

The cytotoxic effects caused by DON at higher concentrations (1-100 µM) after 24 hours lead to a decrease of total protein content around 40 % in comparison to controls (SRB assay). A longer incubation time (48 hours) resulted in even stronger cytotoxic effects decreasing the cellular protein content down to 39,2 ± 7,7 % at 10 µM and around 12-15 % at concentrations of 25, 50 and 100 µM DON. Pizzo *et al.* (2015) identified this biphasic activity of DON as well, since they reported, beside the above mentioned proliferation stimulus, also cytotoxicity and a decrease in cell number of 22 % at a DON concentration of 3,3 µM. In the study conducted by Diesing *et al.* (2011a) in IPEC-J2 cells, nuclei were counted manually using a DAPI staining. An apically exposure of DON did not show an effect on the cell number, but after an incubation for 24 hours with 13,5 µM applied basolaterally a decrease of DAPI stained nuclei was observed, which was evaluated statistically significant after 48 hours (Diesing *et al.*, 2011a). In this respect, the presence of the sulfate-group in the molecule seems to "stabilize" the proliferative effect, possibly due to the absence of the cytotoxicity that limits the effect of DON.

Since drawing conclusions from one single assay would have been very limiting, it was decided to clarify if the results obtained in the SRB assay could be confirmed by other cytotoxicity and proliferation assays not based on measurements of the total protein content. In this respect, the WST-1 assay and also a microscopic confluence analysis were performed using the most relevant concentrations (1 nM-10 µM). Since the WST-1 assay is measuring the mitochondrial activity of the cells, which can further be related to cell viability and number, the results confirmed a significant decrease in absorbance values after treatment with DON at 1 and 10 µM ($p<0,01$). This assay was also performed in a study from Nielsen *et al.* (2009) to evaluate the cytotoxic effects induced by trichothecenes, including DON, in eight different human cell lines. They reported, for instance, after 48 hours of incubation an IC_{50} value of 1 µM in Caco-2 cells and identified Jurkat cells with an IC_{50} value of 0,6 µM as the most sensitive cell line among the eight. HUVEC and HEp-2 cells instead were reported with IC_{50} value of 4,5 µM and 4,9 µM, respectively as the less susceptible cell lines investigated during this study (Nielsen *et al.*, 2009). Obviously the WST-1 assay does not provide information on the protein content, so the outcome of the assay can only be eventually related to a variation of the cell number or of the mitochondrial activity. However, Nielsen *et al.* (2009) found in several cell lines, such as Hep-G2 and U937 a significant induction of the mitochondrial activity after incubations with 0,1 µM DON.

In regard to WST-1 experiments performed with DON3S and DON15S again nothing has been reported yet, but in comparison to the negative control an increase in mitochondrial activity could be observed at DON3S concentrations higher than and equal to 0,1 µM which reached statistical significance at 1 µM ($p<0,05$). Treatment with 0,1 and 1 µM DON15S showed a higher mitochondrial activity compared to the solvent control, but could not be evaluated significantly different.

Similar results could be found in the confluence analysis, where digital phase contrast brightfield images of the growth surface were acquired and the percentage of the area covered with cells was calculated using the image statistics application of the BioTek™ Gen5™ software. The analysis of the confluence is useful to discriminate between an increase of the SRB signal due to an increase of the cellular proteins or to the number of cells. Cells treated 24 hours with DON, for instance, showed again a significant increase at 0,1 µM but beyond that also at 1 µM ($p<0,05$). Also in regard to the increased cell proliferation identified after incubation with the sulfated DON derivatives, a significantly higher confluence could be identified after a treatment with 0,1 and 1 µM of DON3S for 24 hours. DON15S did not show a significant increase in the cell confluence in a range of concentrations at which also in the WST-1 assay no significant differences in the mitochondrial activity were reported. Taken together, these data about cell proliferation seem to point toward an effect of the compounds on the cell number rather than limited to the protein content.

Furthermore in both assays WST-1 and confluence analysis the sulfated derivatives caused at very low concentrations (1 or 10 nM) a significant decrease of the mitochondrial activity and the confluence, respectively. Intriguingly, not only DON seems to show a biphasic activity but also the sulfated DON metabolites. In regard to this decrease in mitochondrial activity and confluence, to date no further experiments or publications have been reported yet.

After this different proliferation and cytotoxicity assays, immunocytochemical experiments were performed and confocal images were acquired using a confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system. These experiments were planned to confirm the proliferation stimulus triggered by DON3S and DON15S in an experimental system that does not require spectrophotometric measurements. To this aim, immunocytochemical stainings and quantification of the protein KI-67, a cellular proliferation marker, were chosen.

In this respect, several studies have already used KI-67 to describe the cytotoxicity of DON and its toxicological potential. For instance, during a feeding study in piglets, the mRNA levels of KI-67 were monitored by qRT-PCR. After a short-term, low-dose exposure to DON the protein was significantly downregulated in the jejunum. Nevertheless, an immunohistochemical staining for KI-67 did not reveal obvious changes in the protein distribution in the jejunum compared to piglets exposed as a control group to uncontaminated feed (Alizadeh *et al.*, 2015). A further study conducted *ex vivo* on intestinal explants of piglets used immunohistochemical staining of the KI-67 protein to evaluate the effect of DON exposure on the treated tissue. To quantify the positive immunoexpression of KI-67, strongly positive stainings in the nuclei were counted and no significant effects were identified after DON-treatment (da Silva *et al.*, 2014). However, during this thesis, in order to perform quantifications of the KI-67 signal, 3D reconstructions of the nuclei have been acquired and the nuclear protein signals were evaluated of maximum intensity projections using the software ImageJ. This approach allows to evaluate the fluorescent signal from the complete nuclear region and not only from a single focus plane. In our experimental system a significant differences in the fluorescence signal could be identified after 24 hours of incubations with 0,1 and 10 µM of DON, DON3S and DON15S. In fact, a treatment with 10 µM DON caused a significant decrease of the KI-67 signal ($p<0,05$), whereas 0,1 µM of both sulfated derivatives DON3S and DON15S caused a significantly increased fluorescence intensity of KI-67 ($p<0,05$). Accordingly, these data support both the cytotoxic effect caused by 10 µM in HT-29 cells and in addition also the activation of cell proliferation after incubation with DON3S and DON15S primarily at a concentration of 0,1 µM.

Since during the performance of these image acquisitions a high incidence of cells in mitosis has been observed, a quantification of this event was performed as a last assay of this thesis. In these experiments confocal images were acquired at a magnification of 10x (zoom 3) and cells were counted manually for the estimation of the percentage of cells in active mitosis. Also in this assay incubations with DON at 10 µM showed a significantly decreased amount of cells in mitosis ($p<0,001$), whereas after 24 hours of incubation only 0,1 µM of DON15S caused a significantly increased number of mitotic cells. Moreover, in addition to bipolar mitotic spindles also a high incidence of multipolar spindles has been identified during these quantifications primarily in cells incubated with DON but they also occurred in wells incubated with DON3S and DON15S. In regard to this, a study conducted in porcine oocytes incubated for 42

hours together with different concentrations of DON (0,02, 0,2, 2 µM) showed significant alterations of the meiotic spindle formation and also a decreased progression through meiosis. Increasing concentrations of DON significantly reduced the number of meiotic cells reaching the M II stage and a higher incidence of chromatin and meiotic spindle malformations were observed (Schoevers *et al.*, 2010). In this respect, the data presented in this thesis open new perspectives for a further and more focused analysis of the effects of the DON3S and DON15S on the mitotic spindle and on their effect on the cell cycle.

6. Summary

The type B trichothecene mycotoxin DON occurs worldwide in cereals and cereal-derived products at concentrations ranging from some micrograms up to more than 40 mg/kg (European Commission, 2003) and, since its toxicological profile has been investigated for decades, the JECFA established a PMTDI of 1 µg/kg bw (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2002). Recently, two metabolites DON-3-sulfate and DON-15-sulfate have been discovered in artificially inoculated wheat and the former one also as a human urinary metabolite (Warth *et al.*, 2015; Warth *et al.*, unpublished). Accordingly, due to their novelty, apart from an *in vitro* translation assay (Warth *et al.*, 2015), further toxicological characterisation of these compounds was not available.

Since the parent compound DON was known to induce oxidative stress (Katika *et al.*, 2015) and to cause statistically significant cytotoxic effects on human intestinal cells (Caco-2 cells) at 10 µM (Kouadio *et al.*, 2005), this thesis aimed to initiate the characterisation of the biological activity of DON3S and DON15S concerning their cytotoxicity, their potential to cause cellular oxidative stress and mechanisms of action in the human colorectal adenocarcinoma cell line HT-29.

As a first step, the DCF assay was performed: in these experiments slightly increased ROS levels primarily induced by DON3S were identified (further explained in 4.1). Such a low intracellular oxidative stress is known to stimulate cell proliferation in cancerogenic cells (Burdon *et al.*, 1996; Liou and Storz, 2010) and thus the increased total protein content (explained in 4.2) and mitochondrial activity (further details in 4.3) determined after incubation with DON3S and DON15S in the SRB and the WST-1 assay, respectively, could be the result of the low cellular oxidative stress. Beside these two photometric assays, a significantly increased cell proliferation induced by DON3S and DON15S was also confirmed after a quantification of the KI-67 signal (shown in 4.5) and the cells in mitosis (indicated above in 4.7) using immunocytochemistry and confocal microscopy. In order to determine if this trigger of proliferative stimulation is a characteristic limited to the cancerogenic nature of HT-29 cells or can also be found in non tumourigenic cells, further experiments are in progress to clarify this aspect.

In addition, during the acquisition and analysis of the confocal images a high incidence of multipolar spindle formations was observed (further explained in 4.7). As far as this phenomenon is concerned, not only a quantification of these mitotic malformations in

the cancer cells, but again the evaluation in non cancerogenic cells needs to be performed, in order to evaluate its toxicological relevance.

Since DON is known, and this effect was also confirmed by the results obtained during this thesis, to cause cytotoxic effects and to induce apoptosis, as already mentioned above, at concentrations around and higher than 1 µM (Kouadio *et al.*, 2005), its sulfo-metabolites DON3S and DON15S, which were expected to be related in their toxicity to the parent compound and which did not show such cytotoxic effects, could be considered detoxification products of DON. Nevertheless, since in the used experimental system the sulfated derivatives do not show the same biological effects of DON, the characterisation of their activity, especially for their potential to trigger cell proliferation, needs to be further investigated. These data will be crucial for a proper evaluation of the risk for humans and animals associated to the exposure to these novel DON-metabolites and their entrance into the food and feed chain.

7. Materials and methods

7.1 Chemicals and devices

7.1.1 Chemicals

• Acetic acid	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Albumin Fraction V/Bovine serum albumin (BSA), ≥98 %, powdered	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• 2', 7'-Dichlorofluorescin diacetate (DCFH-DA), ≥97 %	Sigma Aldrich Corporation, St. Louis, US
• Dimethyl sulphoxide (DMSO), ≥99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Donkey anti-Goat IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 647 conjugate, for IF	Thermo Fisher Scientific, Waltham, US
• Donkey anti-Mouse IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 488 conjugate, for IF	Thermo Fisher Scientific, Waltham, US
• Donkey anti-Rabbit IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 568 conjugate, for IF	Thermo Fisher Scientific, Waltham, US
• Ethanol, denaturated, 96 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Formaldehyde solution, 36,5-38 %	Sigma Aldrich Corporation, St. Louis, US
• Fluorescence Mounting Medium	Agilent Technologies, Santa Clara, US
• Glycine, PUFFERAN®, ≥99 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• HEPES, PUFFERAN®, ≥99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Hydrogen peroxide (H_2O_2), 30 %, ROTIPURAN®, stabilised	Carl Roth GmbH + Co. KG, Karlsruhe, DE

• Hydrochloric acid (HCl), 37 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Immersion oil Immersol 518 F, for 30 °C, fluorescence free	Carl Zeiss Microscopy GmbH, Jena, DE
• KI-67 antibody (H-300), sc-15402, Rabbit polyclonal IgG	Santa Cruz Biotechnology Incorporated, Dallas, US
• Lamin B antibody (C-20), sc-6216, Goat polyclonal IgG	Santa Cruz Biotechnology Incorporated, Dallas, US
• Live Cell Imaging Solution	Thermo Fisher Scientific, Waltham, US
• Methanol (for LC-MS)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Potassium dihydrogen phosphate (KH_2PO_4), ≥99 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Potassium chloride (KCl)	Merck KGaA, Darmstadt, DE
• Sodium chloride (NaCl), ≥99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot H_2O$), ≥99,5 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Sodium hydroxide (NaOH), ≥98 %, in pellets	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Sulforhodamine B sodium salt (SRB), powder	Sigma Aldrich Corporation, St. Louis, US
• Tris(hydroxymethyl)-aminomethane, PUFFERAN®, ≥99,3 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Tris-hydrochloride	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Trichloroacetic acid (TCA), ≥99 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE

- Triton® X 100, extra pure — Carl Roth GmbH + Co. KG, Karlsruhe, DE
- α Tubulin antibody (B-7), sc-5286, Mouse monoclonal IgG — Santa Cruz Biotechnology Incorporated, Dallas, US
- Water (H_2O), LC-MS Ultra CHROMASOLV®, tested for UHPLC-MS — Sigma Aldrich Corporation, St. Louis, US
- WST-1 cell proliferation reagent — Roche Applied Science, Basel, CH

7.1.2 Cell culture reagents

- Dulbecco's Modified Eagle Medium (DMEM), high glucose, phenol red, L-glutamine — Thermo Fisher Scientific, Waltham, US
- Dulbecco's Modified Eagle Medium (DMEM), with 1.000 mg/L glucose and L-glutamine, without sodium bicarbonate and phenol red, powder — Sigma Aldrich Corporation, St. Louis, US
- Fetal bovine serum (FBS), qualified, South America origin — Thermo Fisher Scientific, Waltham, US
- Penicillin-Streptomycin (P/S), with 5.000 U/ml and 5.000 µg/ml — Sigma Aldrich Corporation, St. Louis, US
- Trypan blue solution, sterile filtered — Sigma Aldrich Corporation, St. Louis, US
- Trypsin, 5.000 USP-U/mg, free from salt, from porcine pancreas, lyophilised. — Carl Roth GmbH + Co. KG, Karlsruhe, DE

7.1.3 Cell line

- Human colorectal adenocarcinoma cells HT-29 — German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, DE

7.1.4 Test reagents

- Deoxynivalenol (DON) — Romer Labs Division Holding GmbH, Getzersdorf, AT

- Deoxynivalenol-3-sulfate (DON-3-SO_4/DON3S), — in-house synthesised as published in Fruhmann *et al.* (2014)
- Deoxynivalenol-15-sulfate (DON-15-SO_4/DON15S) — in-house synthesised as published in Fruhmann *et al.* (2014)

7.1.5 Consumable materials

- 8 Chamber polystyrene vessel tissue culture treated glass slide — Corning Incorporated, New York, US
- 96-well assay plate, black plate, clear bottom, tissue culture treated, sterile — Corning Incorporated, New York, US
- 96-well cell culture plate, standard growth surface for adherent cells, sterile — Sarstedt AG & Co, Nümbrecht, DE
- Cell culture flask T-75, standard growth surface for adherent cells, ventilation cap, sterile — Sarstedt AG & Co, Nümbrecht, DE
- Centrifuge Tube (15 ml, 50 ml), for light sensitive samples, sterile — VWR International, Radnor, US
- Combitips (10 µl, 50 µl, 100 µl, 250 µl), sterile — Eppendorf AG, Hamburg, DE
- DURAN® Laborflasche (100 ml, 250 ml, 500 ml, 1.000 ml, 2.000 ml) — DURAN Group GmbH, Wertheim/Main, DE
- Hemocytometer/Neubauer counting chamber — Labor Optik GmbH, Lancing UK
- Light-Duty Tissue Wipers — VWR International, Radnor, US
- Microscope cover glasses 24 x 24 mm, thickness 0.17 ±0.005 mm) — Carl Roth GmbH + Co. KG, Karlsruhe, DE
- Microscope cover glasses 24 x 60 mm, thickness No. 1.5 (0.16 to 0.19 mm) — Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, DE
- Nitrile Gloves, size M, powder free — Kimberly-Clark GmbH, Koblenz-Rheinhafen, DE

• Pasteur pipette, without cotton stoppers, overall length 230 mm	Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Pipette tips (10 µl, 200 µl, 1.000 µl), non-sterile	Sarstedt AG & Co, Nümbrecht, DE
• Pipette tips (5.000 µl), non-sterile	Eppendorf AG, Hamburg, DE
• Reaction tubes (0,5 ml, 1,5 ml, 2 ml, 5 ml), non-sterile	Sarstedt AG & Co, Nümbrecht, DE
• Serological pipette (5 ml. 10 ml, 25 ml), sterile	Sarstedt AG & Co, Nümbrecht, DE
• Tube (15 ml, 50 ml), transparent, sterile	Sarstedt AG & Co, Nümbrecht, DE
• Vacuum filtration unit Filtropur V 50, 500 ml, 0,22 µm pore size, sterile	Sarstedt AG & Co, Nümbrecht, DE

7.1.6 Instruments

• Autoclave	Systec DX-150: Systec GmbH, Weitenberg, DE
• Balances	ML204/01 NewClassic Analytical Balance: Mettler-Toledo, Columbus, US XP26 DeltaRange Microbalance: Mettler-Toledo, Columbus, US ML6001/01 NewClassic Analytical Balance: Mettler-Toledo, Columbus, US
• Drying chamber	ATP Line™ ED (E2): Binder GmbH, Tuttlingen, DE
• Fridge-freezer	Liebherr Premium and Comfort (4°C, - 20°C): Liebherr, Bulle, CH

- Incubator — Heracell 240i, CO_2 Incubator:

  Thermo Fisher Scientific, Waltham, US

- Laminar flow hood — HeraSafe™ KS 18:

  Thermo Fisher Scientific, Waltham, US

  HeraSafe™ KSP 18:

  Thermo Fisher Scientific, Waltham, US

- Magnetic stirrer — IKA® RCT basic:

  IKA®-Werke GmbH & CO. KG, Staufen, DE

- Microscopy — Inverted microscope Axiovert 40C, Objectives: A-Plan 5X/0,12 Ph0, A-Plan 10X/ 0,25 Ph1 Ocular: PL 10X/18:

  Carl Zeiss Microscopy GmbH, Jena, DE

  LSM confocal, with ELYRA-system PS.1
  Objectives: Plan-Neofluar 10X/0,30, Plan-Apochromat 63X/1,4 oil, Plan-Apochromat 100X/1,46 oil:

  Carl Zeiss Microscopy GmbH, Jena, DE

- Multi-channel pipette — Research® plus 300 µl:

  Eppendorf AG, Hamburg, DE

- Multi-mode microplate reader — Cytation™ 3 Cell Imaging Multi-Mode Reader
  Software: Gen5™ Data Analysis Software:

  BioTek Instruments Incorporated, Winoosky, US

- pH meter — S20 SevenEasy™ pH, electrode: InLab 408/120:

  Mettler-Toledo, Columbus, US

• Pipettes	Research® P 2,5-5000 µl: Eppendorf AG, Hamburg, DE
• Pipette filler	pipetus® 100-240 V, battery powered: Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, DE
• Pump	Vacuum Aspiration System: Ditabis, Pforzheim, DE Vacuum pump Laboport®: Integra Biosciences AG, Zizers, CH
• Rocker	Mini Rocker MR-1: VWR International, Radnor, US
• Stepper/Repetitive pipette	Rainin AutoRep M: Mettler-Toledo, Columbus, US
• Tweezers	Precision tweezers, Eco, Inox02: Carl Roth GmbH + Co. KG, Karlsruhe, DE
• Ultra low temperature freezer (- 80°C)	MDF-U53V, VIP series: Sanyo electric Co. Ltd, Moriguchi, JP
• Ultraviolet crosslinker	BIOLINK™ BLX-312: VWR International, Radnor, US
• Vortex mixer	Lab dancer S40: IKA®-Werke GmbH & CO. KG, Staufen, DE
• Water bath	GD 100: Keison Products, Chelmsford, GB
• Centrifuge	MIKRO 220: Andreas Hettich GmbH & Co.KG, Tuttlingen, DE

7.2 Cell culture

7.2.1 Cell line HT-29

HT-29 (Figure 28) is a human colon adenocarcinoma cell line isolated in 1964 from the primary tumour of a 44-year-old Caucasian female patient with colorectal adenocarcinoma by J. Fogh using the explant culture method (ATCC, 2014). The cell line was described to form well-differentiated grade I tumours and further to be herterotransplantable. HT-29 cells have an epithelial morphology and grow adherent with a characteristic doubling time around 40-60 hours both as monolayers and in large colonies (Deutsche Sammlung von Mikroorganismen und Zellkulturen, 2016).

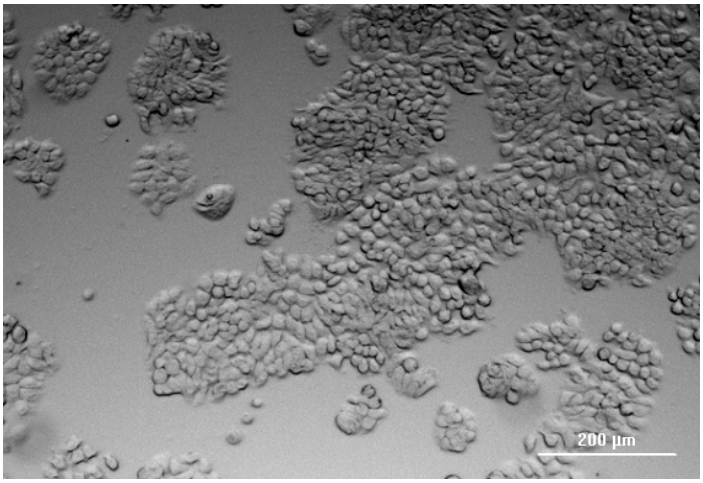

Figure 28: HT-29 cells (scale bar: 200 µm)

7.2.2 Cultivation of HT-29

The tumourigenic cell line HT-29 was maintained in culture in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 1 % v/v penicillin/streptomycin (P/S) and 10 % v/v heat-inactivated foetal calf serum (FCS). DMEM contained glucose, vitamins, inorganic salts and amino acids and was therefore supporting cellular growth. As DMEM contained sodium bicarbonate (3,7 g/L) as a buffer system it requires a 5-10 % CO_2 environment to maintain physiological pH (Thermo Fisher Scientific, 2015a). The addition of FCS was necessary to supply the cells not only with growth factors and hormones but also with a complex array of proteins needed for attachment and spreading (Gstraunthaler and Lindl, 2008).

Cells were cultivated in 75 cm^2 cell culture flasks and, as indicated above, in a 5 % CO_2 environment at 37 °C in a humidified incubator. All the solution used for the cell culture workflow were pre-warmed at 37 °C in the water bath to create for the cells an

atmosphere as regular and as consistent as possible. To prevent contaminations all the operations were performed in laminar flow cabinets (Gstraunthaler and Lindl, 2008).

7.2.3 Subculturing/Passaging of HT-29

Due to the increase in cell number, gradual rise of toxic metabolites and use of nutrients of the culture medium cells should have been subcultured at a confluence, which is the percentage of the growth surface covered by cells, of about 80-90 %. Furthermore another indication that the cells needed to be transferred from the previous culture to fresh growth medium was a change of colour of the pH indicator phenol red from red to yellow (Gstraunthaler and Lindl, 2008).

In the first step the medium was removed using a sterile Pasteur pipette and a vacuum pumping system. Cells were rinsed with 5 ml of phosphate buffered saline (PBS), to remove dead, non-adherent cells and the last traces of serum containing medium, which would affect the activity of the proteolytic pancreas enzyme trypsin. After removing the PBS about 1,5 ml trypsin are added to the cells and the culture flask was placed in the incubator for 3-4 minutes (5 % CO_2, 37 °C) for the optimal activity of trypsin. As Trypsin catalyses the hydrolysis of peptide bonds, during this incubation the proteins bonding the cultured cells to surface were cleaved by the enzyme. Beyond that, if the detachment of the cells was low, the flask could have also been hit gently on the surface of the laminar flow hood. Incubating cells too long or at too high trypsin concentrations will damage cell membranes and kill them. So if most cells were floating further tryptic digestion should have been terminated as soon as possible by adding medium containing serum (Gstraunthaler and Lindl, 2008). In order to ensure homogeneous distribution of the cells in suspension, they were repeatedly gently pipette to break up lumps using a 10 ml sterile pipette. At the same time during this pipetting process cell suspension was in constant contact with the flask surface, so that most of adhering cells could be detached and resuspended.

To use the cell suspension, for carrying on the culture or for different experiments, the total number of cells and also cell viability needed to be determined (see 7.2.4). In case of HT-29, for the maintenance of the culture, about two million cells were added in a new culture flask (75 cm^2) together with about 14-16 ml culture medium (preparation as described above in 7.2.2), with the result that, dependent on their individual growth, they could be subcultured regularly every 3-4 days. After preparing the new culture flask, cells were incubated again at 37 °C and 5 % CO_2 in a humidified incubator while

the remaining cell suspension could be seeded in preparation of the experiments (Gstraunthaler and Lindl, 2008).

7.2.4 Counting and determining the viability of cultured cells

7.2.4.1 Cell counting using a Hemocytometer

Counting and determining the exact number of cells in a cell suspension is not only important in standardisation of culture conditions but also to guarantee the reproducibility of cell experiments. According to this, methods for determination of cell numbers and their viability play an important part in quality control for cell culture systems (Gstraunthaler and Lindl, 2008).

Hemocytometers (Figure 29) are devices used to perform cell counts, made out of a thick glass microscope slide. The surface of the counting chamber has a certain grid, so that, after the correct positioning of a special coverslip, the area bounded by the lines as well as the volume of the chamber is known (Gstraunthaler and Lindl, 2008). The depth of the counting chambers manufactured by Marienfeld-Superior and used to perform the cell count for the following experiments was 0,1 mm (Figure 30) and the volume of the chambers obtained between the coverslip and the Hemocytometer was 10 µl.

Figure 29: Hemocytometer/ Neubauer counting chamber produced by Marienfeld-Superior

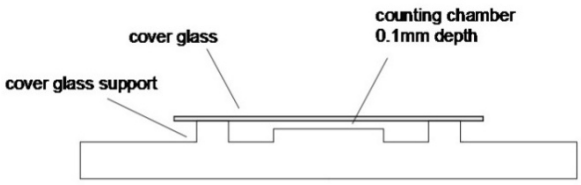

Figure 30: A schematic overview of a Hemocytometer (viewed from the side)

One device closed by the coverslip leads to two chambers each defined by the same counting grid (Figure 31). The gridded area consists of nine, equal squares (1 mm²) and also the sixteen smaller squares inside each of the four corner squares are equal in their size (Marienfeld Superior, 2011). This leads to the conclusion that, counting cells in squares and volumes which are the same size, makes it able to take an average of the counts and to calculate the cell density in the used fluid (Gstraunthaler and Lindl, 2008).

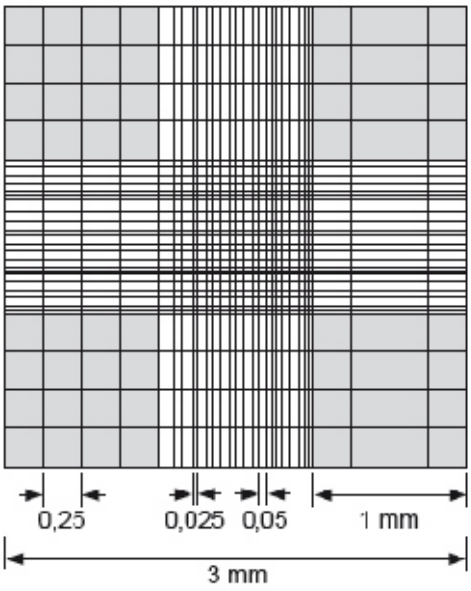

Figure 31: Counting grid of the Hemocytometer

(Marienfeld Superior, 2010)

7.2.4.2 Determination of cell count and cell viability

To determine the cell density in a cell suspension the tube containing the cells should have been swirled a bit to avoid an inhomogeneous distribution of the cells in the solution. The suspension was then immediately diluted 1:5 in a solution of Trypan Blue (0,4 %; Figure 32), so for example 80 µl of the dye solution were mixed with 20 µl of cell suspension (Abcam, 2016).

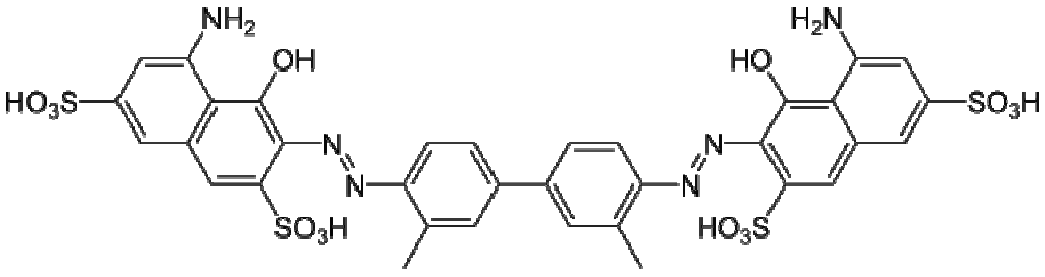

Figure 32: Chemical structure of Trypan Blue

Trypan Blue is commonly used as a cell dye to determine cell viability for the performance of the so called “dye exclusion test”. The test is based on the principle that viable cells still have an intact cell membrane and do not take up the staining solution, whereas dead cells do due to their permeable membranes. As a consequence viable cells appear with a clear, white cytoplasm while nonviable cells or cells with a destroyed membrane will have a blue cytoplasm (Thermo Fisher Scientific, 2015c).

The dilution of Trypan Blue and cell suspension was vortexed briefly to homogenise the solution and then 10 µl were taken twice to fill each chamber of the Hemocytometer. All the cells found in one set of 16 squares in the four corner squares were counted separately using an inverted microscope focused on the grid lines of the Hemocytometer. After the counts of all four sets had been performed, the average cell count from each of the four squares was taken and the number of cells in each millilitre of cell suspension has been calculated as follows:

Formula 1: Number of cells in each millilitre cell suspension

$$N = \overline{x} \times 10.000 \times f \qquad [\text{cells/ml}]$$

- N = numbers of cells per ml
- $\overline{x}$ = average of counted cells
- 10.000 = factor for the counting chamber
- f =dilution factor

Cell viability was calculated as a ratio of viable cells to the total cell count (see Formula 2).

Formula 2: Calculation of cell viability in percentage terms

$$viability = \frac{c_v}{c_t} \times 100 \qquad [\%]$$

- c_v = counted viable cells
- c_t = total cell count (viable + dead cells)

(Gstraunthaler and Lindl, 2008)

7.2.5 Mycoplasma test

7.2.5.1.1 Principle of *Mycoplasma* detection

Mycoplasma species are the smallest free-living bacterial cells yet discovered and they vary in size and shape between 0,2 and 2 µm. *Mycoplasma* contaminations in cell culture is found quite often and as they normally cannot be detected and identified

using common optical microscopy techniques, it is necessary to perform *Mycoplasma* detection test regularly (Gstraunthaler and Lindl, 2008).

All cells used during the experimental work for this thesis were tested regularly performing a 4',6-diamidino-2-phenylindole (DAPI) staining, to avoid bacterial contaminations by different *Mycoplasma* species. DAPI is a fluorescent stain which binds to AT-rich regions in DNA and excited with ultraviolet light (358 nm) its blue fluorescence, with an emission maximum at 461 nm, can be detected through blue or cyan filters. As a consequence *Mycoplasma* contaminations can be detected as small, blue dots concentrated on the cell surface, in the surrounding medium and on the culture dish (Gstraunthaler and Lindl, 2008; Young *et al.*, 2010).

7.2.5.1.2 Method of *Mycoplasma* detection

For the Mycoplasma detection test 2×10^6 HT-29 cells were seeded in a petridish (d = 10 cm) containing a functionalised microscope slide and cultivated for 48 hours in a humidified incubator (37 °C, 5 % CO_2) in DMEM supplemented with 10 % FCS and 1 % P/S. After that adherence phase, confluence was checked optically using an inverted microscope. Cells should not be more confluent than 70 % for the reason that the blue dots of the *Mycoplasma* contamination could not be well-identified in too confluent cells. After one washing step with pre-warmed PBS, the microscope slide was treated four hours at a temperature of – 20 °C with methanol to fix the cells. Cells were stained with DAPI and then examined using an ultraviolet excitation between 330-380 nm.

7.3 Methods

7.3.1 Cellular reactive oxygen species (ROS)

Oxidative stress is defined as an imbalance between the production of ROS and the antioxidant defenses in cells when the concentration of pro-oxidant components exceeds the cellular antioxidant capacity (Sies, 1991)

The usage of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorometric assay was described first by Keston and Brandt (1965) for the analysis of ultramicro quantities of hydrogen peroxide. The method to use DCFH-DA to evaluate the potency of pro-oxidants or also the efficacy of antioxidant compounds against oxidative stress (OS) in cells combined with a fluorescent microplate reader was then developed by Wang and Joseph (1999).

7.3.1.1 Principle of the dichlorofluorescein assay

In the dichlorofluorescein assay, also well-known as DCF assay, the non-fluorescent DCF derivative DCFH-DA is taken up by the cells into the cytoplasm due to its non-polar and non-ionic properties. After having crossed the cell membranes deacetylation of the compound to the still non-fluorescent 2′,7′-dichlorofluorescin (DCFH) is catalysed by intracellular esterases (Figure 33).

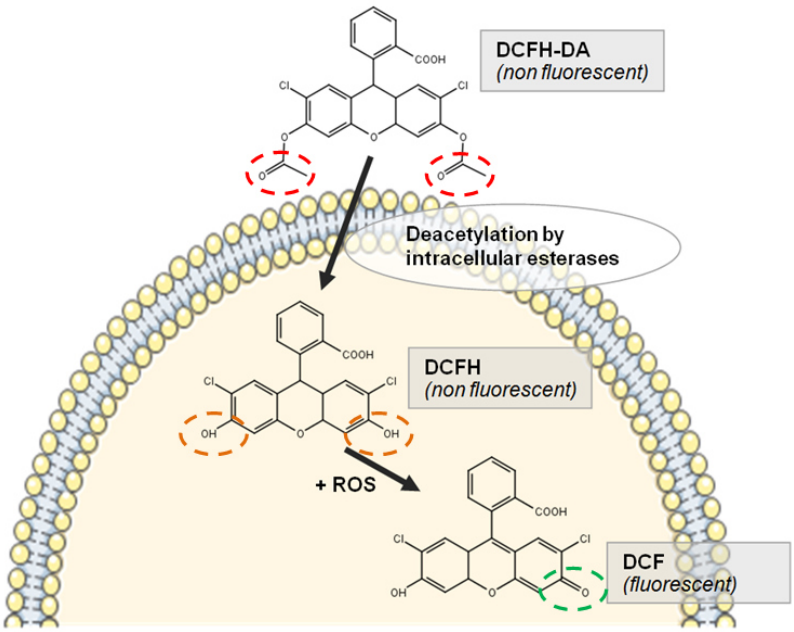

Figure 33: Schematic overview of DCF assay

In the cellular cytoplasm present reactive oxygen species rapidly oxidise DCFH to highly fluorescent DCF, where the emitted fluorescence is directly proportional to the intracellular ROS concentration (LeBel *et al.*, 1992).

7.3.1.2 Protocol for the DCF assay

- Cell seeding

HT-29 cells were seeded at a density of 8.000 cells per well in black 96-well plates with clear bottom to avoid high fluorescent background. Then cells were cultured at standard conditions (37 °C, 5 % CO_2, in a humidified incubator) with 200 µl per well DMEM supplemented with 10 % FCS and 1 % P/S for 72 hours. At least three wells should be left without cells to obtain a cell-free blank.

- Cell preparation and incubation

At a confluence of about 80 % the old culture medium was gently removed using a vacuum pump system. Immediately afterwards with a multi-channel pipetting system cells were washed once with 150 µl pre-warmed PBS. After removing the PBS solution, cells were incubated at standard conditions with 100 µl per well of 50 µM DCFH-DA in PBS for 30 minutes. The stock solution of DCFH-DA is at 5 mM in the solvent dimethyl sulfoxide (DMSO) and diluted 1:100 with PBS.

During the DCFH-DA incubation period all the dilutions of the compounds should be prepared to avoid, since the reaction between ROS and DCFH is relatively quick, additional delays in the workflow. All the incubation solutions of the compounds were prepared by the following constituents:

- phenol red-free DMEM to exclude interactions of the pH indicator with the measurements (10 % FCS, 1 % P/S, 25 mM HEPES)
- 1 % water (LC-MS grade)
- substances in different incubation concentrations (dilution factor 1:100)

As a positive control cells were incubated with hydrogen peroxide at a concentration of 500 µM, again diluted 1:100 from a 50 mM stock solution. For the measurements of the blank only culture medium was used, whereas for the negative control 1 % water (LC-MS grade) was added to the culture medium.

- Measurements

After 30 minutes of incubation the DCF-DA solution was removed and cells were rinsed with 150 µl of pre-warmed PBS per well. At this point the first measurement of the fluorescence intensity, the so called “pre-incubation measurement”, was taken at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Afterwards, the washing buffer was quickly replaced by all the prepared incubation and control solutions (100 µl/well). Fluorescence values were measured then after 5, 10, 30, 60, 90, 120 and 180 minutes of incubation with a multi-mode microplate reader.

- Data analysis

To analyse the measured values of the fluorescence intensity the average of the blanks and also the pre-incubation data needed to be subtracted from the values measured at different time points.

7.3.2 Cytotoxicity/Proliferation

In order to evaluate the cytotoxic capacity of the tested compounds or also their stimulation of cell proliferation two different assays were performed, namely the WST-1 (Water Soluble Tetrazolium) assay and the Sulforhodamine B (SRB) assay. These assays were based on different cell functions which allowed a better comprehension about the cytotoxic or proliferating effects of the compounds.

7.3.2.1 Principle of the WST-1 assay

The WST-1 assay is a colorimetric method to quantify cellular proliferation, viability, and cytotoxicity. For its performance a ready-to-use kit, containing WST-1 (sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt) and an electron coupling reagent, is used (Berridge *et al.*, 2005; Roche Diagnostics, 2016). WST-1, a negatively charged disulfonated inner salt containing an iodine residue, is normally utilised in conjunction with an intermediate electron acceptor (IEA). This IEA promotes a dye reduction of the slightly red coloured WST-1 to a reduced derivative, a dark red, soluble formazan (Figure 34). The amount of reduced WST-1 directly correlates with the number of viable cells in exponential growth phase.

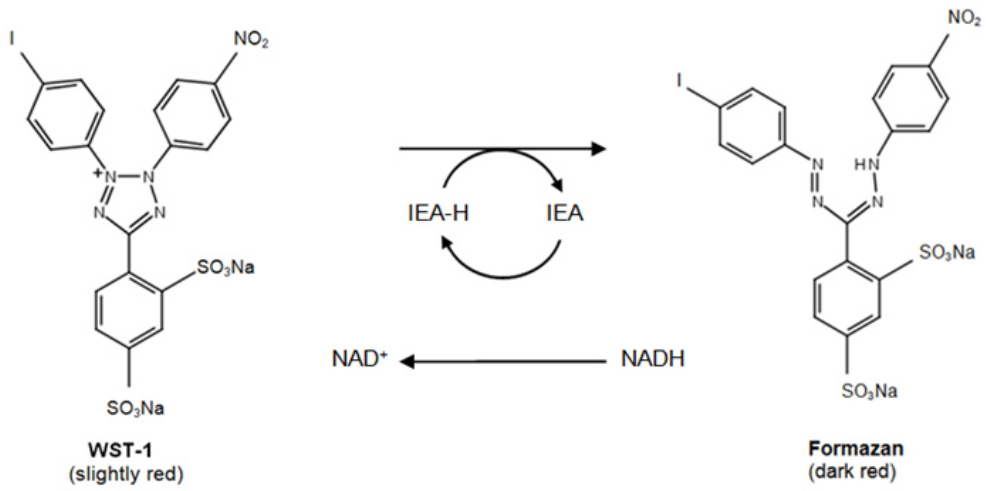

Figure 34: Reduction of WST-1 to Formazan by IEA

In comparison to other tetrazolium salts (e.g. MTT) WST-1 is not able to cross the plasma membrane due to it is negatively charged sulfonate groups (Figure 34) but it is reduced extracellularly by electron transport across the plasma membrane via the electron carrier. The cellular reductant is NADH mainly deriving from the mitochrondial TCA cycle (Berridge *et al.*, 2005). Therefore the amount of formazan dye formed by

reduction correlates not only with the number of metabolically active cells but is also proportional to the mitochondrial activity of the cells (Figure 35).

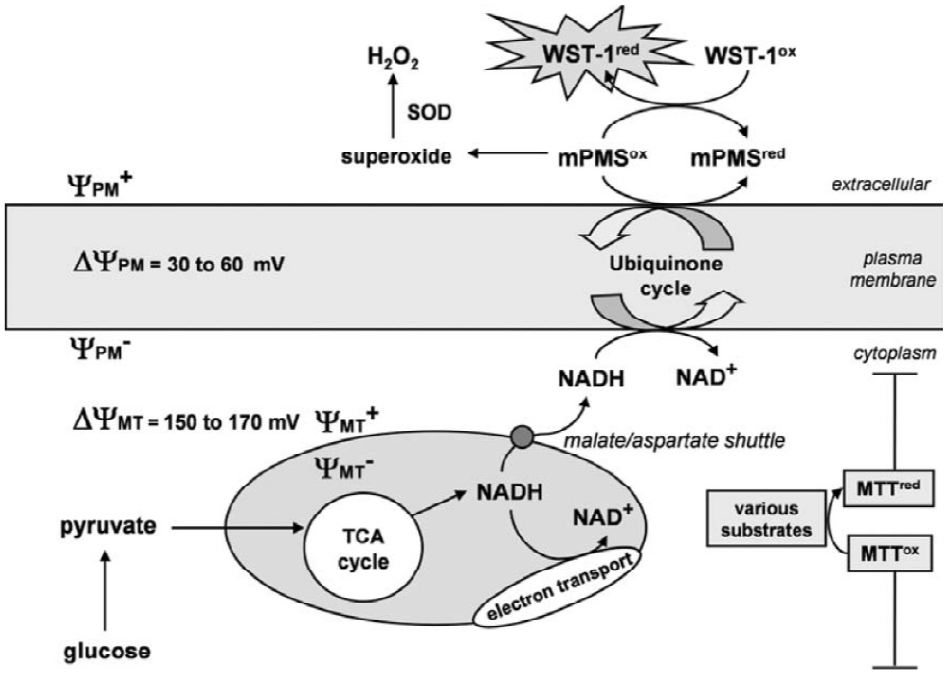

Figure 35: Schematic overview of the mechanisms of WST-1 and MTT (Berridge et al., 2005)

7.3.2.2 Protocol for the WST-1 assay

- Cell seeding

HT-29 cells were seeded in 96-well plates at a density of 7.500 cells per well and cultured for 48 hours in 200 µl DMEM per well supplemented with 10 % FCS and 1 % P/S at the standard conditions. At least three wells should have been left without cells to obtain a cell-free blank.

- Cell preparation and incubation

At a confluence of about 70 %, the culture medium was removed gently and the incubation was conducted in DMEM (10 % FCS, 1 % P/S) containing the corresponding concentration of the tested compounds and a final concentration of 1 % of the solvent water (LC-MS-grade). Solutions with 1 % water (LC-MS grade) and 1 % Triton-X 100 were used as negative and positive control, respectively. After 24 hours in a humidified incubator at 37 °C and 5 % CO_2 the incubation

solutions were aspirated using a vacuum pump system and cells were washed once with 150 µl pre-warmed PBS per well. This washing step was required to avoid inferences between the FCS and the WST-1 reagent.

- Measurements

PBS was replaced then by 100 µl per well detection solution composed of serum-free DMEM and 10 % WST-1 reagent. At 37 °C and 5 % CO_2 the cells were incubated for 35 minutes in a humidified incubator. Before reading the plate, it was shaked gently for five minutes to exclude an inhomogeneous distribution of the colour. Immediately afterwards the absorbance was measured at 450 nm and 650 nm (reference wavelength) using a multi-mode microplate reader.

- Data analysis

To analyse the measured absorbance values first the average of at least three cell-free blank values was subtracted, followed by the subtraction of the values taken at the higher wavelength from the absorbance values at 450 nm.

7.3.2.3 Principle of the Sulforhodamine B assay

The SRB assay is a method for measuring the cellular protein content of adherent cultures and it was developed by Skehan *et al.* (1990). This method is based on the ability of the protein-binding dye Sulforhodamine B (Figure 36) to bind under mild acidic conditions electrostatically to protein basic amino acid residues of cells fixed with trichloroacetic acid (TCA) (Voigt, 2005).

The protein-bound dye can be dissolved then in TRIS base (tris (hydroxymethyl-) aminomethane) to perform a photometrical measurement. The absorbance results are directly proportional to the total cellular protein (Skehan *et al.*, 1990).

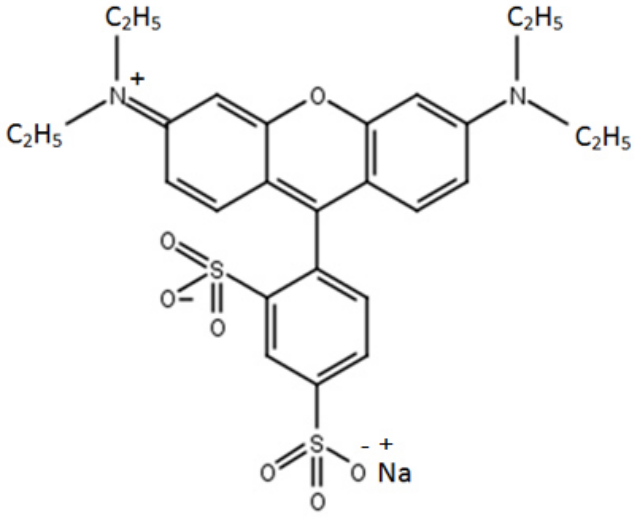

Figure 36: Chemical structure of Sulforhodamine B as a sodium salt

7.3.2.4 Protocol for the Sulforhodamine B assay

From the experimental point of view it is possible to conduct both cytotoxicity assays, WST-1 followed by the SRB assay, on the same plates. This approach allows both a reduction of the costs and a direct correlation of the toxicity endpoints (mitochondrial activity and protein content) in the same cells. In fact, the obtained proliferation or cytotoxicity data of both assays can be compared well as the experiments are performed at unchanged cellular conditions.

- Preparation of the used solutions

Solutions needed to conduct the SRB assay were prepared as shown in Table 22.

Table 22: For SRB assay required solutions

Solution	Preparation
TCA	50 % (w/v) in distilled water
SRB dye solution	0,4 % (w/v) in 1 % acetic acid
Acetic acid	1 % in distilled water
Tris	10 mM in distilled water, pH = 10

- Cell seeding

If no WST-1 was performed before and the cells have already been prepared, cells should be seeded in 96-well plates at a density of 7.500 cells per well and cultured for 48 hours in 200 µl DMEM supplemented with 10 % FCS and 1 % P/S standard conditions.

- Cell preparation and incubation

At a confluence of about 70 %, the culture medium was removed gently and the incubation solutions, prepared with DMEM (10 % FCS, 1 % P/S) containing the corresponding concentration of the tested compounds and a final concentration of 1 % of the solvent water (LC-MS-grade), were added. Solutions with 1 % water (LC-MS grade) and 1 % Triton-X 100 were used as negative and positive control, respectively. After incubation times of 24 or 48 hours at 37 °C and 5 % CO_2 in humidified conditions the solutions are aspirated using a vacuum pump system and cells were washed once with 150 µl pre-warmed PBS per well.

- Cell fixation

The incubation solutions or, after the performance of a WST-1 assay the reagent, was removed from the cells and a washing step using 200 µl pre-warmed PBS per well was conducted. Again PBS (100 µl per well) was put on the cells followed by the addition of 10 µl of trichloroacetic acid (preparation described in Table 22) at a concentration of 50 % (w/v). After one hour of incubation in the dark at 4 °C, the plate was flicked and washed four times with water to remove excess fixative. Then the plate was dried overnight at room temperature

- Cell staining

For the staining of the proteins, 50 µl of the SRB dye solution (0,4 % w/v) were added to each well and incubated for one hour in the dark at room temperature. The plate was rinsed again, four times with water and two times with 1 % acetic acid to remove the unbound dye.

- Measurements

After drying the plates completely, the dye was dissolved by adding 100 µl of 10 mM Tris buffer (pH 10. To guarantee a homogeneous distribution of the colour, plats were shaked for five minutes and afterwards a photometrical measurement was performed at a wavelength of 570 nm.

- Data analysis

The average of at least three cell-free blank values was subtracted from the measured absorbance data.

7.3.3 Proliferation and confluence analysis using the digital phase contrast of brightfield images

Brightfield imaging followed by a digital phase contrast processing step is a non-destructive and non-phototoxic method which allows the user to perform both qualitative and quantitative measurements of living cells (El-Schich *et al.*, 2015). The digital phase contrast application corrects, by creating a much more consistent contrast of the image (e.g. shown in Figure 37), unsteady illuminations of brightfield images (BioTek Instruments, 2016). This microscopic method, in comparison to techniques that measure extracellular release of markers or cell staining methods, enables to non-invasively obtain data on cellular confluence (El-Schich *et al.*, 2015).

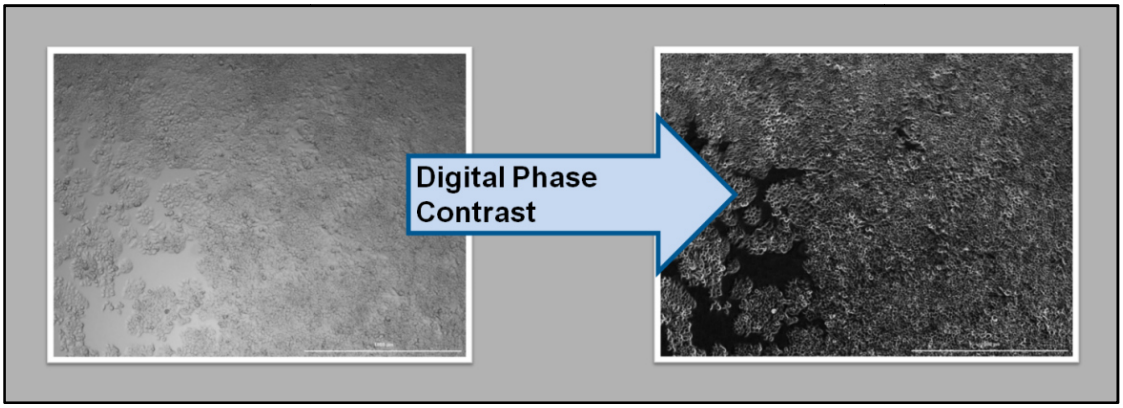

Figure 37: Digital phase contrast application (scale bar: 1000 µm)

7.3.3.1 Protocol for the confluence analysis using digital phase contrast images

- Cell seeding

HT-29 cells were seeded in 96-well plates at a density of 7.500 cells per well in 200 µl DMEM supplemented with 10 % FCS and 1 % P/S.

- Cell preparation and incubation

After 48 hours at standard conditions the culture medium was replaced by the required incubation solutions prepared in DMEM (10 % FCS, 1 % P/S) containing the corresponding concentration of the tested compounds and a final concentration

of 1 % of the solvent water (LC-MS grade). As negative and positive control 1 % of the solvent water (LC-MS grade) and 1% Triton-X 100 were used, respectively.

- Image acquisition and confluence analysis
  The incubation solutions were removed after 24 hours at 37 °C and 5 % CO_2 in a humidified incubator and a washing step with 200 µl pre-warmed PBS per well was performed.

In order to provide better clarity and signal-to-noise ratio (Thermo Fisher Scientific, 2015b), after removing the PBS, 150 µl pre-warmed Live Cell Imaging Solution per well were added to the cells. For the purpose to cover the whole well, the brightfield images were acquired as 3x4 image montages with a Cytation™ 3 cell imaging multi-mode reader using a 4X objective (see Figure 38). Further image editing and analysis was conducted by the data collection and analysis software BioTek™ Gen5™ 2.09.2. Image montages were stitched and a digital phase contrast image was created (see Figure 37), followed by a threshold analysis. Determined and set the right threshold, the image statistics application of the BioTek™ Gen5™ software was used to calculate the confluence of each well.

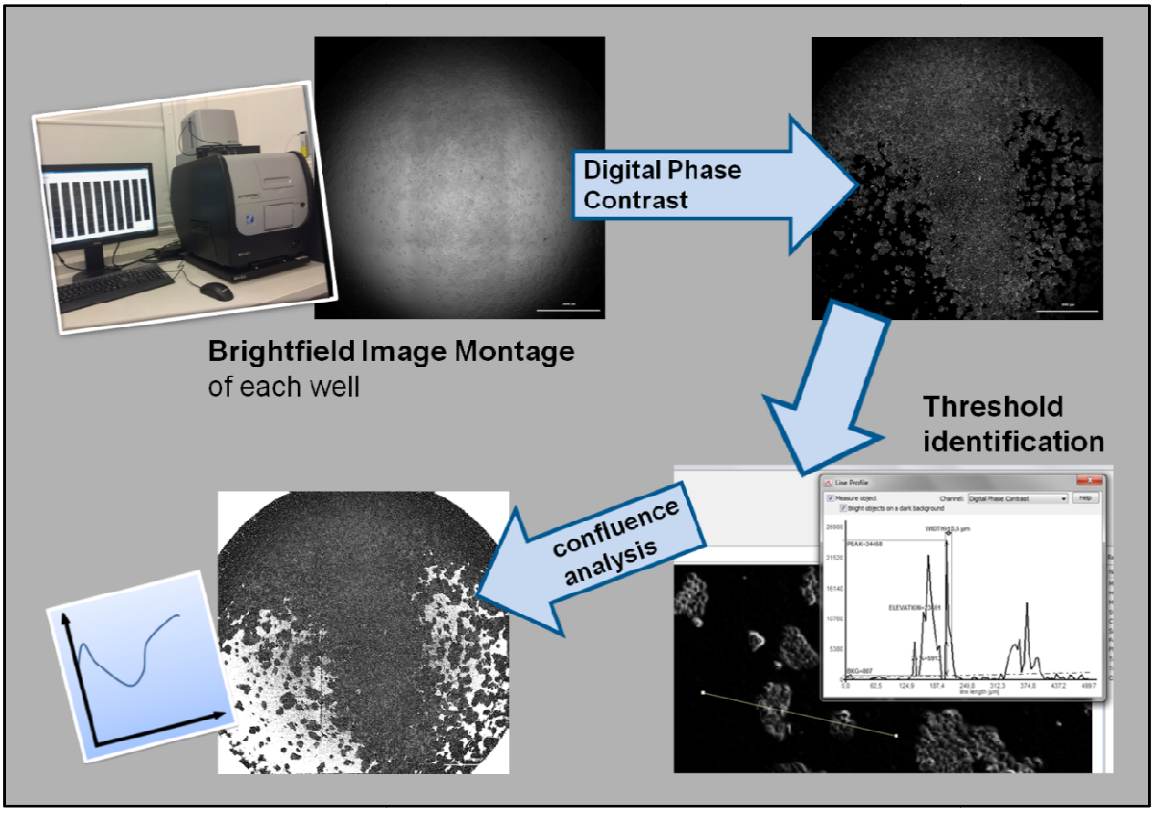

Figure 38: Workflow of the confluence analysis (scale bar: 3000 µm)

7.3.4 Proliferation analysis by immunocytochemistry (ICC)

Immunofluorescent techniques were first conceptualised and implemented by Coons *et al.* (1941), using specific antibodies labelled with fluorescent markers to identify substances in tissue sections.

Immunocytochemistry refers to the process of detecting antigens (e.g. proteins) in cells based on the principle of antibodies binding specifically to antigens (Van Noorden and Polak, 1983). The interaction is highly sensitive and as the antibody is tagged with a visible label it can be used, in conjunction with a photonic microscope, to detect and to visualise both the presence and subcellular location of a particular molecule of interest (Sternberger, 1979).

Immunocytochemical experiments conducted for this thesis were all focused on the analysis of cell proliferation and therefore one specific protein, namely KI-67, was investigated. KI-67 is a non-histone nuclear protein (Gerdes *et al.*, 1983) and as it is present in all proliferating cells (normal and tumour cells) and absent in resting ones (Gerdes *et al.*, 1984), it is regularly used as a marker for cellular proliferation e.g. in Etemad-Moghadam *et al.* (2013) or (Urbanska *et al.* (2015)).

7.3.4.1 Protocol for immunocytochemical analysis of cell proliferation and KI-67

- Preparation of the used solutions

Solutions needed to conduct the immunocytochemical staining were prepared as shown in Table 23:

Table 23: For immunocytochemical staining required solutions

Solution	Preparation
FA-FIX 3,7 %	dilution 1:10 of Formaldehyde solution (36,5-38 %) in PBS-A
PBS-A	3mM KH_2PO_4, 5,4 mM KCl, 0,27 M NaCl, 25 mM $Na_2HPO_4 \cdot 2\ H_2O$ in distilled water, pH = 7,3
PBS-BSA 1 %	1 % Albumin Fraction V/BSA in PBS-A
0,2 TX-100 Permeabilisation buffer	0,2 % Triton® X 100 (extra pure) in PBS-A
0,05 TX-100 Washing buffer	0,05 % Triton® X 100 (extra pure) in PBS-A
PBS-G	100 mM glycine in PBS-A

- Cell seeding

For immunocytochemical analysis HT-29 cells were seeded in 8-chamber microscope slides at a density of 50.000 cells per well and allowed to grow for 48 hours in 500 µl DMEM supplemented with 10 % FCS and 1 % P/S at standard conditions.

- Cell preparation and incubation

Before the cells were incubated with the respective concentrations of the tested compounds, prepared with DMEM (10 % FCS, 1 % P/S) and a final concentration of 1 % of the solvent water (LC-MS grade), culture medium was removed gently using a vacuum pump system. Solutions with 1 % water (LC-MS grade) were used as negative control.

- Cell fixation

At the end of the 24 h incubation phase the cells were washed with pre-warmed PBS and fixed at the dark with using the fixing solution FA-FIX 3,7 % (see Table 24) for 15 minutes. Afterwards the cells were washed again three times with PBS and the slide was stored in the fridge at 4 °C until the immunocytochemical staining.

- Immunocytochemical staining

Cells were permeabilised with 250 µl per well 0,2 TX-100 Permeabilisation buffer (Table 23) for 15 minutes on a rocker in a humidified chamber and washed once with 250 µl PBS-A per well for five minutes on the shaker. After removing the washing solution using a vacuum pump system, the cells were blocked for one hour on the rocker at room temperature with 500 µl PBS-BSA 1 % per well. Afterwards the primary antibodies diluted in PBS-BSA 1 % were added to the cells and incubated under static conditions for two hours at room temperature in a humidified chamber. For the purposes of this thesis the primary antibodies prepared as listed in Table 24 were used according to the specification of the supplier.

Table 24: Dilution of the used primary antibodies for the immunocytochemical staining

Antibody	Dilution
KI-67 antibody (H-300), sc-15402, Rabbit polyclonal IgG	1:500 in PBS-BSA 1 %
Lamin B antibody (C-20), sc-6216, Goat polyclonal IgG	1:350 in PBS-BSA 1 %
α Tubulin antibody (B-7), sc-5286, Mouse monoclonal IgG	1:1000 in PBS-BSA 1 %

After removal of the primary antibodies, three washing steps were performed each of five minutes on the rocker using 250 µl 0,05 TX-100 Washing buffer per well. Then fluorescent labelled secondary antibodies were prepared as annotated in Table 25 and incubated in a dark humidified chamber for one and a half hours at room temperature and static conditions.

Table 25: Dilution of the used secondary antibodies for the immunocytochemical staining

Antibody	Dilution
Donkey anti-Goat IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 647 conjugate	1:1000 in PBS-BSA 1 %
Donkey anti-Mouse IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 488 conjugate	1:1500 in PBS-BSA 1 %
Donkey anti-Rabbit IgG (H+L) polyclonal secondary antibody, Alexa Fluor® 568 conjugate, for IF	1:1500 in PBS-BSA 1 %

The antibody-solutions were removed and followed by six washing steps on the shaker, three performed with 0,05 TX-100 Washing buffer (250 µl/well) and three with PBS-A (250 µl/well). The slides were then post-fixed for 15 minutes with about 500 µl per well FA-FIX 3,7 % at room temperature protected from light. The fixing solution was removed and two further PBS-A-washing steps are performed. To mask reactive sites slides were incubated with PBS-G for five minutes at room temperature (250 µl/well). Before the slides were mounted and sealed with

Fluorescence Mounting Medium, they were washed again with 250 µl PBS-A to remove excess PBS-G. Until the image acquisition the samples were stored at 4 °C protected from light.

- Image acquisition for the analysis of KI-67quanitification

Confocal images for the proliferation analysis focused on a quantification of the cellular proliferation marker KI-67 were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system using a Plan-Apochromat 63X/1,4 oil objective (zoom 2). At least four different cell preparations were used for the analysis of the KI-67 signals and for each experiment at least three optical fields were acquired. To obtain a complete 3D reconstruction at least 25 images were acquired at intervals of 0,625 µm.

- Image analysis for the KI-67 quantification

For the purpose of this analysis, the evaluation of the KI-67 signals was performed in the cellular nuclei, taking as reference the nuclear structure provided by lamin B. In order to avoid biased selection of the images and also of the analysed areas, the KI-67 acquisition channel was disabled during the selection of the optical fields and of the areas later used for further image analysis. To ensure the comparability of the data, the acquisition of all the experimental conditions of the same biological replicate was acquired the same day, maintaining constant laser parameters (power, digital gain), as well as background levels.

A Maximum Intensity Projection of the 3D reconstructions was performed by the microscope and imaging software Zeiss ZEN 2012 SP1 and the single acquisition channels were exported separately to JPEG format (see Figure 39). Further signal analysis was then conducted by the public domain, Java-based image processing program ImageJ.

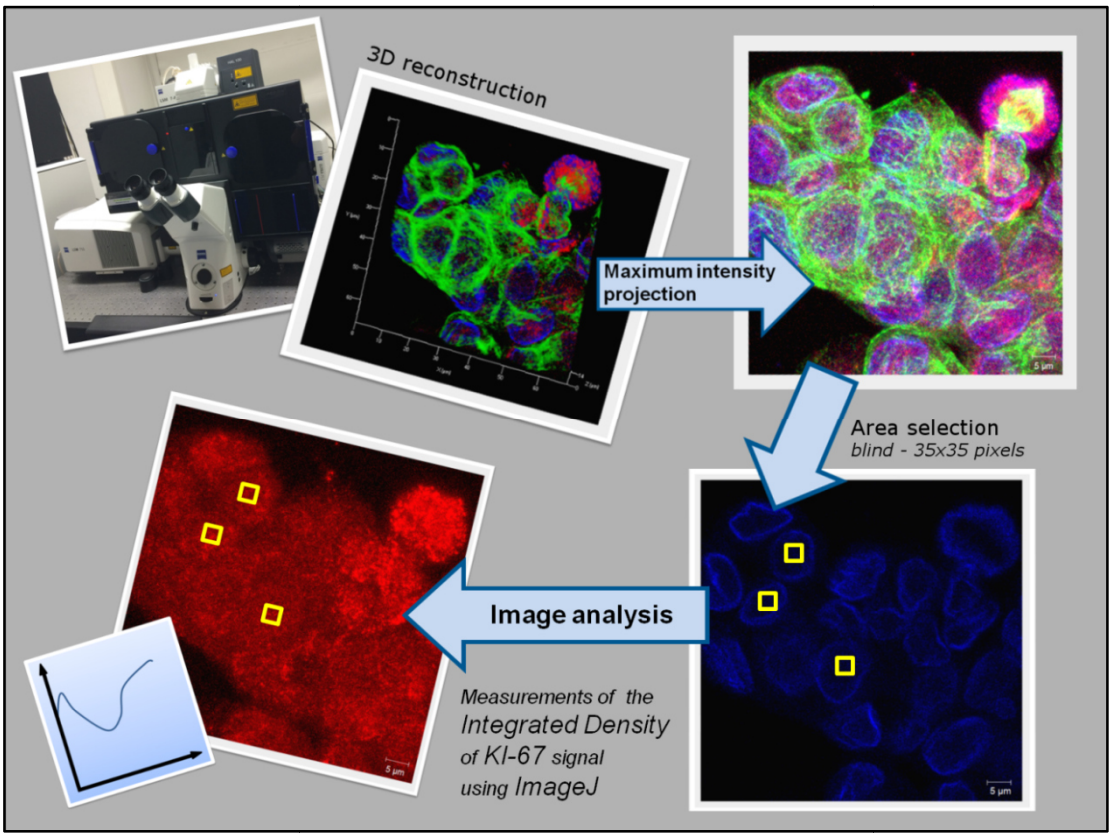

Figure 39: Workflow of the image analysis and quantification of the KI-67 signal (scale bar: 5 µm)

For the estimation of the KI-67 signals the integrated density of at least three different nuclear areas (35x35 pixels) per image was measured, using the respective ImageJ function. As already indicated above, all the analysed nuclear areas were selected randomly in the optical fields.

- Image acquisition for the quantification of cells in mitosis

For the proliferation analysis regarding a quantification of cells in mitosis, the confocal images were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system using a Plan-Neofluar 10X/0,30 objective (zoom 3). At least four different biological replicates were prepared for the analysis of cells in mitosis and for each experiment at least two optical fields were acquired resulting in the analysis of at least eight different optical fields for each experimental condition.

- Image analysis for the quantification of cells in mitosis

The acquisition of all the experimental conditions of the same biological cell preparation was conducted the same day, using constant laser parameters (power, digital gain) and background levels, to guarantee the comparability of the obtained signals. The acquisition channels were exported both single and merged to JPEG format using the microscope and imaging software Zeiss ZEN 2012 SP1. For the analysis of cells in mitosis found in every optical field, the numbers of all the single nuclei provided by lamin B and also of the mitotic structures (e.g. as shown in Figure 40) were gathered by manual counting.

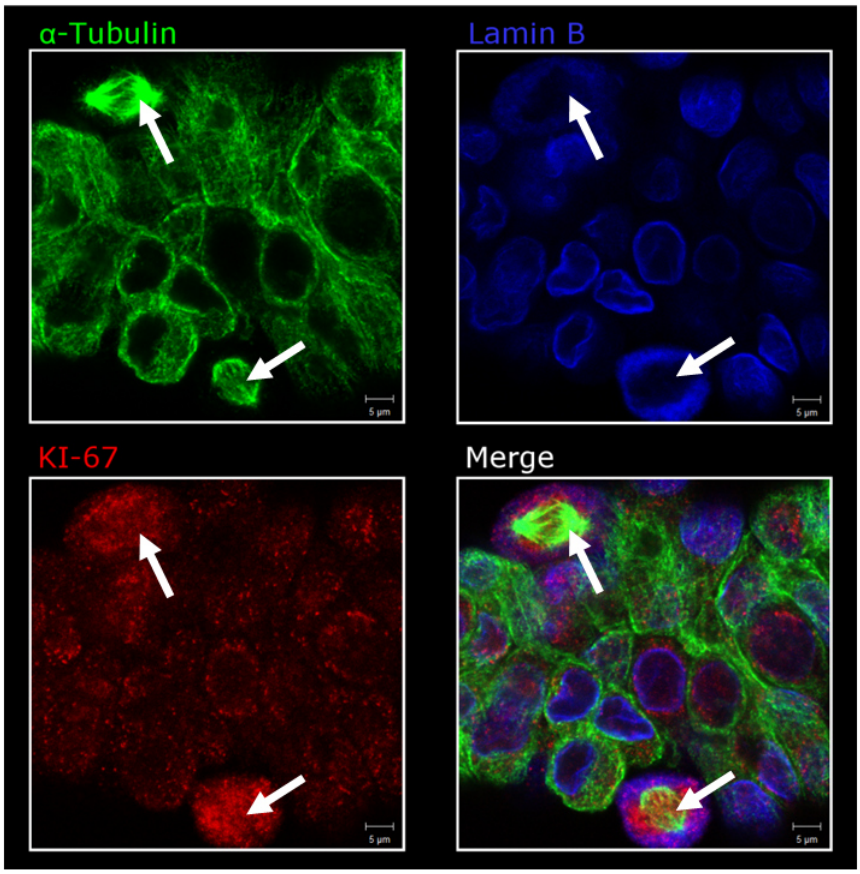

Figure 40: Example for different mitotic structures indicated with arrows (scale bar: 5 µm)

The resulting quantification of cells in mitosis derived from the ratio of cells showing mitotic structures to the total cell number in each optical field expressed in percentages (Figure 41).

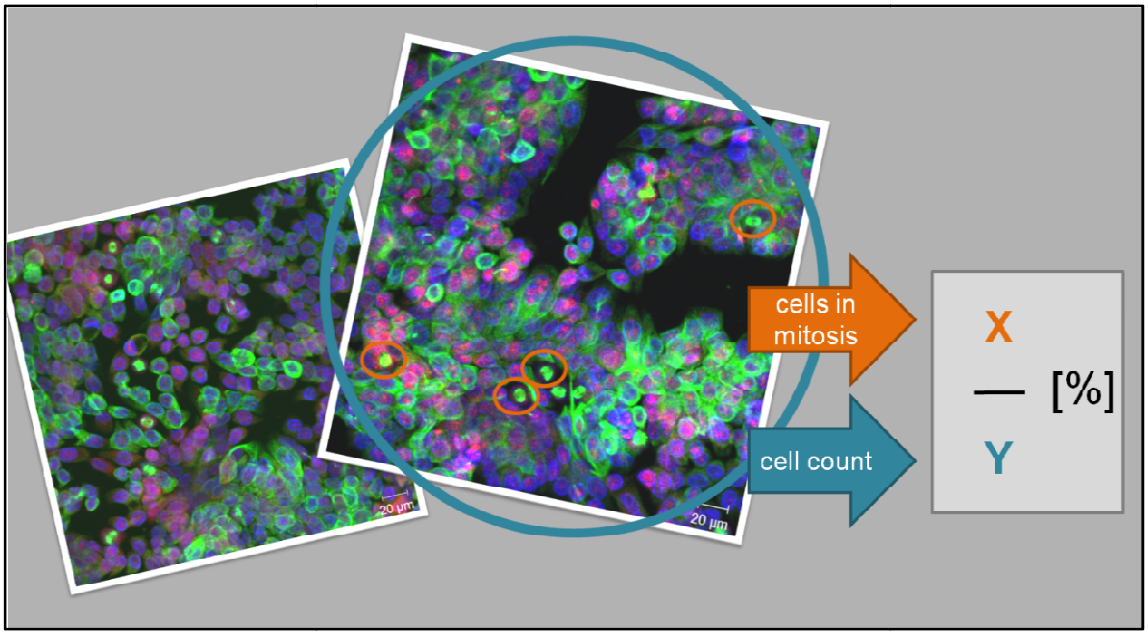

Figure 41: Workflow of the image analysis and quantification of cells in mitosis (scale bar: 20 µm)

7.3.4.2 Structure illumination Microscopy (SIM) images of the cell proliferation marker KI-67 and lamin B

In order to illustrate and to support the quantification data SIM images of the KI-67 signal in reference to the nuclear structure provided by lamin B were acquired with a Confocal LSM Zeiss 710 equipped with ELYRA PS. 1 system using a Plan-Apochromat 100X/1,46 oil objective (zoom 2, rotations 5, phases 5). In order to obtain a complete 3D reconstruction at least 25 images were acquired at intervals of 0,672 µm. Further rendering steps were conducted by the microscope and imaging software Zeiss ZEN 2012 SP1.

7.3.5 Statistical evaluation of the data

All the experiments for this thesis were at least conducted in three independent biological replicates and in technical duplicates (see Table 26). All data were presented as the means ± SEM and their statistical analysis was performed with Origin Pro 9.1G. For all the calculations statistical levels are set to 5 % (*/§/# $p < 0,05$; **/§§/## $p < 0,01$; ***/§§§/### $p < 0,001$) and the normal distribution of the data was tested using the Shapiro Wilk test. Statistical evaluation of the concentration series of single substances, performed in at least five independent biological cell preparations, was conducted using the one-way analysis of variance (ANOVA) and in case of a significant difference ($p < 0,05$) post-hoc Fisher's least significant difference (LSD) test was used.

If less than five independent biological replicates were performed of an experiment a non-parametric method, namely the Kruskal-Wallis ANOVA, was applied.

Table 26: Overview of the performed experiments and their biological and technical replicates

Experiment	Indipendent biological replicates	Technical replicates in each biologically independent experiment
DCF assay	at least n = 5	performed at least in hexuplicates
SRB assay 24 hours incubation	at least n = 3	performed at least in quadruplicates
SRB assay 48 hours incubation	at least n = 3	performed at least in quadruplicates
Immunocytochemical staining for **KI-67** quantification	at least n = 4	performed at least in triplicates
Immunocytochemical staining for the quantification of **mitotic structures**	at least n = 4	performed at least in duplicates
WST-1 assay	at least n = 5	performed at least in quadruplicates
Confluence analysis	at least n = 3	performed at least in quadruplicates

8. Indices

8.1 Bibliography

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9. Appendix

9.1 Abstract

Deoxynivalenol (DON) is a natural-occurring type B trichothecene mycotoxin mainly produced by certain *Fusarium* spp. which colonise worldwide food and feed. Thus DON constitutes a major issue for global food an feed safety and a provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg body weight was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2001 (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2002). Recently, two novel sulfo-metabolites of DON were reported in wheat (Warth *et al.*, 2015), namely DON-3-sulfate (DON3S) and DON-15-sulfate (DON15S) and the former one was identified also as a human urinary metabolite (Warth et al., unpublished).

Since these compounds could have a similar biological activity as their parent compound DON, their characterisation is of crucial importance in order to evaluate their toxicological relevance. Thus, during this thesis, the biological activity of DON3S and DON15S was investigated in comparison to DON in the human colorectal adenocarcinoma cell line HT-29. For this reason, a panel of assay was selected on the basis of the pre-existing data about the parent compound DON. As a first step, the impact of DON, DON3S and DON15S on the intracellular oxidative stress was investigated through the DCF assay. Both DON (10 µM) and DON3S (1 and 10 µM) increased significantly the ROS levels in a quite fast and transient reaction (3 min). In fact, after two hours of incubation cells recovered to basal level again. DON15S only triggered a slight increase effect after 3 hours of incubation.

In order to verify if the increase of oxidative stress affected cell viability and proliferation, the SRB, the WST-1 assay and a confluence analysis were performed. After 24 hours of incubation, DON showed a biphasic response, inducing cell proliferation at 0,1 µM and resulting in significant cytotoxic effects at concentrations of 10 µM and higher. DON3S and DON15S, on the other hand, showed a significant stimulating effect on cell proliferation at concentrations from 0,1 µM to 25 µM. The induction of cell proliferation and also the cytotoxicity of DON at 10 µM were further confirmed and supported by the results of immunocytochemical quantification of the proliferation marker KI-67 and of cells in mitosis.

Overall, in the experimental system used during this thesis the two sulfo-metabolites did not seem to share the same toxicological activities as their parent compound DON, but still the impact of DON3S and DON15S on the cellular proliferation characterised

during this thesis open new possibilities to evaluate the risk associated to the entrance of these compounds into the food and feed chain.

9.2 Kurzfassung

Das Typ B Trichothecene Deoxynivalenol (DON) ist ein sehr bekanntes Mykotoxin, welches als Stoffwechselprodukt von verschiedenen Pilzarten der Gattung *Fusarium* produziert wird und dadurch als Kontaminant in verschiedenen Nahrungs- und Futtermittel, vor allem jedoch auf befallenem Getreide und daraus verarbeiteten Produkten vorkommt. Neben vielen weiteren toxischen Effekten führt die Einnahme akut toxischer Dosen von DON in Menschen und Tieren zu starker Übelkeit und Erbrechen (Forsyth *et al.*, 1977), weshalb gemäß des Joint FAO/WHO Expert Committee on Food Additives (JECFA) eine provisorische maximal tolerable tägliche Aufnahme von 1 µg pro kg Körpergewicht für DON und dessen acetylierte Metabolite festgelegt wurde (Food and Agriculture Organisation and World Health Organisation (FAO/WHO), 2002). Eine biologische Charakterisierung von zwei erst kürzlich identifizierten DON-Metaboliten, DON-3-Sulfat und DON-15-Sulfat (Warth *et al.*, 2015), welche ähnliche biologische Aktivitäten wie DON aufweisen könnten, ist deshalb von großer Bedeutung um deren toxikologische Relevanz evaluieren zu können.

Im Zuge dieser Diplomarbeit wurde demnach, unter Berücksichtigung der bereits vorhandenen Datenlage zu DON, eine initiale Charakterisierung der biologischen Aktivität von DON-3-Sulfat und DON-15-Sulfat vergleichend zu DON in der humanen Colon-Adenokarzinom Zelllinie HT-29 durchgeführt. Zu Beginn wurde der Einfluss von DON und dessen Sulfo-Metaboliten auf die zelluläre Produktion von reaktiven Sauerstoffspezies (ROS) im Dichlorofluorescein-Assay erhoben. DON (10 µM) und DON-3-Sulfat (1 und 10 µM) zeigten dabei eine sehr rasche und kurzweilige Reaktion und führten zu einem signifikanten Anstieg des intrazellulären, oxidativen Stresslevels nach drei Minuten Inkubationszeit, wohingegen DON-15-Sulfat (10 µM) erst nach drei Stunden Inkubation zu einem leichten Anstieg von ROS führte.

Um darüber hinaus den Einfluss dieses leicht erhöhten oxidativen Stresslevels auf Zytotoxizität, Zellviabilität und zelluläre mitochondriale Aktivität zu erheben, wurden die Substanzen weiter im SRB Assay, WST-1 Assay und mittels Konfluenzanalyse untersucht. Inkubationen mit DON resultierten dabei in einer zweiphasigen Reaktion, in welcher 0,1 µM DON zu erhöhter Zellproliferation führte, 10 µM und höhere DON Konzentrationen jedoch bereits nach 24 Stunden zytotoxische Effekte hervorriefen. Um die erhobenen Zytotoxizitäts- und Proliferationsdaten zu bestätigen bzw. zu unterstützen, wurden weiters immunzytochemische Analysemethoden unter Einsatz von Konfokal-Mikroskopie verwendet und eine Quantifizierung des Proliferationsmarkers KI-67 und von Zellen in aktiver Mitose durchgeführt.

In dem für diese Diplomarbeit gewählten, experimentellen Rahmen zeigten die beiden Sulfo-Metabolite DON-3-Sulfat und DON-15-Sulfat kein dem DON ähnliches toxikologisches Verhalten. Deren starker Einfluss auf Zellproliferation und Viabilität in HT-29 Zellen eröffnet jedoch einen ersten Einblick in die toxikologische Wirkungsweise der Sulfate und darüber hinaus, neben ersten Ansätzen in Bezug auf Risikobewertung, auch die Basis für weiterführende Forschungsfelder.