



universität
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DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„Interactions of selected polyphenols with drugs and
food contaminants“

verfasst von / submitted by

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

Doktor der Naturwissenschaften (Dr. rer. nat.)

Wien, 2017 / Vienna 2017

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on the student
record sheet:

A 796 605 419

Dissertationsgebiet lt. Studienblatt /
field of study as it appears on the student record sheet:

Chemie / Chemistry

Betreut von / Supervisor:

Univ.-Prof. Dr. Doris Marko

Acknowledgements

To Doris Marko for the ongoing support with scientific challenges and for motivating me to address ever the next question.

To the crazy crew for all the fun we had.

To my family, including my parents and my wife Moni, for the constant support over a decade of studying.

To Elija, whose ability to pull me out of my darkest mood with a few happy words is beyond scientific comprehension.

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1 Introduction

1.1 Scope

At all times our organism is simultaneously exposed to different bioactive compounds. Some of them might occur in our food, as native food constituents or even contaminants. Others – like drugs - are deliberately consumed to exert certain effects. Over decades, much research has been dedicated to describe the impact of those compounds on cells, tissue and organisms. But less attention has been paid to the interplay of multiple chemicals targeting similar cellular pathways. However, the existence of antagonistic or synergistic interactions may completely change the risk of a toxic compound, or decide the outcome of a drug therapy.

One group of compounds that has a high potential to cause such interactions is the class of polyphenols. Those wide-spread food constituents from fruits and vegetables have been described to exert a wide range of biological effects. Especially their outstanding capability to counteract oxidative stress by either scavenging free radicals or inducing the cellular oxidative defense system is well documented. Thus, polyphenols in general are linked to chemopreventive, anti-oxidative and anti-inflammatory effects that are beneficial for human health[1]. Besides those attributes, some polyphenols have also been shown to exert cytotoxic, cytostatic or even genotoxic effects and to affect crucial enzymes like topoisomerases (Topo) and receptor tyrosine kinases (RTK), which represent potential targets in chemotherapy [2, 3].

1.1.1 Objectives

This thesis was dedicated to explore interactions between selected dietary polyphenols and certain chemotherapeutic drugs and food contaminants that use cellular pathways known to be affected by the former. The following questions have been addressed:

1. Are polyphenols able to modulate the cytostatic properties of chemotherapeutic RTK inhibitors, exemplified by erlotinib (ERL), a drug inhibiting the epidermal growth factor receptor (EGFR)?
 - a. If so, what are the mechanisms behind the interactions?
 - b. What are the resulting perspectives for therapeutic treatment?

2. Do polyphenols modulate the toxicity of mycotoxins produced by *Alternaria spp.* and if so, which biological pathways are responsible?

1.2 Polyphenols

Polyphenols, defined as compounds with at least two hydroxyl groups attached to an aromatic ring system, represent a vast class of secondary plant metabolites. A high number of studies have addressed their potential beneficial effects on human health[1].

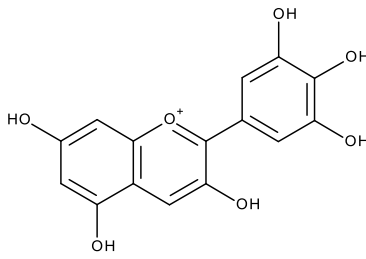
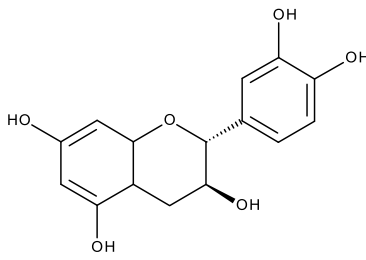
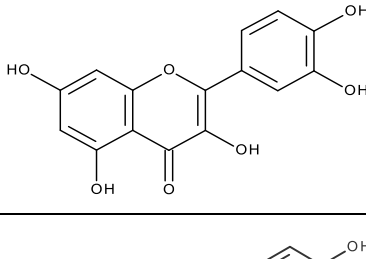
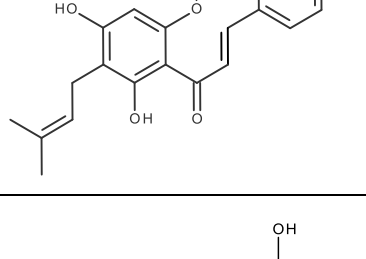
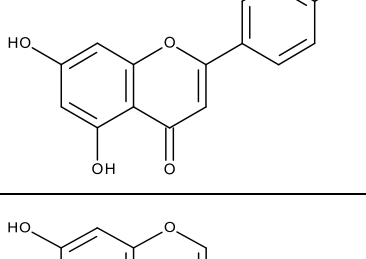
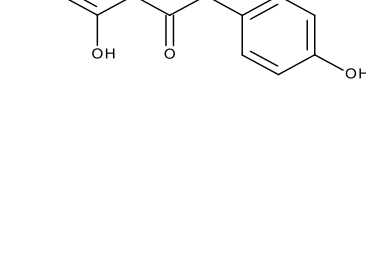
Most dietary polyphenols belong to the class of flavonoids, which can in turn be divided in subclasses that differ in substitution pattern and position of the benzene moieties (Table 1).

1.2.1 Isoflavones

1.2.1.1 Occurrence and bioavailability

Isoflavones occur in several plants, but the main human dietary sources are soy-based products like tofu, where the major isoflavones genistein (GEN) and daidzein (DAI) are abundant. While they occur glycosylated in raw soy beans, fermentation causes the cleavage of the sugar moiety, leading to the ingestion of the more lipophilic aglycons, which are able to pass the gastrointestinal barrier and are therefore taken up systemically [4]. A human intervention study administering soy food to healthy women recovered up to 63.2% of the given DAI and 25.2% of the given GEN, with peak plasma concentrations around 2-3 μM [5]. Of note, this study used realistic soy amounts that can easily be reached with a normal diet, so one has to consider that upon the intake of isoflavone-containing food supplements, higher plasma concentrations could be reached.

Table 1: Subclasses of flavonoids

subclass	exemplary compound
anthocyanidins	delphinidin
	
flavanols	(+)-catechin
	
flavonols	quercetin
	
chalcones	xanthohumol
	
flavones	luteolin
	
isoflavones	genistein
	

1.2.1.2 Bioactivity

Isoflavones are known for their estrogenic activity, as some of them structurally mimic 17 β -estradiol (E2), and thus are able to bind to and activate estrogen receptors [6]. This has brought much attention to soy isoflavones and the application as food supplements for women suffering from a decrease of E2 levels during menopause.

On the other hand, concerns on their impact on health has grown as the most potent isoflavone, GEN, has been shown to possess genotoxic and clastogenic (i.e. “chromosome-breaking”) properties in high concentrations, probably due to its ability to interfere with mammalian topoisomerases (Topos) [7-10], enzymes crucial for the regulation of DNA topology. While it is highly unlikely to reach systemic amounts high enough for GEN to exert those effects with a soy-rich diet, the intake of isoflavone-containing food supplements could result in concentrations high enough for a genotoxic impact on cells, in particular in the gut [11].

GEN was also reported to inhibit receptor tyrosine kinases (RTKs), including the human growth factor receptor (EGFR), and thus to inhibit the growth of cancer cells which are often overexpressing those receptors [12].

1.2.2 Anthocyanins

1.2.2.1 Occurrence and bioavailability

Anthocyanins consist of a flavonoid aglycon, the anthocyanidin, and at least one glycosidically bound sugar. They are deeply red to blue colored compounds present in the epithelia of leaves, flowers or fruits. Humans are exposed to these compounds via ingestion of fruits that are rich in pigments, like bil- and blueberries, elderberries, aubergines or red wine [13]. After the uptake, the glycosidic bond is hydrolyzed by the gut microbiota and the free anthocyanidin is subject to chemical degradation due to the pH-neutral environment in the intestine, resulting in the formation of phloroglucinol aldehyde (PGA) and a phenolic acid [14, 15].

Thus, systemic bioavailability of anthocyanidins is low and their site of action is mainly limited to the gastrointestinal tract. However, it should be considered that degradation products, which might be taken up in a higher amount, have also been shown to exert biological effects. For example, PGA was shown to induce the cellular defense system against oxidative stress via the Nrf2 pathway [16].

1.2.2.2 Bioactivity

Anthocyanins and their aglycons are often associated with chemoprevention and anti-oxidative properties. Many of them are acting as free radical scavengers, enabling them to quench reactive oxygen species (ROS). Thus, they are preventing oxidative stress, the state of a cell where pro-oxidative species increase to a level where the innate anti-oxidative defense system is “overloaded”, which in turn leads to damage of proteins, lipids or the DNA [17].

Some anthocyanidins were also shown to inhibit RTKs and to reduce the growth of cancer cells. The most potent compounds in this regard were the catechols delphinidin (DEL) and cyanidin [18-20]. In addition, DEL was reported to act as a catalytic inhibitor of topoisomerase II (Topo II) by preventing the binding of the enzymes to the DNA [21].

1.3 Interactions of polyphenols with drugs and food contaminants

1.3.1 Erlotinib

Several important proteins involved in cell growth and proliferation signalling belong to the group of RTKs. One of the most prominent representatives is the epidermal growth factor receptor (EGFR), which is often overexpressed in cancer cells. One drug targeting this receptor is ERL (Figure 1), which is approved for the treatment of late-stage non-small cell lung cancer and pancreatic cancer [22]. As some dietary polyphenols were recently shown to inhibit the EGFR as well – albeit much less potently – the question whether those compounds would influence the activity of ERL arose. Initial *in vitro* studies combining the polyphenols (-)-epigallocatechin-3-gallate (EGCG) and genistein (GEN) with ERL treatment found those compounds to enhance the activity of ERL and to delay the acquirement of resistance against the drug in different cancer cell lines [23-25]. However, in the following clinical trial GEN failed to improve patient survival, even as it reduced the incidents of severe skin rash, one of the common side effects of ERL [26]. As also some anthocyanidins were reported to act as RTK inhibitors, interactions of the latter with ERL seem conceivable.

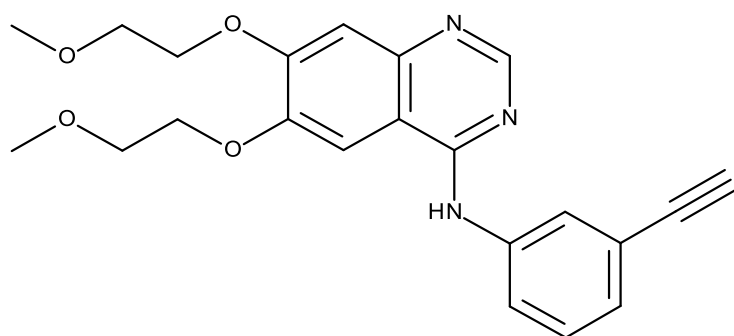


Figure 1: Erlotinib (ERL)

1.3.2 *Alternaria* toxins

Apart from drugs there are other bioactive compounds that target pathways which may be modulated by polyphenols, e.g. food contaminants, or more specifically mycotoxins. Mycotoxins are toxic secondary metabolites of molds and may enter the food chain when food or food raw materials are contaminated by fungi. One emerging group of mycotoxins are produced by *Alternaria spp.* Those are characteristically black molds growing under a wide range of conditions, and they simultaneously produce a variety of different toxins, amongst others alternariol (AOH, Figure 2) and its monomethyl ether (AME), tenuazonic acid, altertoxins (ATX) I-III (Figure 3), stemphyliotoxins and altenuene. The kind of toxins that are produced and their relative concentrations are differing depending on the fungal strain and the respective growth conditions, which makes risk assessment of *Alternaria* toxins even more challenging.

Several recent studies showed dietary polyphenols to protect against the toxicity of different mycotoxins. The anthocyanin cyanidin-3-*O*-glucoside (C3G) [27], green tea polyphenols (GTPs) [28] and a polyphenol-rich cocoa extract [29] were found to antagonize cytotoxic and pro-oxidative effects of ochratoxin A in different cell lines. The toxicity of aflatoxin B1 (AFB1) was shown to be tempered *in vitro* by C3G [27], a rosemary extract [30] or resveratrol (RES) [31]. *In vivo* studies confirmed those results for RES in broiler birds [32] and found curcumin to protect against AFB1-induced liver damage in rats [33]. Furthermore, GTPs were found to protect against toxic effects of patulin in mice [34] and deoxynivalenol *in vitro* [35]. Concerning *Alternaria* toxins, Chiesi et al. observed a protective effect of the polyphenol-rich fraction of native olive oil against the cytotoxic and pro-oxidative effects of AOH *in vitro* [36]. However, cytotoxicity is not the main issue with AOH exposure, as the compound is considered to be

genotoxic due to its Topo II-poisoning properties [37]. As mentioned briefly in 1.2.1.2, Topo II enzymes are involved in regulating DNA topology. They help with the unwinding process by forming a covalent complex with the DNA, inducing a double strand break, allowing a second DNA strand to pass through, and then religating and releasing the DNA. Topo II poisons are compounds that are able to stabilize the covalent complex between the enzyme and the DNA, which leads to a prolongation of the induced strand break and thus to genotoxic effects. As described in 1.2.2.2, some anthocyanidins have been identified as catalytic inhibitors of Topo II, i.e. they prevent the binding of Topo II to the DNA and the induction of strand breaks. Interestingly, recent publications found those catalytic inhibitors to diminish complex-stabilizing and genotoxic effects of chemotherapeutic Topo poisons *in vitro* [38-40] and *in vivo* [41]. Thus, a similar interaction might take place regarding the genotoxicity of AOH.

Another *Alternaria* toxin, the altertoxin II (ATX-II), was recently discovered as the major genotoxic compound in an extract of *Alternaria alternata* cultured on rice [42]. It does by far exceed the DNA-damaging properties of other toxins like AOH. At the low concentrations where DNA damage can be detected *in vitro*, the compound is not influencing the activity of Topos [43] or causing oxidative stress [44]. As ATX-II carries an epoxy group, it might be speculated that the mode of action includes the ability to form DNA adducts [42]. However, this hypothesis is still awaiting experimental validation.

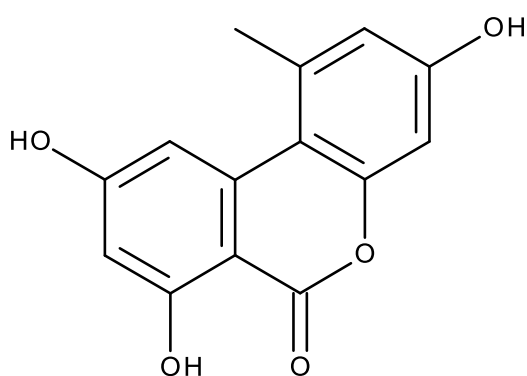


Figure 2: Alternariol (AOH)

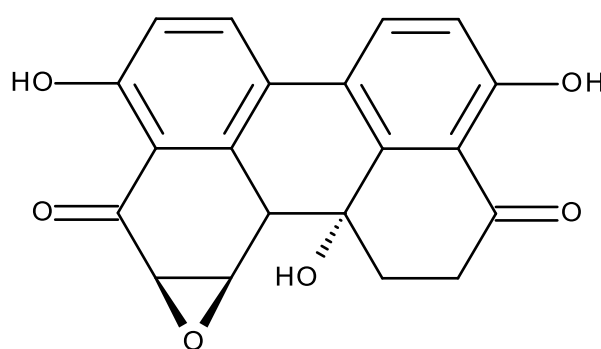


Figure 3: Altertoxin-II (ATX-II)

1.3.3 Discussion of key results

The first objective of this thesis was to address the question whether polyphenols are able to modulate cytostatic properties of ERL, and to find mechanisms for the observed effects. Thus, and for the reasons described in 1.3.1, it was decided to combine a bilberry extract (BE), its main anthocyanin delphinidin-3-*O*-glucoside (D3G), the corresponding anthocyanidin delphinidin (DEL) and its potential degradation products – the phloroglucinol aldehyde (PGA) and gallic acid (GA) - with ERL. GEN was included to compare obtained results to previous studies. Experiments to assess the impact of those food constituents on ERL's cytostatic and EGFR-inhibiting effects were carried out in A431 epithelial carcinoma cells that overexpress the EGFR and are therefore commonly used in studies involving effects on RTKs. Contrary to the original hypothesis, the food constituents were found to antagonize the cytostatic effects of the drug. In the conducted SRB assays, a 72 h co-incubation with either GEN, the BE, D3G or DEL lead to higher IC₅₀ values of ERL. For GEN and BE, a combination index (CI) analysis (see chapter 3.3) was possible and revealed antagonistic interactions of each of the components with the cytostatic potency of ERL. Of note GA and PGA did not exert antagonistic combinatory effects in that regard, so it can be assumed that these degradation products are not responsible for the observed interactions. GEN was additionally found to interact with ERL's ability to inhibit the EGFR in SDS-PAGE/Western Blot experiments, as the inhibition of receptor phosphorylation - i.e. activation – was found be significantly lower than additive when both compounds were present. Thus it can be assumed that an interplay with this pathway is an involved mechanism for the observed reduction of growth-inhibiting effects. For the berry constituents, no such mechanism was found. Here, other mechanisms should be considered to explain the impairment of ERL's cytotoxicity. These results were published in Food&Function, 2016 (publication 1) [45].

The second objective was to investigate a potential impact of polyphenols on the toxicity of mycotoxins produced by *Alternaria spp.* In accord with the considerations described earlier (1.3.2), it was decided to assess the influence of the catalytic Topo inhibitor and anti-oxidative agent DEL on the toxicity of the pro-oxidative and Topo-poisoning mycotoxin AOH. Again, the isoflavone GEN, which was also described as a Topo poison [46], but in addition is sometimes also attributed effects on the cellular redox status [47], was included. For experiments, HT-29 colon carcinoma cells were used as the systemic bioavailability of the compounds, in particular of DEL, might be very low and the highest concentrations are to be expected in the

gastrointestinal tract. While a 1 h incubation of those cells with AOH (50 μ M) or with DEL (>25 μ M) resulted in a significantly increased rate of DNA strand breaks, the combination of the compounds did not further increase the genotoxic effect, which was significantly lower than the calculated expected effect (see 3.2.1), indicating an antagonistic interaction. GEN, while not being genotoxic itself in the applied concentrations and incubation times, also lead to significantly lower combinatory effects than expected. As a mechanism, both polyphenols were found to antagonize the stabilization of DNA/Topo II complexes that was significantly induced by 50 μ M AOH in the “*in vivo* complex of enzyme” (ICE) assay. While GEN was antagonistic in that regard only at the highest concentration of 100 μ M, DEL showed significant antagonistic effects at concentrations of 25 μ M and above, and even lead to a significant reduction of DNA/Topo II complex levels at 50 μ M. Additionally, DEL lead to a significant reduction of AOH-induced intracellular oxidative stress in the dichlorofluorescein (DCF) assay, which might further contribute to the protective effect against the mycotoxin’s genotoxicity. These results were published in Molecular Nutrition and Food Research, 2017 (publication 2) [48].

Another *Alternaria* toxin, the ATX-II, has an exceptionally high genotoxic potential. While AOH targets the Topo II, ATX-II causes DNA damage via a different pathway, assumedly due to the high reactivity of its epoxy moiety. Thus, DEL was also tested for possible interactions with ATX-II. Surprisingly, very strong protective effects of the anthocyanidin against ATX-II-induced genotoxicity occurred when both compounds were incubated simultaneously. After an impact on oxidative stress and topoisomerases could be excluded, the presence of DEL, but also of its degradation products GA and PGA, were found to significantly decrease the amount of ATX-II in aqueous solutions in a cell-free system analyzed by LC-MS. This indicates that DEL and/or PGA and GA spontaneously react with ATX-II under physiological conditions, thus enhancing its chemical degradation and producing a detoxifying effect in the comet assay. The manuscript containing those results was submitted to Toxicology Letters and is currently under review (publication 3).

2 Overview of publications and manuscripts

publication 1 [45]

<u>status:</u>	published
<u>title:</u>	Bilberry extract, its major polyphenolic compounds, and the soy isoflavone genistein antagonize the cytostatic drug erlotinib in human epithelial cells
<u>authors:</u>	Georg Aichinger ^a , Gudrun Pahlke ^a , Laura J. Nagel ^a , Walter Berger ^b and Doris Marko ^a
<u>affiliations of authors:</u>	a: University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria b: Department of Medicine I, Institute of Cancer Research, Borschkegasse 8A, A-1090 Vienna, Austria
<u>published in:</u>	Food & Function, Volume 7, pages 3628-3636
<u>year:</u>	2016
<u>accepted for publication:</u>	July 16 th , 2016
<u>DOI</u>	10.1039/c6fo00570e
<u>author's contribution:</u>	The study design was developed by Doris Marko, Gudrun Pahlke, Walter Berger and Georg Aichinger. Laura Nagel conducted preliminary experiments. Georg Aichinger performed <i>in vitro</i> tests, evaluated the data, applied statistics and wrote the initial draft. Doris Marko and Gudrun Pahlke supervised the project and refined the manuscript.

publication 2 [48]

<u>status:</u>	published
<u>title:</u>	Genistein and delphinidin antagonize the genotoxic effects of the mycotoxin alternariol in human colon carcinoma cells
<u>authors:</u>	Georg Aichinger ^a , Julia Beisl ^a and Doris Marko ^a
<u>affiliations of authors:</u>	a: University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria
<u>published in:</u>	Molecular Nutrition and Food Research, 61(2)
<u>year:</u>	2017
<u>accepted for publication:</u>	Sept. 11 th , 2016
<u>DOI</u>	10.1002/mnfr.201600462
<u>author's contribution:</u>	Georg Aichinger was involved in the planning of the studies, conducting ICE assay and DCF assay experiments, statistical analysis and writing. Julia Beisl carried out SRB/WST-1 and comet assays. Doris Marko was involved in study planning, supervision of the project and refining the manuscript.

publication 3

<u>status:</u>	submitted
<u>title:</u>	Delphinidin protects colon carcinoma cells against genotoxic effects of the mycotoxin altertoxin II.
<u>authors:</u>	Georg Aichinger ^a , Hannes Puntischer ^a , Julia Beisl ^a , Mary-Liis Kütt ^a , Benedikt Warth ^a and Doris Marko ^a
<u>submitted to:</u>	Toxicology Letters
<u>affiliations of authors:</u>	a: University of Vienna, Department of Food Chemistry and Toxicology, Waehringer Str. 38, A-1090 Vienna, Austria
<u>accepted for publication</u>	N/A
<u>DOI</u>	N/A
<u>author's contribution:</u>	Georg Aichinger was involved in the planning of the studies, conducting comet and DCF assays, statistical analysis and writing. Julia Beisl carried out SRB/WST-1 assays. Hannes Puntischer und Mary-Liis Kytt carried out LC-MS experiments. Benedikt Warth refined the manuscript. Doris Marko was involved in study planning, supervision of the project and refining the manuscript.

3 Theoretical background

3.1 Concepts of additivity and interactions

Two or more compounds per definition interact with each other when their combinatory effect is not additive, but rather synergistic (i.e. “more than additive”) or antagonistic (“less than additive”). Thus, to assess an interaction one has to calculate an expected additive effect for the compounds and then compare it to the actually measured combinatory effect. The challenge this provides to scientists is to calculate additive effects of two compounds a and b, as the seemingly obvious formula 1 only applies if both effects follow a linear dose-response relationship, a situation that is hardly ever the case in biological systems. Thus, mathematical models were created to assess interactions more accurately.

$$\text{expected additive effect } (f_{ab}) = \text{effect a } (f_a) + \text{effect b } (f_b) \quad (1)$$

3.2 Early Models

3.2.1 Independent joint action (IJA)

The IJA model (also referred to as “Webb’s fractional product” or “Bliss Independence”) was originally described by C. I. Bliss. In a preliminary publication, he observed that the toxicity of most compounds follows a non-linear, but rather hyperbole-like dose-response relationship [49]. A perfect hyperbole is described by the formula:

$$f(x) = 2x - x^2 \quad (2)$$

Bliss suggested that upon logarithmizing the dose, $f(x)$ would behave linear for a useful concentration range. Thus, in 1939, he proposed that when two poisons that are joint in application, but act independently, their combined effect is best estimated by addition of the effects at the corresponding doses of the single compounds at a logarithmic scale [50].

In 1963, J.R. Webb, who was mostly interested in joint action of enzyme inhibitors, further refined the model by expanding it to any inhibitory process [51]. He concluded that while any inhibition of a biological process is obviously capped, as the maximal inhibitory effect cannot exceed 100%, a simple addition of f_a and f_b would often result in f_{ab} values above 100% and

that therefore this equation could not picture the reality of interactions in inhibitory systems. Thus, Webb suggested a hyperbole-mimicking calculation of f_{ab} as following:

$$f_{ab} = f_a + f_b - f_a * f_b \quad (3)$$

This formula provided an easy tool to assess additive behaviour for many biological systems and was (and is up to date) widely used in different scientific fields.

3.2.2 Simple similar joint action (SSA) and Loewe's isobologram

The SSA model is another model that is widely used to calculate additivity. While in the IJA model the compounds are exerting effects via independent modes of actions, for the SSA it is assumed that they share their mode of action, i.e. (in the words of Bliss, 1939) they “produce similar but independent effects, so that one component can be substituted at a constant proportion to the other” [50]. With SSA, the expected additive effect is expected to follow the equation:

$$g(f_{ab}) = h(C_a + x * C_b) \quad (4)$$

C_a and C_b are the concentrations of components a and b, x is a numerical constant that describes the different relative effect strengths of the components, g and h are any functions describing the curve shape. After parameters g and x have been experimentally determined, f_{ab} can be calculated for any combination of the two compounds [52].

In 1928, S. Loewe invented the isobologram (Figure 4) as a graphical and mathematical tool to assess interactions based on the foundations of the SSA [53]. Here, effective dose (ED) values of two compounds that share a similar mode of action are experimentally determined. ED values of the same effect strength (most commonly ED₅₀ values) are then plotted at two axis, and connected with a line that represents f_{ab} for every possible combination ratio of the compounds, called the “isobole”. Then, corresponding ED values for constant ratios of those two compounds are determined and their concentrations are plotted as well. If a measured combinatory ED_x lies inside the triangle of axis x, axis y and isobole the combination is considered synergistic, if it lies outside antagonistic, and if it lies very close to the isobole, it is considered additive. Loewe also expressed these ideas by suggesting the calculation of an “interaction index” (T) by:

$$T = \frac{a}{A} + \frac{b}{B} \quad (5)$$

A, B represent ED_x values of the single components and a, b the concentrations of the components at the ED_x value of the combination (C). T<1 indicates a synergistic, T>1 an antagonistic interaction [53, 54].

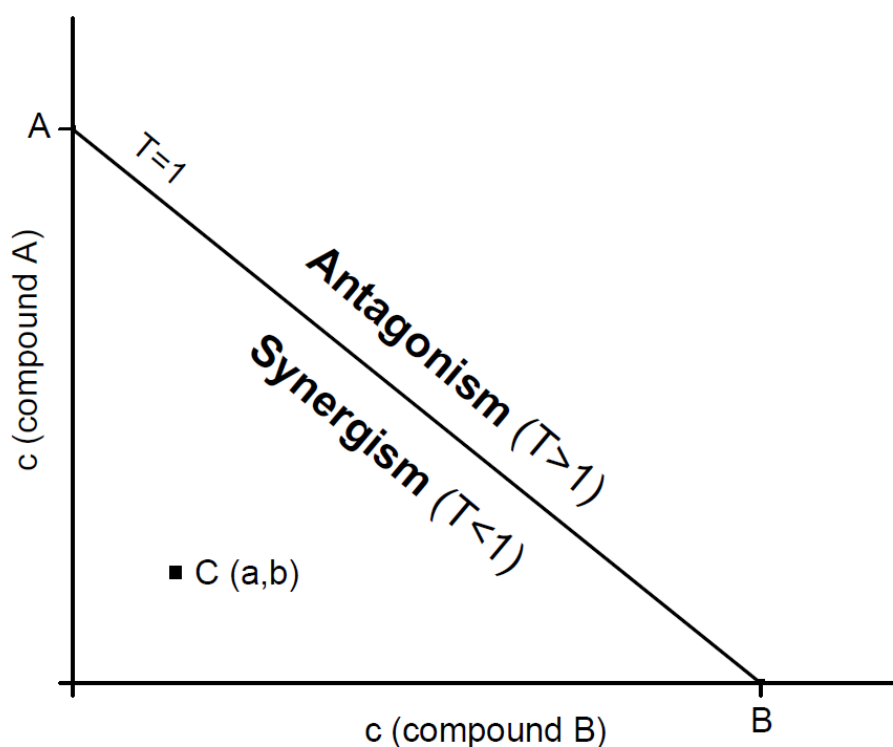


Figure 4: Loewe's isobologram. A, B are ED_x values of compounds A and B, the line connecting the two points is the isobole, where T=1. The measured ED_x value for the combination of the compounds (C) with concentrations a, b can either indicate synergistic (T<1, "under the isobole"), additive (T≈1, "at the isobole") or antagonistic (T>1, "above the isobole") behavior [53].

3.2.3 Limitations of conventional models

The models described above have been extensively used throughout the 20th century, especially in pharmacological research, but also in toxicology. They have, however, significant limitations [55]. First, each model is only valid for a certain relationship between the modes of action of two compounds. IJA is only allowed for compounds with totally independent effects, while for application of SSA or the isobologram, the modes of actions must be similar. Sometimes the mode of action is not clear, forcing researchers to guess which model is appropriate. Furthermore, the IJA is only valid for effects following a hyperbolic dose-response

relationship, a requisition that is sometimes not fulfilled. Over time, researchers have again and again bent those rules and – lacking alternatives - used the models even though they could often not guarantee that the requirements for their application were met. Thus, in 1983, Chou and Talalay established an exact and universal mathematical model for interaction analysis, which has since become known as the “combination index theorem” [56].

3.3 The combination index theorem

3.3.1 Median-effect equation

While other models were based on experimental observations, Chou and Talalay based their calculations on the mass-action law, thus gaining universal validity regardless of modes of action and curve shapes [55]. Deriving from the latter law, Chou et al. found that any dose-response relationship could be described in an equation that was called “median-effect equation” ((6) [57].

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m} \right)^m \quad (6)$$

f_a describes the “affected fraction” and f_u the “unaffected fraction”, e.g. if 9 out of 10 animals would die during a study, f_a would be 90% and $f_u = 1 - f_a = 100\% - 90\% = 10\%$. When dealing with *in vitro* assays, f_a is defined as the effect and $f_u = 1 - f_a$. D is the applied dose of a compound, D_m the median-effect dose (most commonly the ED₅₀) and m is a variable describing the order, i.e. the shape, of the dose-response curve. The equation can also be expressed as:

$$\log \left(\frac{f_a}{f_u} \right) = m * \log(D) - m * \log(D_m) \quad (7)$$

As D_m is constant, this equation describes the effect to be a linear function of the applied dose. Thus, if the experimentally determinable $\log \left(\frac{f_a}{f_u} \right)$ is plotted against $\log(D)$ (the so-called “median-effect plot”), D_m and m can be calculated for any dose-response curve.

3.3.2 The combination index

Subsequently, these parameters can be used to calculate effective doses (ED_x or D_x) for any $f_a = x\%$ and those can be used to plot an isobologram and to calculate the combination index (CI) [56]. The CI of two compounds (a, b) is similar to Loewe’s interaction index and is described as:

$$CI_x = \frac{(D)_a}{(D_x)_a} + \frac{(D)_b}{(D_x)_b} \quad (8)$$

As with the interaction index, $CI < 1$ indicates synergistic, $CI = 1$ additive and $CI > 1$ antagonistic behavior of the two compounds. The CI theorem is also applicable for mixtures of three or more compounds, by using the more general definition:

$${}^nCI_x = \sum_{j=1}^n \frac{(D)_j}{(D_x)_j} \quad (9)$$

As experimental deviations of results are not taken into account by this model, Chou suggested a semi-quantitative scheme to evaluate the strength of an interaction (Table 2) [55]. Here, a CI between 0.9 and 1.1 is considered “nearly additive”. A CI below 0.9 is considered synergistic with increasing strength the lower the CI drops, and a CI above 1.1 is considered antagonistic with increasing strength the higher the CI rises.

Table 2: Description of interactions depending on CI values[55]

Range of CI	Description
<0.1	very strong synergism
0.1-0.3	strong synergism
0.3-0.7	synergism
0.7-0.85	moderate synergism
0.85-0.9	slight synergism
0.9-1.1	nearly additive
1.1-1.2	slight antagonism
1.2-1.45	moderate antagonism
1.45-3.3	antagonism
3.3-10	strong antagonism
>10	very strong antagonism

3.3.3 Additional plots and indices

Deriving from the CI calculation there are some additional meaningful graphs and indices that are commonly shown in publications about interactions. Beside the traditional isobolograms, f_α -CI plots, as also displayed in my publications [45, 48], are used to highlight the change of an interaction depending on the effect strength. The dose-reduction index (DRI) is a useful tool

for drug interaction studies and states how-many fold the dose of an agent can be reduced to get the same effect in combination with another drug. f_a -DRI plots show the shift of the DRI with changing effect strength. And polygonograms can be used to increase the visibility of different interactions between a group of compounds [55].

3.3.4 Advantages and limitations

The CI theorem possesses several advantages compared to the early models. It is universally valid regardless of curve shape and mode of action. Experimental results usually fit well to the median-effect equation, and the CI delivers not only a qualitative statement if an interaction exists, but is also a measure for the strength of the interaction. The method also provides additional parameters and possibilities of graphical display [55].

However, it should be noted that there are also some practical limitations to this model. First, fitting results to the median-effect equation requires all single compounds and mixtures to reach ED_{50} values. If one of the combined components is not effective enough, the model is not applicable as an extrapolation of D_m would inevitably cause errors. Second, CI calculation is only possible if all compounds share the general direction of their effects. It is, for example, not possible to calculate an interaction between an activator and an inhibitor of cell growth, as it would be possible with the SSA model. And third, the testing of constant combination ratios between the compounds is required, something that is possible for high-throughput assays, but is very demanding for more complex assays.

3.4 Application in this thesis

For the reasons described in section 3.3.4, CI analysis was applied whenever it was possible, i.e. for all assays on cytotoxicity where IC_{50} values could be reached by administration of the single compounds. For all other methods, including low-throughput assays like the ICE or comet assay the IJA model was used to assess interactions.

4 Conclusion

The objective of this thesis was to search for interactions and their underlying mechanisms between selected polyphenols and two groups of other bioactive compounds, on the one hand RTK-inhibiting drugs as exemplified by ERL and on the other hand mycotoxins produced by *Alternaria spp.*, represented by AOH and ATX II (see section 1.1.2.). This thesis demonstrates the high potential of dietary polyphenols to interact with these substances through a variety of different mechanisms.

In A431 cells, the isoflavone GEN was shown to antagonize the ability of the chemotherapeutic drug ERL to inhibit the EGFR, leading to a reduction of its cytostatic strength. Also an anthocyanin-rich bilberry extract, its main anthocyanin D3G and the corresponding anthocyanidin DEL were found to impair ERL's ability to inhibit cell growth (publication 1) [45]. In HT-29 cells, GEN and DEL were found to antagonize Topo II poisoning and resulting genotoxic effects of the mycotoxin AOH (publication 2). For DEL, also its strong radical-scavenging and thus anti-oxidative properties pose a possible source of interactions with AOH [48]. Furthermore, DEL and its degradation products might also represent possible reaction partners for reactive food contaminants like ATX-II, enhancing the chemical degradation of the toxin and therefore protecting cells from genotoxic damages (publication 3).

Of note, most interactions that were observed during the course of this thesis were antagonistic, which is in line with recent literature, where mostly protective effects against contaminants are described [27-36]. Thus, polyphenols are understood as promising agents to protect against – or at least to temper – toxic effects of food contaminants like mycotoxins. The finding of protective effects of anthocyanidins against *Alternaria* toxins is of special relevance as those compounds might often co-occur in food [58]. Future studies will expand the survey to other toxins of that family as well as to *Alternaria* extracts and also address the situation *in vivo*. Also, this thesis proposes a potential hypothesis to explain the phenomena that ATX-II was until now hardly ever found in real food matrices, but found in comparably high concentrations in extracts of cultured *Alternaria alternata*, as the chemical degradation of the toxin is shown to be considerably enhanced in the presence of anthocyanins, a very common situation *in planta* [58]. Furthermore, the hypothesis that catalytic Topo inhibitors reduce the toxic impact of Topo poisons could again be affirmed, as DEL and GEN were found to impair the stabilization of Topo/DNA complexes and the induction of DNA strand breaks by

AOH, which is in line with previous studies on protective effects of anthocyanidins against chemotherapeutic Topo poisons [38-41].

However, while undergoing a chemotherapy, protective effects of secondary food constituents against cytostatic drugs are generally less desirable. Here, the main question is whether the systemic bioavailability of the relevant polyphenols is high enough to exert these antagonisms at the target organs of the affected drugs. If this is not the case, a polyphenol-rich diet or the intake of respective food supplements during therapy could even reduce gastrointestinal side effects that are very common during treatment, and thus increase the well-being or even the survival chances of patients. Especially anthocyanins are promising in that respect, as their systemic bioavailability is extraordinarily low due to hydrophilicity and limited chemical stability [59]. Of course it has to be considered that chemotherapy often causes damages to the gastrointestinal barrier, which could in turn increase the bioavailability of anthocyanins. Thus, *in vivo* studies are urgently required to address these questions.

Taken together, this thesis provides an initial base on interactions of polyphenols and especially anthocyanidins with drugs and food contaminants, providing a starting point for further investigations on the *in vivo* situation.

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List of abbreviations

AME	alternariol monomethyl ether
AOH	alternariol
ATX-II	altertoxin II
BE	bilberry extract
C3G	cyanidin-3- <i>O</i> -glucoside
CI	combination index
D3G	delphinidin-3- <i>O</i> -glucoside
DAI	daidzein
DCF	dichlorofluorescein
DEL	delphinidin
DNA	deoxyribonucleic acid
DRI	dose reduction index
E2	17 β -estradiol
ED	effective dose
EGCG	(-)-epigallocatechin gallate
EGFR	epidermal growth factor receptor
ERL	erlotinib
EV	expected additive value
f_{ab}	expected additive effect
ICE	<i>in vitro</i> complex of enzyme
IJA	independent joint action
GA	gallic acid
GEN	genistein
GTP	green tea polyphenols
Nrf2	nuclear factor erythroid 2-related factor 2
PGA	phloroglucinol aldehyde
RES	resveratrol
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SRB	sulforhodamine B

SSA	simple similar joint action
Topo	topoisomerase
WST-1	water soluble tetrazolium 1

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Publication 1:

Cite this: *Food Funct.*, 2016, **7**, 3628

Bilberry extract, its major polyphenolic compounds, and the soy isoflavone genistein antagonize the cytostatic drug erlotinib in human epithelial cells

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Erlotinib (Tarceva®) is a chemotherapeutic drug approved for the treatment of pancreatic cancer and non-small cell lung cancer. Its primary mode of action is the inhibition of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK). Recently, RTK-inhibiting polyphenols have been reported to interact synergistically with erlotinib. Furthermore some anthocyanidins and anthocyanin-rich berry extracts have been reported to inhibit tyrosine kinases, including the EGFR, which raises the question of potential interactions with erlotinib. Polyphenol-rich preparations such as berry- or soy-based products are commercially available as food supplements. In the present study we tested a bilberry extract, its major anthocyanin and potential intestinal degradation products, as well as genistein, with respect to possible interactions with erlotinib. Cell growth inhibition was assessed using the sulforhodamine B assay, while interactions with EGFR phosphorylation were analyzed by SDS-PAGE/western blotting with subsequent immunodetection. Genistein, bilberry extract, delphinidin-3-O-glucoside and delphinidin were found to antagonize erlotinib whereas phloroglucinol aldehyde was found to enhance cytostatic effects of the drug on human epithelial A431 cells. Genistein also antagonized the EGFR inhibitory effects of erlotinib, whereas bilberry anthocyanins showed no significant interactions in this regard. Our data indicate that different polyphenols are potentially able to impair the cytostatic effect of erlotinib *in vitro*. Genistein interacts *via* the modulation of erlotinib-mediated EGFR inhibition whereas bilberry anthocyanins modulated the growth-inhibitory effect of erlotinib without affecting EGFR phosphorylation, thus indicating a different mechanism of interference.

Received 21st April 2016,
Accepted 16th July 2016
DOI: 10.1039/c6fo00570e
www.rsc.org/foodfunction

Introduction

Erlotinib (ERL, Fig. 1A) is a potent tyrosine kinase inhibitor, primarily targeting the epidermal growth factor receptor (EGFR).¹ Upon ligand binding to the extracellular domain of the EGFR, an autophosphorylation sequence of tyrosine residues in the intracellular domain is initiated, activating the subsequent “mitogen activated protein kinase” (MAPK) cascade, which in turn triggers the transcription of genes related to cell growth, proliferation and differentiation. ERL competes with ATP for the intracellular binding domain of the receptor and thus inhibits its phosphorylation, leading to a suppression of growth signals and hence reduced cell proliferation. It is approved for use as a chemotherapeutic agent for EGFR-positive non-small cell lung cancer (NSCLC) and as a

second line therapeutic approach for pancreatic cancer.² Previously, synergistic effects of ERL with EGFR-inhibiting polyphenolic food constituents on tumor cell growth inhibition have been reported. The green tea polyphenol (–)-epigallocatechin gallate enhanced cell growth inhibition of ERL in NSCLC cell lines and in squamous cell carcinoma *in vitro*, as well as in a mouse xenograft model.³ The soy isoflavone genistein (GEN, Fig. 1B) potentiated cell growth inhibition of ERL in NSCLC and pancreatic cancer cell lines *in vitro*,^{4,5} but failed to enhance the effect of ERL on tumor growth and patient survival *in vivo*.⁶ Furthermore it was contemplated that GEN could protect from adverse side effects of the drug, as fewer patients required a dose reduction than in a comparative study without GEN.⁶

Anthocyanins are naturally occurring deeply colored compounds imprinting the color of a multitude of flowers, fruits and vegetables. In particular berries represent a rich source of anthocyanins. They consist of a flavylum-based aglycon – the anthocyanidin – and a sugar residue that is bound *via* O-glycosylation. The general term “anthocyanins” refers to the sub-

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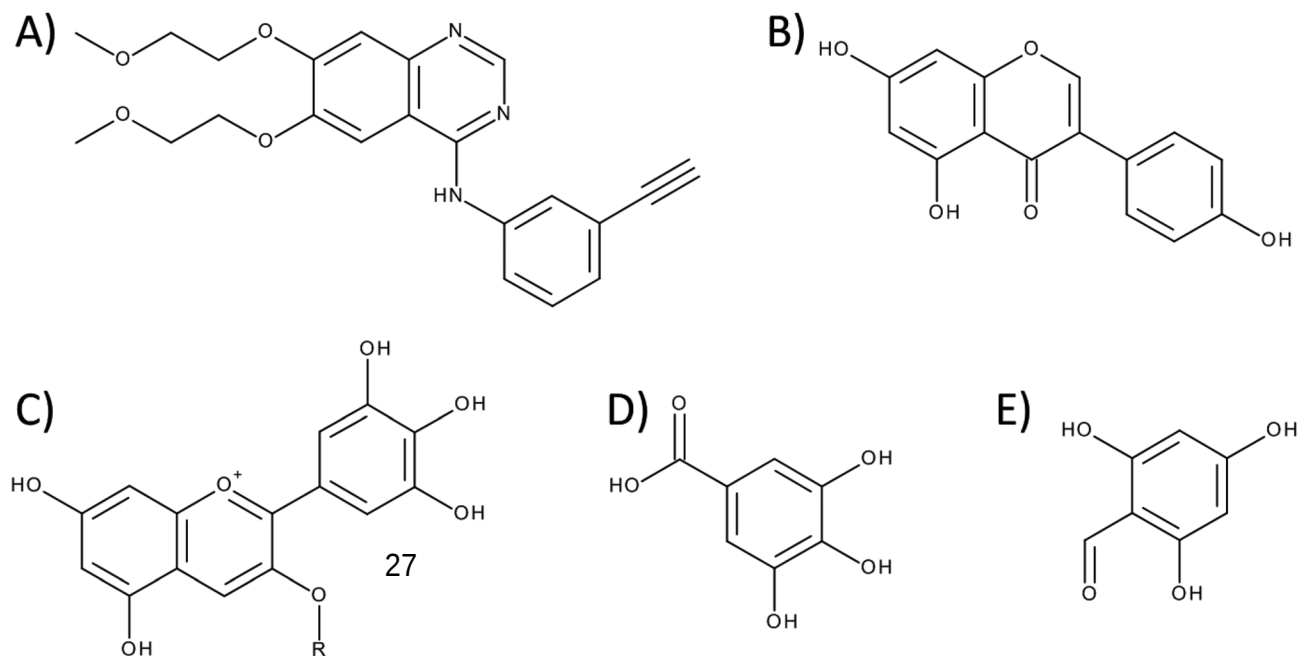


Fig. 1 Chemical structures of (A) erlotinib, (B) genistein, (C) delphinidin (R = OH) and delphinidin-3-O-glucoside (R = glucose), (D) gallic acid and (E) phloroglucinol aldehyde.

stance class that consists of both anthocyanins and anthocyanidins. Anthocyanins possess anti-oxidative, anti-inflammatory and chemopreventive properties and thus they are not only linked with cancer prevention, but also discussed as possible food supplements for patients undergoing chemotherapy.^{7,8} At best, anthocyanins could help in reducing common side effects of chemotherapeutic agents such as diarrhea without diminishing the effect of drugs at the target organ. In line with this hypothesis, a recent *in vitro* study reported that an anthocyanin-rich bilberry extract protects against oxidative stress triggered by the drug mitomycin c without impairing its cytotoxic abilities against bladder cancer cells.⁹ Nevertheless, it cannot be excluded that those compounds might also help to protect cancer cells from cytostatic drugs. Reports about antagonistic interactions with topoisomerase-targeting drugs showed that both anthocyanidins and anthocyanin-rich berry extracts reduce the DNA-damaging effects of camptothecin and doxorubicin in colon carcinoma cells.^{10–12} However, the systemic bioavailability of anthocyanins is expected to be very low, with most studies conducted on this topic finding urinary recoveries of less than 0.2%.^{13–15} This limits their site of action mainly to the gastrointestinal tract (GIT). Thus it seems possible that gastrointestinal side effects of those drugs might be reduced whereas systemic therapeutic effectiveness remains unaffected. However, it has to be taken into account that patients often suffer from impaired gastrointestinal barrier function after preceding chemotherapeutic treatments, which could in turn lead to higher systemic concentrations of compounds than commonly observed in healthy individuals.

Anthocyanins, especially the free aglycons, have been reported to be quite unstable in cell culture medium.¹⁶ Chemi-

cal degradation, which is enhanced by the neutral pH of the culture medium, leads to the formation of phenolic acids (*e.g.* gallic acid in the case of delphinidin) and phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde, PGA).¹⁷ Thus it cannot be excluded that biological effects attributed to anthocyanins are in part produced by their degradation products.

Furthermore, both anthocyanidins and anthocyanin-rich berry extracts have been reported as inhibitors of various RTKs, including the EGFR.^{18,19} As described above, other RTK-inhibiting polyphenols were already reported to interact with ERL. Thus we decided to test a commercially available bilberry extract (BE), its major anthocyanin delphinidin-3-O-glucoside (D3G, Fig. 1C), the corresponding anthocyanidin delphinidin (DEL) and its two major degradation products, gallic acid (GA, Fig. 1D) and phloroglucinol aldehyde (PGA, Fig. 1E), with respect to potential interactions with ERL. GEN was added to our study for comparative reasons as it was already reported to interact with ERL treatment. We assessed interactions at the level of cell growth inhibition and at the primary cellular target of ERL, EGFR phosphorylation, in A431 human epithelial cells, a common model cell line for effects involving the EGFR due to its significant overexpression of this RTK.

Experimental

Materials

Genistein (purity $\geq 95\%$), delphinidin chloride (purity $\geq 97\%$) and delphinidin-3-O-glucoside (myrtillin) chloride (purity $\geq 95\%$) were purchased from Extrasynthese (France). Bilberry extract “600761 Bilberry Extract 25%” was obtained from

Symrise (Hamburg, Germany). The anthocyanin composition of the extract has been analyzed previously by our group.¹⁸ Antibodies for immunodetection were purchased from Santa Cruz (Heidelberg, Germany). EGF and cell lysis buffer were obtained from New England Biolabs (Frankfurt, Germany). Erlotinib, gallic acid, phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde) and catalase (from bovine liver) were purchased from Sigma-Aldrich (Taufkirchen, Germany). ECL detection reagents were obtained from GE Healthcare (Buckinghamshire, UK).

Methods

Cell culture and treatment. The human epithelial carcinoma cell line A431 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were grown in Minimum Essential Medium (MEM) without sodium pyruvate, supplemented with L-glutamine (4.5 g L⁻¹), 10% (v/v) heat inactivated fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin. Media and supplements were obtained from Invitrogen™ Life Technologies (Karlsruhe, Germany). The cells were cultivated in humidified incubators (37 °C, 5% CO₂) and routinely tested for mycoplasma contamination.

All test substances were dissolved in DMSO and added to the incubation media, resulting in the final concentrations of the test compounds and 1% (v/v) DMSO. As anthocyanidins were found to generate H₂O₂ in culture medium in previous studies, catalase (100 U mL⁻¹) was added to the incubation solution to prevent oxidative substance degradation or cell damage.

Cell growth. The sulforhodamine B assay (SRB assay) was performed according to a modified method of Skehan *et al.*²⁰ Briefly, 5000 A431 cells per well were seeded into 96 well plates and allowed to attach for 24 h. For “co-incubation” experiments, the cells were incubated with solvent control and different concentrations of polyphenols, erlotinib and polyphenol/erlotinib mixtures for 72 h. For “serial incubation” experiments, the cells were incubated with polyphenols for 24 h, washed with PBS, and incubated with ERL for 48 h. After incubation, the cells were fixed by addition of trichloroacetic acid to the incubation medium and subsequently stained with SRB solution (0.4% w/v). After washing with water and 1% v/v acetic acid, the color was eluted with a 10 mM TRIS buffer at pH 10. Extinctions were measured at 570 nm with a Perkin-Elmer Victor³V multiple plate reader. Effects on cell growth were referred to the solvent control (1% v/v DMSO).

EGFR phosphorylation. Western blot analysis of EGFR and pEGFR levels was performed as described previously.²¹ Briefly, 1 × 10⁶ A431 cells were seeded in Petri dishes (*d* = 10 cm) and allowed to grow for 72 h. The medium was changed to serum-reduced medium (1% FCS) 24 h prior to the incubation. The incubations were performed in a FCS-free medium. The cells were incubated with test compounds or solvent control for 1 h, washed twice with PBS, and treated with ERL for another 1 h. Epidermal growth factor (EGF, 1 µg mL⁻¹) was added for the final 15 min of incubation to stimulate the EGFR.

After incubation, the cells were washed twice with PBS and lysis was performed by adding 200 µl cell lysis buffer containing 1 mM phosphatase inhibitors, followed by cell harvesting and sonication for 15 s. SDS-PAGE and western blot analysis were carried out as described previously.²¹ Primary antibodies for EGFR, phospho-EGFR (pEGFR, Tyr 1173) and α-tubulin (loading control), as well as secondary HRP-coupled anti-goat and anti-mouse antibodies were used for immunodetection with ECL. For quantification, chemoluminescence was captured by using a CCD-imager (Fujifilm LAS4000, Dusseldorf, Germany) and quantified by Multi Gauge 3.2 (Fujifilm). Effects on EGFR expression and phosphorylation were referred to the solvent control (1% v/v DMSO).

Statistical analysis. The terms “synergism” and “antagonism” are defined as the combinatory effect of two or more compounds being higher, respectively lower, than the additive effect of the single compounds. As dose-response curves are rarely linear, the additive effect usually cannot be calculated by simply adding up single effects. Thus several mathematical models exist for the assessment of interactions.

For cell proliferation data, measured values at the same concentration of ERL were compared by one-way ANOVA. IC₅₀ values were obtained by using a hyperbolic curve fit for all raw data points and were compared with Student's *t*-test. If IC₅₀ values could be obtained for the cell growth inhibition by the polyphenol alone, dose-response curves were analyzed by Chou's “multiple drug effect equation” (MDEE),²² where a combination index (CI) is calculated. A CI < 0.9 indicates a synergistic interaction, 0.9 < CI < 1.1 “nearly additive” effects and CI > 1.1 an antagonistic interaction.²³

Results on EGFR inhibition were analyzed by using the model of independent joint action (IJA, also referred to as “Webb's fractional product method”),²⁴ as adapting the method of western blot analysis to the requirements of MDEE analysis was too complex. With IJA, an expected value (EV) is calculated for each combination by using the formula: $f_{ab} = f_a + f_b - f_a \times f_b$, with f_{ab} being the expected combinatory effect and f_a and f_b being the measured effects of the single compounds. The EV was compared to the measured combinatory value with Student's *t*-test.

Results

Genistein

GEN was found to diminish the cytostatic effect of ERL at a molar ratio of 1 : 10 (Fig. 2A). While the significant difference between cell growth inhibition of ERL alone and in a 1 : 10 combination with GEN at 10 µM ERL can mainly be attributed to the additional effect of 100 µM GEN, permitting only a cell growth of about 33%, the combination index at the effect level of 50% (CI₅₀) was 1.90, which indicates an antagonism according to Chou.²³ The effect-CI plot reveals that this interaction becomes even more potent at higher effect levels (Fig. 3A). Combination data for the 1 : 1 combination ratio seem almost perfectly additive at the IC₅₀ (CI = 0.96), but also develop

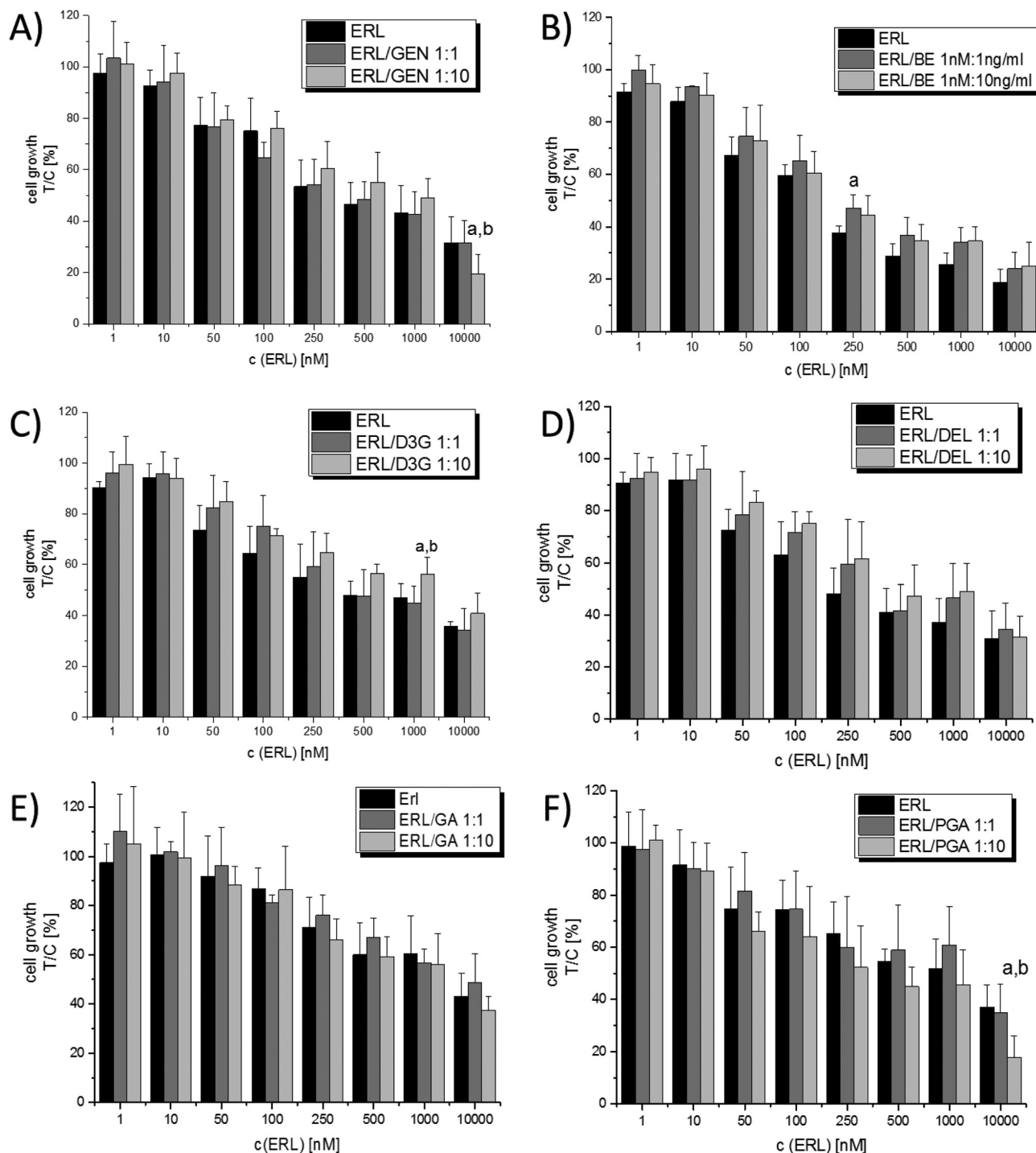


Fig. 2 Influence of GEN (A), BE (B), D3G (C), DEL (D), GA (E) and PGA (F) on the inhibition of cell growth by ERL, measured with the SRB assay. A431 cells were incubated with single substances and the combinations at molar (A, C–F) ratios of 1 : 1 and 1 : 10 for 72 h. Values are shown as percentage of test/control (1% v/v DMSO) T/C + SD. Significant differences between the same concentration of ERL were calculated by one-way ANOVA, followed by Fisher's LSD test ($p < 0.05$). Significant differences to the respective concentration of ERL are referred to as "a", differences to the respective concentration of the 1 : 1 combination as "b".

towards an antagonistic interaction with an increasing effect. The antagonism was observed as well when the cells were pre-incubated with GEN for 24 h and subsequently incubated with ERL for 48 h (Fig. 4A), yielding significantly diminished cell

growth inhibition for the 1 : 10 combination containing 100 nM ERL.

Concerning the potential mechanism, an antagonistic interaction was observed for the inhibition of EGFR phosphoryl-

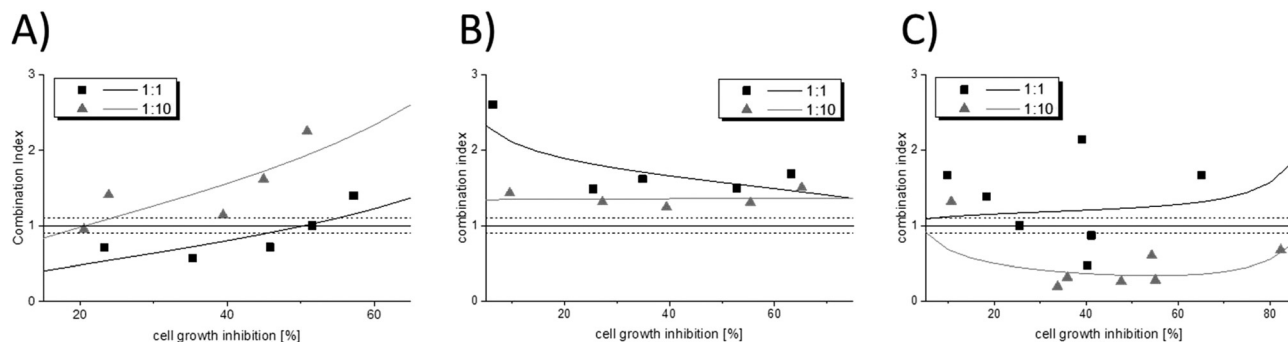


Fig. 3 Effect–combination index (CI) plots for the combination of GEN (A), BE (B) and PGA (C) with ERL, regarding cell growth inhibition after 72 h of co-incubation. CIs at effectively measured concentrations are shown as squares for the 1 : 1 combination ratio or as triangles for the 1 : 10 ratio. Lines represent the CI calculation by Chou's MDEE over the whole effect range, which is based on the median-effect equation by Chou and Talalay.²² A CI > 1.1 is considered antagonistic, a CI < 0.9 synergistic. These key values are indicated as dotted lines.

ation (Fig. 5A). A pre-incubation with 1 μM GEN significantly impaired the EGFR-inhibiting effect of 10 nM ERL. At a concentration of 100 μM , GEN itself led to a significant inhibition of EGFR phosphorylation. Thus, the pEGFR level measured for the combination of 100 μM GEN and 10 nM ERL was not significantly higher than the pEGFR level of 10 nM ERL. However, the combinatory value differed significantly from the calculated expected value, indicating an antagonistic interaction by definition of this term.

Anthocyanins

The BE, its major anthocyanin D3G and the corresponding aglycon DEL interacted also antagonistically with ERL regarding cell growth inhibition (Fig. 2B–D). ERL/BE mixtures with a 1 : 1 ratio resulted in less growth inhibition at 250 nM ERL as compared to the drug alone, and the obtained IC_{50} for the 1 : 1 (ng : nmol) combination (described as mean $\pm$ SD, 226.5 ± 62.3 nM ERL) was significantly higher than for ERL alone (137.5 ± 23.9 nM) (Fig. 2B). The presence of 10 μM D3G caused significantly less cell growth inhibition at 1 μM ERL and again, the obtained IC_{50} for the combination at a molar ratio of 1 : 10 (1091.1 ± 737.4 nM ERL) was significantly higher than for ERL alone (377.5 ± 196.2 nM), as well as the IC_{50} for the 1 : 10 combination of ERL and DEL (combination: 415.1 ± 153.5 nM; ERL: 244 ± 77.2). Unfortunately, the single compounds did not reach 50% cell growth inhibition at the highest applicable concentrations (data not shown), so Chou's MDEE was not applicable for data analysis. For BE, where a growth inhibition of 38% was obtained at the highest concentration of 1 mg ml^{-1} , CI analysis was performed despite the risk of inexactness due to the necessary extrapolation in the median-effect plot. The calculated CI_{50} values were 1.57 for the 1 : 1 combination ratio and 1.36 for the 1 : 10 ratio, which are considered “antagonistic” and “moderately antagonistic”, respectively. The effect–CI plot (Fig. 3B) also shows that the potency of these interactions is quite constant over the measured effect range.

The observed antagonistic interactions were also found in the serial incubation experiments (Fig. 4B–D). While the antag-

onistic trend was not significant for pre-incubations with BE, a pre-incubation with 1 μM D3G significantly impaired the cytostatic effect of ERL at 100 nM ERL, as well as pre-incubations with 1 μM and 100 μM DEL at 100 nM and 10 μM ERL, respectively.

Regarding the impact on EGFR phosphorylation, BE and DEL were found to suppress phosphorylation (Fig. 5B and C). Both substances also enhanced the inhibition of EGFR phosphorylation by ERL. However, measured combinatory pEGFR levels did not significantly differ from the expected values, thus no interaction was observed – *i.e.* the combinatory effects have to be considered “additive”. D3G did not inhibit EGFR phosphorylation and also did not alter EGFR inhibition by ERL (Fig. 5D).

Gallic acid and phloroglucinol aldehyde

As anthocyanins have been reported to be quite unstable in cell culture medium,¹⁶ it might be speculated that bioactive degradation products might at least contribute to cellular effects attributed to the parent compound. To consider this option, we screened the two major degradation products of DEL, namely GA and PGA, for interactions with erlotinib at the level of cell growth inhibition. The antagonistic effects observed for combinations of ERL with the parent substances were not found for combinations with GA and PGA (Fig. 2E and F). On the contrary, for the combination of ERL and PGA at a ratio of 1 : 10, even a potent synergistic inhibition of cell growth was observed with a CI_{50} of 0.35. Furthermore the CI is close to constant over the measured effect range (Fig. 3C). GA was not found to interfere with ERL-induced cell growth inhibition. A 24 h pre-incubation with PGA also did not alter the cytostatic effect of ERL (Fig. 4E).

It also seems unlikely that an interaction at the EGFR occurs, as pEGFR concentrations of ERL-treated cells were not influenced by PGA (Fig. 5E).

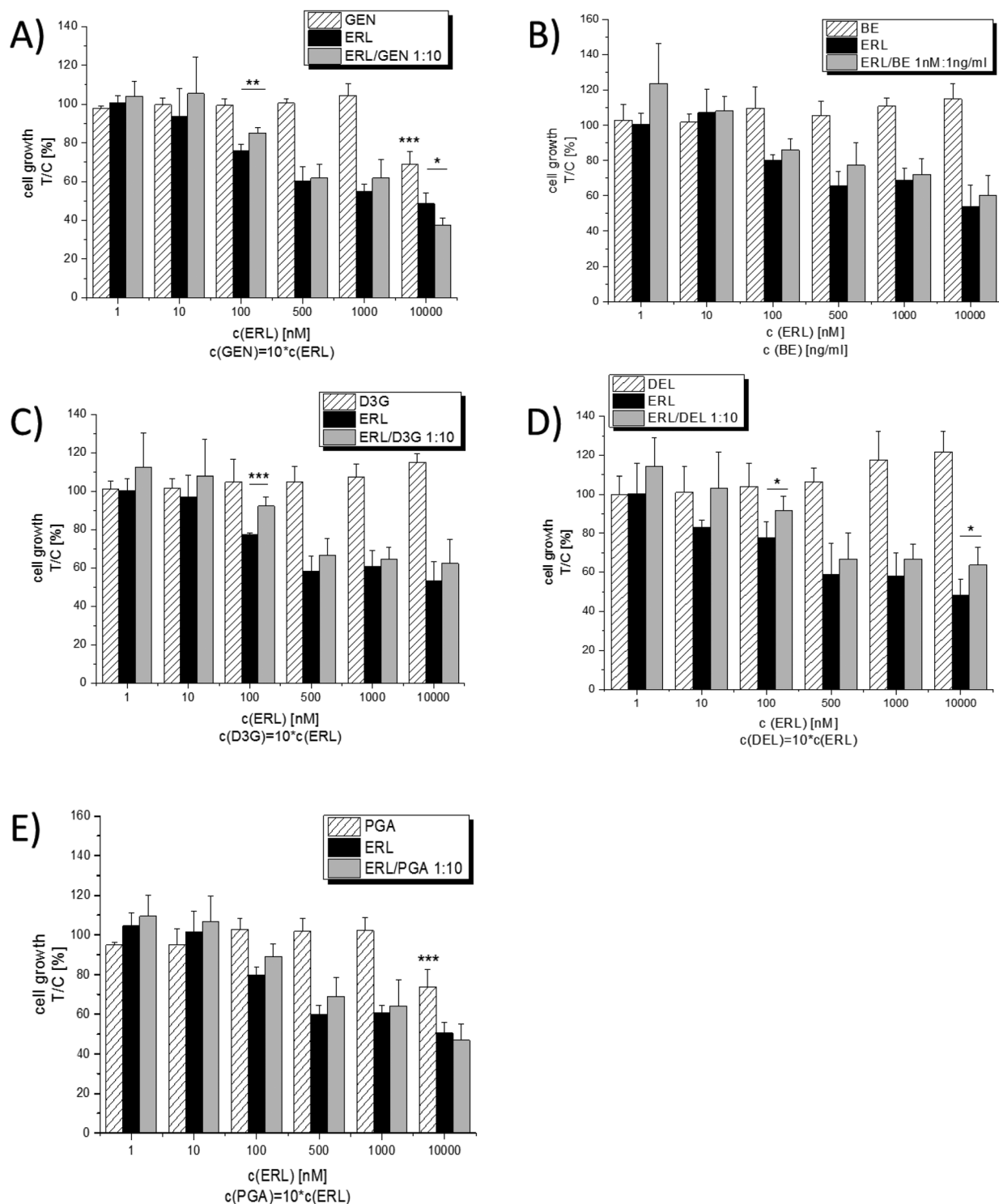


Fig. 4 Influence of a pre-incubation with GEN (A), BE (B), D3G (C), DEL (D) or PGA (E) on the cytostatic effect of ERL, measured with the SRB assay. Cells were pre-incubated with the polyphenolic compounds for 24 h, washed with PBS and then incubated with ERL for 48 h. Values are shown as test/control (1% v/v DMSO) T/C + standard deviation of at least three independent experiments. Statistics was performed with one-way ANOVA, followed by Fisher's LSD test using the measured values for the polyphenols in comparison to the solvent control. Values for ERL and the combination at specific concentrations were compared with Student's *t*-test (**p* < 0.05; ***p* < 0.01; ****p* < 0.001).

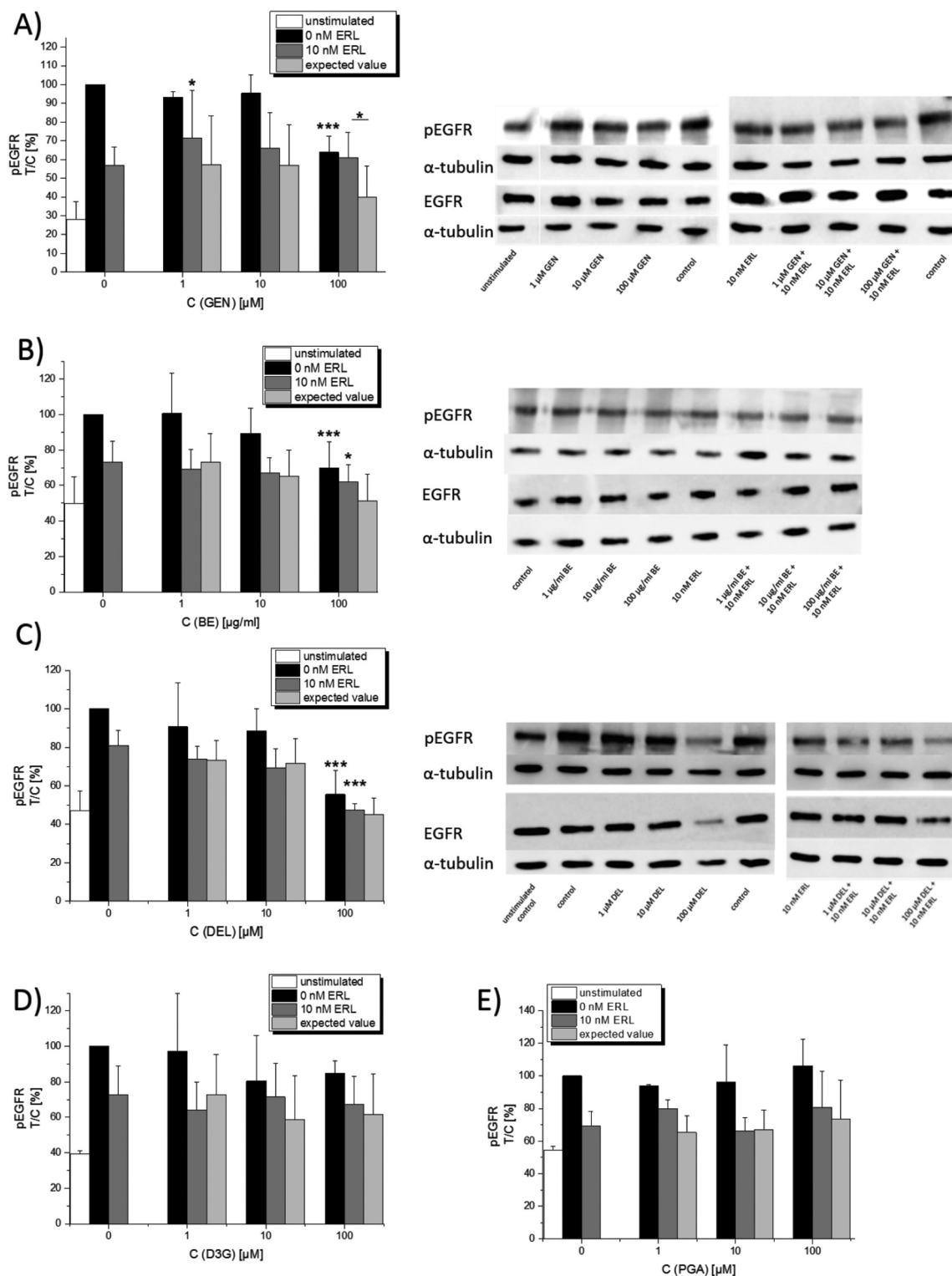


Fig. 5 Effect of GEN (A), BE (B), DEL (C), D3G (D) and PGA (E) on EGFR phosphorylation and inhibition of EGFR phosphorylation by ERL. For GEN, also a representative western blot is shown. A431 cells were incubated with different concentrations of the test compounds for 1 h, washed twice with PBS and incubated with 0 or 10 nM ERL for 1 h. EGFR was stimulated by addition of EGF for the last 15 min of incubation. Diagrams show pEGFR levels expressed as percentage of the solvent control (1% v/v DMSO), as mean values + SD of at least three independent experiments. Significant differences to the respective control (pre-incubation with 1% v/v DMSO) within the series of either 0 nM or 10 nM ERL were calculated by one-way ANOVA, followed by Fisher's LSD (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$). Expected values were calculated using Webb's model of independent joint action and were compared to the measured combination values by Student's t -test (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

Discussion and conclusions

The present study shows that natural polyphenolic RTK inhibitors occurring in food and present in comparably high concentrations in respective food supplements are able to interfere with the cytostatic effect of ERL. Contrary to our expectations, genistein interacted antagonistically regarding the EGFR-inhibiting properties of ERL, which was also reflected at the stage of cell growth inhibition. BE, its major anthocyanin D3G and the corresponding anthocyanidin DEL, but not their main degradation products GA and PGA, interacted antagonistically with the cytostatic properties of ERL. However, the underlying mechanism remains to be clarified, as none of those compounds showed an antagonistic interaction with ERL regarding the inhibition of EGFR phosphorylation as shown by western blot experiments.

One possibility for interactions could be an influence on the EGFR signaling cascade further downstream of the receptor. As polyphenols have been reported to also inhibit various other RTKs in addition to EGFR, and many kinases are involved in EGFR signaling, this possibility might be considered. GEN, for example, has been shown to inhibit Akt and NF- κ B activation, which could impact a crosstalk with the MAPK pathway.⁴

Furthermore, the influence of anthocyanins on the activation of transporter proteins or on metabolism might be considered as a mode of action. ERL is extensively metabolized by cytochrome P450 (CYP) 3A4 (70%) and 1A2 (30%), resulting in the formation of less active compounds.²⁵ An influence on the catalytic activity of CYP3A4 by anthocyanidins and D3G has recently been shown in human hepatocytes.²⁶ So far no data on CYP3A4 expression by A431 cells exists, but the protein is expressed by skin cells²⁷ and it is likely that A431 cells might show an expression as well. The cellular export of the drug is undertaken by the ATP binding cassette transporters ABCB1 and ABCG2, and different polyphenols, including GEN, have been reported to affect the activity and the expression of those proteins.²⁸ The obtained results of the serial incubation experiments, where the cells were treated with ERL for 48 h after a 24 h pre-treatment with the polyphenols, are in line with these hypotheses, as the antagonistic effects were found to remain prominent after the polyphenols were washed off.

Also, ERL was recently reported to inhibit human topoisomerases,²⁹ enzymes needed for the regulation of DNA topology. As mentioned earlier, anthocyanins have been reported to antagonize topoisomerase inhibitors like camptothecin and doxorubicin,^{10–12} so a similar interaction with ERL might be conceivable.

Furthermore, the results show that the antagonistic interactions of BE, D3G and DEL with ERL are not related to their two major degradation products, GA and PGA. While GA did not affect the cytostatic properties of ERL at all, PGA even interacted synergistically, which might be of interest for further research. However, the results let assume that PGA and ERL have to be present at the same time, as a 24 h pre-incubation with PGA failed to reproduce the synergistic effect. As

no synergistic effects were observed regarding the suppression of EGFR phosphorylation, the mechanism of this interaction remains to be clarified.

As described earlier the low systemic bioavailability of polyphenols limits their site of action mainly to the GIT. Thus it is conceivable that the tested compounds could alleviate gastrointestinal side effects of ERL without impairing chemotherapy. In their phase II study combining GEN and ERL, El-Rayes *et al.*⁶ described that only 10% of their patients required a dose reduction of ERL, while in a phase III study without the use of GEN almost 50% of the patients required a reduction because of severe side effects. Our results may provide some evidence for an explanation for this observation. Concerning the combination of anthocyanins with ERL treatment, especially in regard to potential interference of supplemented polyphenols with chemotherapy, more research is required, considering such interactions and the potential mechanisms involved.

Acknowledgements

We thank Giorgia Del Favero for proof-reading the manuscript.

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RESEARCH ARTICLE

Genistein and delphinidin antagonize the genotoxic effects of the mycotoxin alternariol in human colon carcinoma cells

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Scope: Although associated with anti-oxidative properties, genistein has been reported to induce DNA strand breaks, whereby oxidative stress and topoisomerase poisoning are considered as potential mechanisms. In contrast, delphinidin, a catalytic topoisomerase inhibitor, is known to suppress the DNA-damaging properties of several topoisomerase poisons. Recently, alternariol, a mycotoxin produced by *Alternaria spp.*, was found not only to induce oxidative stress but also to act as a topoisomerase poison. As both, polyphenols and mycotoxins, might occur in our nutrition simultaneously, the question was addressed whether potential combinatory effects on DNA integrity have to be considered.

Methods and results: We determined combinatory effects of either genistein or delphinidin with alternariol in HT-29 cells. Cytotoxicity was assessed by WST-1 and SRB assays, whereby only weak interactions were observed. The comet assay revealed significant antagonistic interactions of both polyphenols with the genotoxicity of AOH. The underlying mechanism comprises the suppression of alternariol-mediated stabilization of DNA/topoisomerase-II-intermediates, as observed in the ICE assay. Furthermore, DEL but not GEN was found to suppress AOH-mediated oxidative stress.

Conclusion: Our data indicate that a respective polyphenol-rich diet might aid to protect against genotoxic damages caused by AOH, whereby bioactive concentrations of DEL are predominantly expected locally in the intestines.

Keywords:

Anthocyanin / Food contaminant / Genotoxicity / Oxidative stress / Synergism

Received: June 6, 2016

Revised: September 8, 2016

Accepted: September 11, 2016

1 Introduction

Molds of the genus *Alternaria* produce a variety of mycotoxins which frequently contaminate foodstuffs. The proportions in which those toxins are produced largely depend on the respective *Alternaria* strain and environmental influences like temperature, pH and light conditions [1]. However, tenuazonic acid, alternariol (AOH, Fig. 1A) and alternariol monomethyl ether (AME) are among the major *Alternaria* mycotoxins with respect to quantity [2]. Several *Alternaria* toxins were identified to be genotoxic, among them AOH, AME and altertoxin II [2–4]. Regarding the underlying mechanism, AOH was

found to target human topoisomerases (TOPO) [4], but also to cause oxidative stress [5].

TOPOs (Fig. 2) are the enzymes essential for the regulation of DNA topology, a process necessary to provide access of proteins during replication and transcription. Compounds that interfere with TOPOs can be divided into two major groups. “Catalytic inhibitors” decrease the enzyme’s affinity for DNA binding and therefore its activity. On the other hand, “topoisomerase poisons” trap the enzyme at the DNA, i.e. they stabilize the covalent TOPO/DNA intermediates (the so-called “cleavable complexes”) which are formed during the catalytic cycle and cause DNA strand breaks after the subsequent degradation of the intermediates. Therefore, TOPO poisons like AOH are regarded as genotoxic [6].

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Abbreviations: AOH, Alternariol; DCF, dichlorofluorescein; DEL, delphinidin; FPG, formamidopyrimidin-DNA-glycosylase; GEN, genistein; ICE, *in vitro* complex of enzyme; SRB, sulforhodamine B; TOPO, topoisomerase; WST, water soluble tetrazolium

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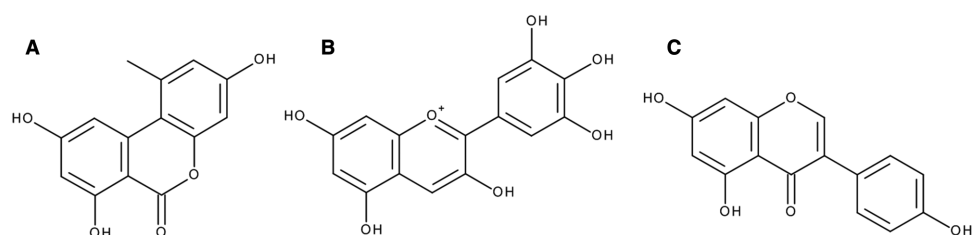


Figure 1. Chemical structures of (A) alternariol, (B) delphinidin and (C) genistein.

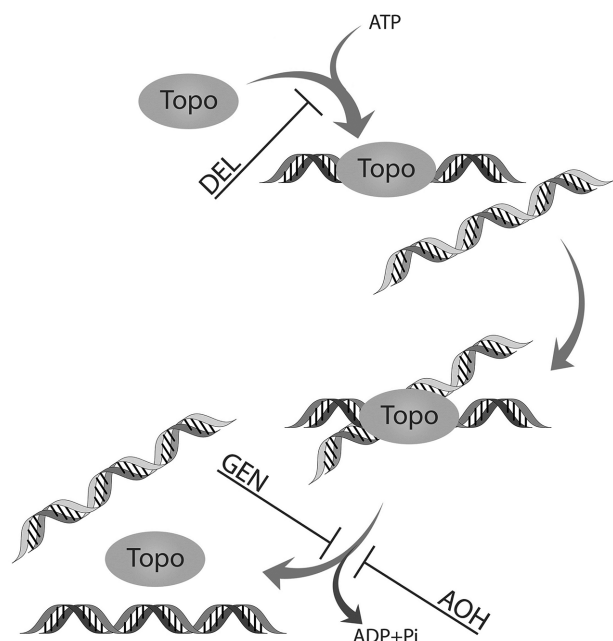


Figure 2. Schematic overview of the catalytic circle of topoisomerase II (TOPO). TOPO binds covalently to a DNA double strand and—upon ATP binding—cleaves it. This step is suppressed by so-called “catalytic TOPO inhibitors” like DEL. In a next step, a second DNA strand is passed through the generated gap. Subsequently, ATP is hydrolyzed to ADP + Pi as the enzyme rejoins the strand and dissociates from the DNA. Compounds inhibiting this step, e.g. AOH or GEN, are referred to as “TOPO poisons” and result in trapping the covalent TOPO/DNA (i.e. “cleavable”) complexes which in turn causes the persistence of DNA double strand breaks.

Recently, our group reported the interference of several TOPO-inhibiting polyphenolic compounds like delphinidin, resveratrol or anthocyanin-rich berry extracts with clinically used TOPO poisons, regarding both their ability to stabilize cleavable complexes and to induce DNA strand breaks [7–10]. Catalytic TOPO inhibitors might interfere with TOPO poisons, as they reduce the activity of the enzyme and therefore the intracellular level of cleavable complexes that are targeted by those poisons.

Delphinidin (DEL, Fig. 1B) belongs to the class of anthocyanidins. In its glycosylated form, as anthocyanin, it occurs in many red and blue colored fruits and vegetables such as berries, plums or aubergines. Anthocyanidins and anthocyanins have been reported to possess anti-oxidative proper-

ties and to act as free radical scavengers [11]. Thus they are described as chemopreventive agents both *in vitro* and *in vivo* [12, 13]. Regarding the mechanism of anti-cancer effects, both the inhibition of receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) and the catalytic inhibition of topoisomerases might contribute to this effect [14, 15]. However, there is also some contradiction, as the application of DEL as a free aglycon has been found to promote tumor growth and metastasis formation in rats [16].

Anthocyanins, and especially anthocyanidins, are light-sensitive compounds and unstable in non-acidic environments [17, 18]. Thus, their systemic bioavailability is very low [19] and their site of action is mainly limited to the gastrointestinal tract. Anyway, these polyphenols have become increasingly popular as food supplements, which enhances the possibilities to reach sufficient amounts to mediate biological effects—particularly in the gut.

Another well-investigated polyphenol, the isoflavone genistein (GEN, Fig. 1C), is typically found in red clover and soy, the latter being the primary source of human intake. Beside its function as a phytoestrogen [20], GEN has been reported to induce a large variety of biological effects, including both pro-oxidative and anti-oxidative effects [21], and the inhibition and poisoning of TOPOs [22–24]. In several cell models GEN was shown to possess clastogenic (i.e. “chromosome-breaking”) and genotoxic effects [25–28]. Thereby, the ability to poison TOPOs seems to play an important role in the mechanism of these effects.

In spite of those concerns, isoflavones in general and GEN in particular became very popular as food supplements and are especially advertised for women in menopause, potentially resulting in an intake of far higher amounts of the compounds than expected for the habitual Western diet.

If AOH is ingested by individuals with high consumption of polyphenol-containing food or even polyphenol-rich food supplements, it seems conceivable that the toxic properties of the mycotoxin might be altered by those compounds. One obvious possibility could be the interaction with TOPO-poisoning properties of the toxin. As mentioned above, the catalytic TOPO inhibitor DEL was reported to interfere with other TOPO-poisons, so one could expect a similar interplay with AOH. GEN was also described to inhibit TOPOs via the stabilization of cleavable complexes, so an interaction might be possible as well. Furthermore, there is ample evidence for anti-oxidative properties of polyphenols, while AOH was reported to induce cellular oxidative stress. Such interactions would of course affect the risk assessment of *Alternaria* toxins,

but also enlighten the question if a polyphenol-rich diet or the intake of polyphenol-rich food additives could help to protect the human organism from mold-induced genotoxicity.

Therefore, we combined the polyphenols DEL and GEN with AOH, and investigated possible interactions in the HT-29 colon carcinoma cell model. Cytotoxic and genotoxic effects were investigated and the influence of both polyphenols on topoisomerase-poisoning and ROS-generating properties of AOH was assessed in detail.

2 Materials and methods

2.1 Chemicals, antibodies and enzymes

Genistein and delphinidin chloride were purchased from Extrasynthese (Genay, France). Alternariol, catalase (from bovine liver), sulforhodamine B and DCF-DA were obtained from Sigma-Aldrich (Taufkirchen, Germany). Antibodies for immunostaining were purchased from Santa Cruz (Heidelberg, Germany), ECL detection reagents from GE Healthcare (Buckinghamshire, UK). WST-1 reagent was obtained from Roche (Basel, Switzerland) and formamidopyrimidin-DNA-glycosylase (FPG) from New England Biolabs (Frankfurt, Germany).

2.2 Cell culture and treatment

HT-29 human colon carcinoma cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). For cell culture, Dulbecco's Modified Eagle's Medium, supplemented with 10% (*v/v*) heat inactivated fetal calf serum and 1% (*v/v*) penicillin/streptomycin was used. Media and supplements were obtained from Invitrogen™ Life Technologies (Karlruhe, Germany). Cells were cultivated at 37°C, under humidified conditions with 5% CO₂ and were routinely tested for mycoplasma contamination. Polyphenols, AOH and all other tested compounds were dissolved in dimethylsulfoxide (DMSO) and added to the incubation media, resulting in the final concentrations of the test compounds and 1% (*v/v*) DMSO. As there were concerns about solubility problems of AOH in certain cell culture media, we confirmed the full solubility and stability of the highest used concentrations in both stock and media solutions by HPLC-DAD (data not shown). Polyphenols were previously reported to generate H₂O₂ in cell culture, so 100 u/mL catalase was added to the incubation media to prevent oxidative cell damage and substance degradation.

2.3 Coupled WST-1 and SRB cytotoxicity assay

Per well, 7500 HT-29 cells were seeded in 96-well plates and allowed to grow for 48 h. Incubation was carried out for 24 h

with different concentrations of GEN, DEL and AOH plus the respective combinations at a constant substance ratio of 1:1. The constant ratio was applied to be able to perform accurate combination index analysis as described below. The wells were then washed with PBS, and incubated with a 1:10 mixture of WST-1 reagent and serum-free DMEM. After exactly 45 min, absorption was detected at 450 and 650 nm with a PerkinElmer Victor³V multiple plate reader. For quantification, values at 650 nm were subtracted from those at 450 nm, and measured data was referred to the solvent control (1% *v/v* DMSO).

Furthermore, the same plates were then washed twice with PBS and cells were fixed by addition of a solution of 5% trichloroacetic acid. Upon washing, the plates were allowed to dry overnight at room temperature. Then they were stained by incubation with a solution of 0.4% (*w/v*) sulforhodamine B (SRB) for 1 h. After washing with water and 1% *v/v* acetic acid, the color was eluted with a 10 mM TRIS buffer at pH 10. Extinctions were measured at 570 nm and effects on cell growth were referred to solvent control (1% *v/v* DMSO).

2.4 Single cell gel electrophoreses ("comet assay")

The assay was performed according to the guidelines of Tice et al. [29]. Briefly, 1.5×10^5 HT-29 cells were seeded in petri dishes (diameter 3.5 cm) and allowed to grow for 48 h. Cells were pre-incubated with the polyphenols for 30 min to allow cellular uptake before the addition of the more lipophilic mycotoxin with the subsequent co-incubation for 60 min. UV-B radiation was used as positive control. Afterwards, cells were washed twice with PBS and trypsin was added to displace and singularize the cells. Cells were counted and their viability was assessed by trypan blue exclusion. For each sample, two slides were prepared. Cell suspensions were aliquoted to contain 30 000 cells each, pelleted by centrifugation, resuspended in low melting agarose and embedded on object slides. Lysis was carried out overnight at 4°C in a buffer containing Triton X and lauroyl sarcosinate, followed by FPG-treatment of half of the slides for 30 min at 37°C. Upon equilibration in the alkaline buffer (pH 10) for exactly 20 min at 0°C, electrophoresis was performed for 20 min at 25 V and 300 mA. The slides were neutralized and stained with a solution of 0.02 g/mL ethidium bromide. Microscopic analysis of DNA damages was carried out with a Zeiss Axioskop ($\lambda_{\text{ex}} = 546 \pm 1$ nm; $\lambda_{\text{em}} \geq 590$ nm). For image analysis, the "Comet Assay IV" system (Perceptive Instruments, Suffolk, UK) was used to score 50 images per gel pad.

2.5 In vitro complex of enzyme (ICE) assay

The assay was performed as described previously with slight modifications [30]. Briefly, 6.5×10^6 HT-29 cells were seeded in petri dishes (diameter 10 cm) and allowed to grow for 48 h. Cells were pre-incubated with different concentrations of

GEN, DEL or the respective control for 30 min and subsequently incubated with AOH, GEN, DEL, combinations or controls for 60 min. After washing with PBS, lysis buffer was added and cells were harvested. 4 mL of the lysate were put on 8 mL of a cesium chloride gradient (0.75–1.6 mg/mL) and centrifuged for 24 h at 100 000 g to separate free TOPOs from the covalent TOPO/DNA complexes. The gradient was fractionized and the fractions blotted on a nitrocellulose membrane. TOPO II β was detected via immunostaining with HRP-coupled antibodies, and chemiluminescence was captured with an LAS-4000 imager (Fujifilm Life Science, Cleve, Germany).

2.6 Dichlorofluorescein (DCF) assay

The assay was performed according to the method of Keston and Brandt [31]. Briefly, 20 000 HT-29 cells per well were seeded in black 96-well plates and allowed to grow for 48 h. Cells were washed with PBS and incubated with a 50 μ M solution of DCF-DA in PBS for 30 min. After washing with PBS again, the cells were incubated with the substances alone or in combinations in non-colored DMEM, containing 1% (v/v) DMSO and 100 u/mL catalase. Fluorescence was measured at 485/535 nm (excitation/emission) after 0 and 1 h. A solution of 1 mM H₂O₂ in non-colored DMEM without catalase was used as a positive control.

2.7 “Protective” DCF assay

Per well, 20 000 HT-29 cells were seeded in black 96-well plates and allowed to attach for 24 h. Cells were incubated with different concentrations of the polyphenols in DMEM (containing 10% FCS, 1% v/v DMSO and 100 u/mL catalase) for another 24 h.

The wells were then washed with PBS and incubated with a 50 μ M solution of DCF-DA in PBS for 30 min. After being washed with PBS again, the cells were incubated for 1 h with either 1 mM H₂O₂ or 50 μ M AOH in non-colored DMEM containing 1% v/v DMSO and no catalase for 1 h. Measurements and quantification were performed as described above.

2.8 Statistical analysis

An “interaction” is defined as a combinatory effect of two or more substances being different to the additional effect of the single compounds. To assess interactions of substances that do not follow a linear dose-response relationship, mathematical models are needed to calculate this “additive effect”. Probably the most accurate model is Chou’s “multiple drug effect equation” (MDEE) [32] that we used for assessing interactions at the level of cytotoxicity like recently described [33]. Here, a combination index (CI) is calculated

as a measure for the combinatory properties of two compounds, thus the method is often referred to as “CI analysis”. A CI < 0.9 indicates a synergistic interaction, 0.9 < CI < 1.1 “mostly additive” effects and CI > 1.1 an antagonistic interaction.

However, the experimental demands of MDEE were too challenging for application in the other assays. Therefore, in addition, the model of “independent joint action” [34] (also referred to as “Webb’s fractional product method”) was used. Here, an expected additive value (EV) is calculated from the effects of the single compounds using either the formula $f_{ab} = f_a + f_b$ for data that can theoretically exceed an effect of 100% or $f_{ab} = f_a + f_b - f_a f_b$ for data capped between 0% and 100%, with f_{ab} being the combinatory effect and f_a , f_b being the effects of the single compounds. The EV was then compared to the measured combinatory value with Student’s t-test.

3 Results

3.1 Cytotoxicity

AOH showed cytotoxic effects in HT-29 cells, starting at a concentration of 25 μ M in the WST-1 assay (Fig. 3A and C) and 50 μ M in the SRB assay (Fig. 3B and D). DEL induced significant cytotoxic effects in the WST-1 assay at $\geq$ 25 μ M, and GEN in both the WST-1 ($\geq$ 25 μ M) and SRB assay ($\geq$ 50 μ M).

Combinatory measurements were analyzed regarding possible interactions with both the “combination index” and the “independent join action” model. As CI analysis requires the single compounds to reach IC₅₀ values, it could not be applied for combinations with DEL, with the highest measured concentration (100 μ M) causing cytotoxicity of approximately 15%.

However, both DEL and GEN showed synergistic effects with AOH with respect to cytotoxicity. The combination of DEL and AOH at 50 μ M each was significantly more toxic in the SRB assay than the expected value (EV). Cytotoxicity of the combination of GEN and AOH was significantly higher than the respective EV at 25 μ M and 50 μ M in the WST-1 assay and at 25 μ M in the SRB assay. Moreover, most CI values at the measured concentrations and also the CI₅₀ values (0.84 for SRB, 0.81 for WST-1 data) were in the synergistic range (CI < 0.9). It was clearly visible from the effect-CI plots that with increasing cytotoxicity the CI values are rising, i.e., “moving out” of the synergistic range (Fig. 4).

3.2 Genotoxicity

Since not cytotoxicity but much more genotoxicity is a major concern for the toxicological impact of mycotoxins, the combinatory effects of DEL/GEN with AOH on DNA-integrity of HT-29 cells was investigated in the comet assay. A representative concentration of 50 μ M AOH, known

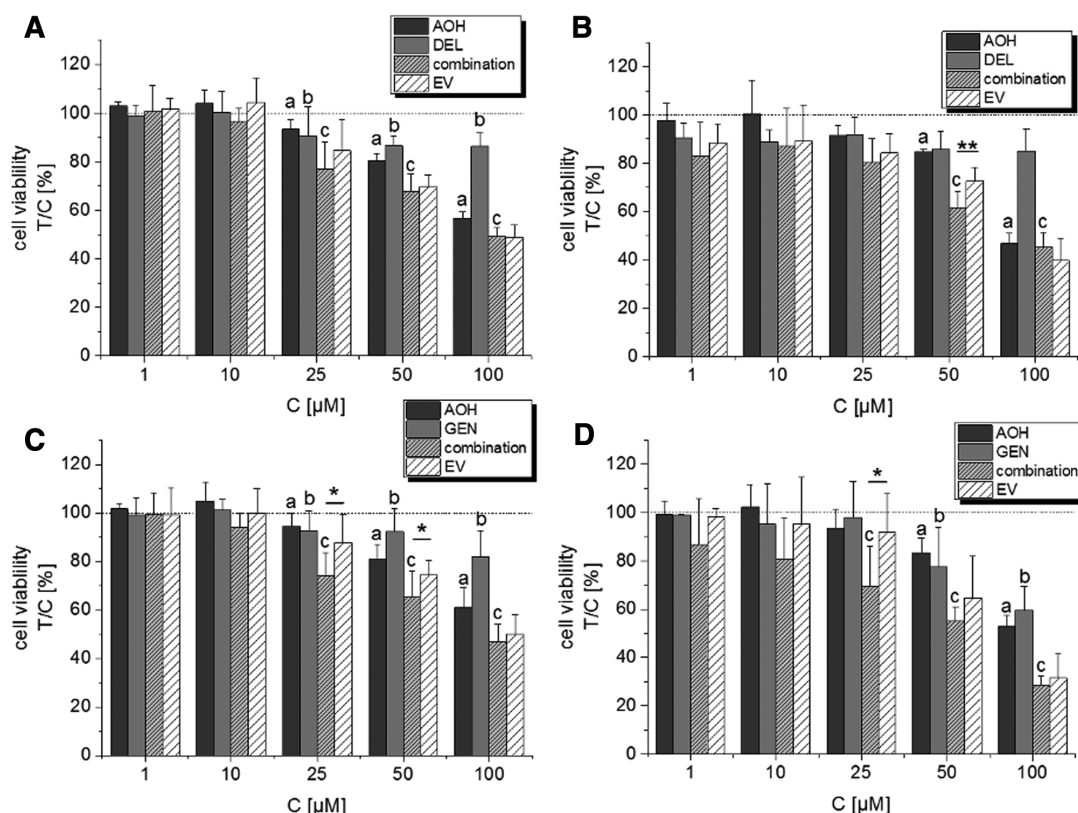


Figure 3. Effects of the polyphenols on the cytotoxicity of AOH in coupled WST-1 and SRB assays. (A) and (B) show the results of the WST-1 and the SRB assay for the combination of AOH with DEL, respectively. (C) and (D) show WST-1 and SRB data for the combination of AOH with GEN. HT-29 cells were incubated for 24 h with the single compounds or co-incubated for 24 h with a 1:1 combination of them. Shown cell viability results are expressed as mean + SD of at least four independent experiments. Expected values (EV) were calculated by applying the model of independent joint action as stated in the methods section. Significant differences between concentrations of the same compound with the respective no-effect level at 1 μM were calculated by one-way ANOVA, followed by Fisher's LSD ($p < 0.05$), and are indicated by a, b and c for AOH, the polyphenol and the combination, respectively. Significant differences between the measured combinatory cytotoxicity and the respective EV were calculated by Student's *t*-test, and are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$)

to mediate significantly enhanced levels of DNA-strand breaks in the comet assay [4], was selected. As expected, incubation with AOH for 60 min increased the rate of DNA strand breaks (Fig. 5), an effect that was further enhanced by inclusion of formamidopyrimidine-DNA-glycosylase (FPG) in the comet assay protocol, indicating oxidative DNA damage.

Treatment of HT-29 cell for 30 + 60 min with DEL as a single compound also mediated enhanced levels of DNA strand breaks at concentrations $\geq 50 \mu\text{M}$ without FPG treatment, and $\geq 25 \mu\text{M}$ with FPG treatment, also indicating the formation of FPG-sensitive DNA sites such as, e.g. oxidative DNA damage (Fig. 5A). In combination, we observed antagonistic interactions between DEL and AOH at $50 \mu\text{M}$ without FPG treatment, manifested in a significantly lower measured tail intensity compared to the calculated EV. Also for the combinations of $25 \mu\text{M}$ and $100 \mu\text{M}$ DEL with $50 \mu\text{M}$ AOH, we found the combinatory value to be significantly lower than the EV, albeit only with respect to FPG-sensitive sites.

We did not observe genotoxic effects of GEN after 30 + 60 min of incubation in the tested concentration range (Fig. 5B). However, we observed an influence on the formation of FPG-sensitive sites by AOH, with measured values for the combinations being lower than the EV at $25 \mu\text{M}$ GEN and $100 \mu\text{M}$ GEN after treatment with FPG.

3.3 Topoisomerase poisoning

Since DEL is known to prevent the formation of covalent DNA-TOPO-intermediates, so-called “cleavable complexes”, whereas GEN and AOH as single compounds act as TOPO poisons, stabilizing already formed cleavable complexes, we assessed the consequences of respective combinations on the level of these DNA-TOPO-intermediates in HT-29 cells using the ICE assay. The analysis was performed exemplarily for TOPO II β isoform. In accordance with previous studies [7,8], the incubation with $50 \mu\text{M}$ AOH for 60 min enhanced the

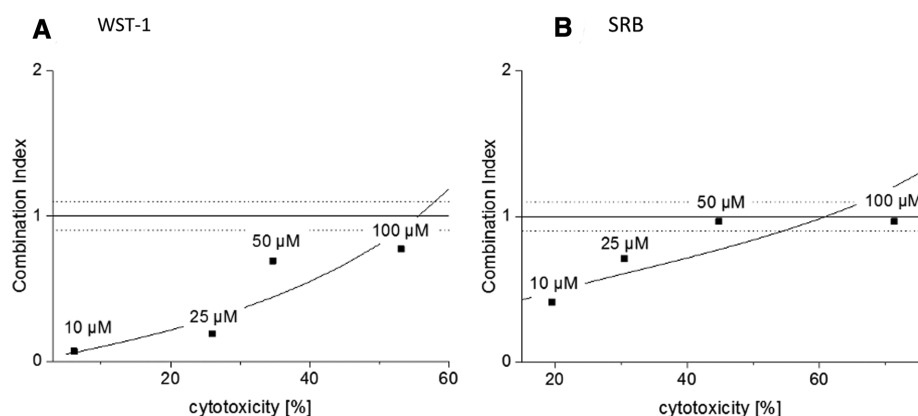


Figure 4. Effect-CI plots for combinatory cytotoxic effects of GEN and AOH. Combination Index (CI) values for 1:1 combinations of GEN and AOH expressed as a function of effect strength. CI values of actually measured combinations (squares) and the calculated effect-CI plot (line) for the results of our 24 h WST-1 (A) and SRB (B) co-incubation assays are shown. The dotted lines incline the borders of additivity (0.9 and 1.1) according to Chou.[32].

level of cleavable complexes in the cells (Fig. 6). Incubation of HT-29 cells with GEN as a single compound for 30 + 60 min enhanced the level of cleavable complexes only at a quite high concentration of 100 μM . Of note, also incubation with DEL apparently affected the level of cleavable complexes at 50 μM . However, this effect did not further increase at 100 μM .

Regarding potential interactions, GEN did not cause a reduction of AOH-induced TOPO poisoning. But also the stabilizing effect of AOH on cleavable complexes was not further increased by GEN, and the measured combinatory value for 100 μM GEN and 50 μM AOH was significantly lower than the expected value that was calculated via independent joint action, thus describing a significant antagonism of the two compounds regarding cleavable complex stabilization. DEL shows a trend for decreasing the TOPO-poisoning effect of 50 μM AOH, at concentrations of 25 μM or higher, which is statistically significant for the combination of 50 μM of each compound. When comparing measured and expected combi-

natory values, we found significant antagonistic interactions for the combination of 50 μM AOH with 25, 50 and 100 μM DEL.

3.4 Oxidative stress

The impact of the single compounds and respective combinations on intracellular ROS levels was investigated in the DCF assay. Direct anti- or pro-oxidative activity can be assessed by short-time incubations. Effects mediated via the modulation of anti-oxidant defense systems including impact on gene expression can be monitored by applying a 24 h pre-incubation period with the respective polyphenol. According to previous publications a concentration of 50 μM AOH was chosen, known to enhance the intracellular ROS levels [5] and corresponding with the concentration used in the comet and the ICE assay.

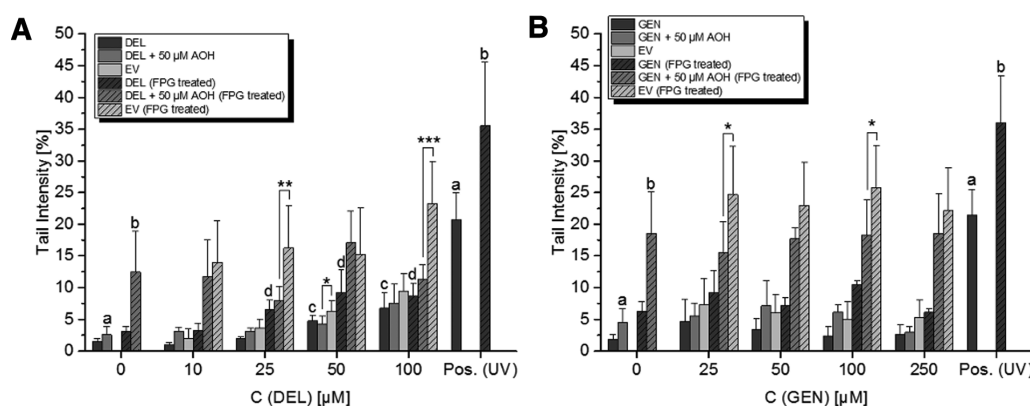


Figure 5. Comet assay: interactions of DEL and GEN with the genotoxic properties of AOH. Cells were pre-incubated with the polyphenolic compounds or the solvent control for 30 min and co-incubated with polyphenols and AOH for 1 h. Graphs show measured tail intensities of single substances and the combinations as well as expected values (calculated via independent joint action—see methods section), both with and without FPG treatment. All values are expressed as mean + SD, and result from at least 5 independent experiments. Significant differences between varying concentrations of the same single compound or combination were calculated by one-way ANOVA, followed by Fisher's LSD ($p < 0.05$), with "c" and "d" indicating a significant difference to the negative control without or with FPG treatment, respectively. Differences between the solvent control and both 50 μM AOH and the positive control were calculated by Student's t -test ($p < 0.05$), and indicated with "a" (not treated) or "b" (FPG treated).

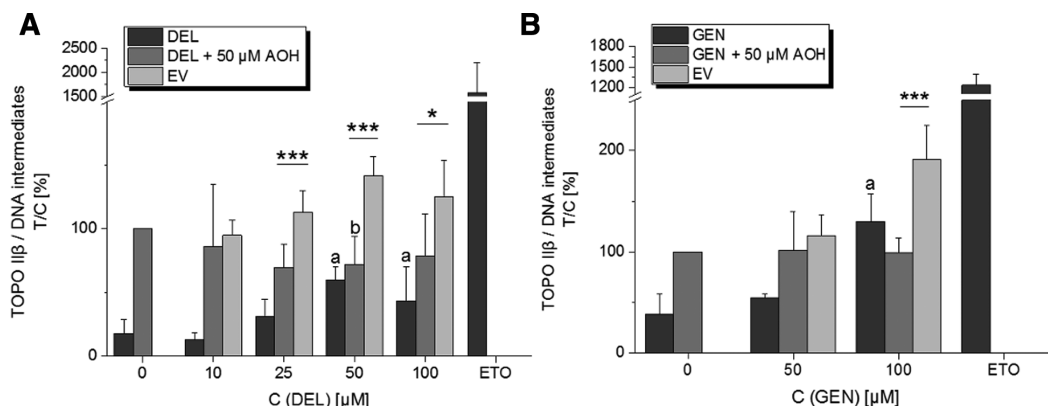


Figure 6. ICE assay: Interactions of polyphenols and AOH regarding cleavable complex stabilization. Cells were preincubated with DEL (A) or GEN (B) for 30 min and co-incubated with the polyphenol and 0 or 50 μM AOH for 1 h. Shown are the detected levels of TOPO IIβ in the DNA-rich fractions after ultracentrifugation (i.e. the amount of cleavable complexes), related to the sample of cells incubated with 50 μM AOH alone. Data are presented as mean + SD of at least four independent experiments. To calculate significant differences, one-way ANOVA, followed by Fisher's LSD, was conducted for series with the same concentration of AOH (0 or 50 μM). "A" and "B" indicate a significant difference ($p < 0.05$) to the respective value at 0 μM DEL (0 or 50 μM AOH). Significant differences between the combinations and the EV (calculated via independent joint action, see methods section) were calculated using Student's *t*-test, and are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

In the short-time assays, DEL significantly reduced ROS-generation of 50 μM AOH at a concentration as low as 1 μM with the effect further pronounced at higher DEL concentrations (Fig. 7A). In contrast, GEN induced oxidative stress itself, and did not suppress the pro-oxidative effects of AOH (Fig. 7B). Also there was no significant difference to the expected additive effects, which were calculated using the independent joint action model.

A pre-incubation of HT-29 cells for 24 h with the polyphenols prevented the cells efficiently from ROS formation induced by the addition of 1 mM H₂O₂ at concentrations ≥ 10 μM DEL and ≥ 25 μM GEN (Fig. 7C and D). It has not escaped our attention that the latter effect might in reality be attributed to the cytotoxicity of GEN at those concentrations rather than to an induction of the cellular antioxidative response system. However, 24 h pre-incubation with DEL or GEN did not affect

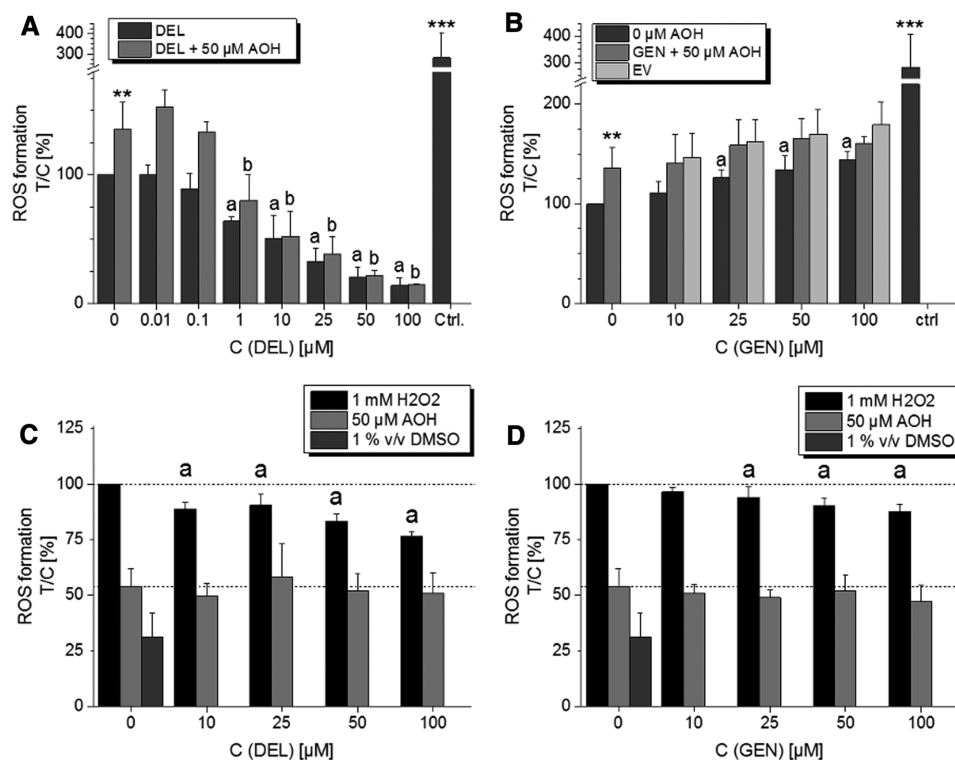


Figure 7. DCF assay: Influence of DEL and GEN on the ROS generation by AOH. Figures (A) and (B) show the influence of DEL (a) or GEN (b) on the generation of reactive oxygen species (ROS) by AOH in HT-29 cells after 1 h of co-incubation, while figures (C) and (D) show the protective influence of a 24 h pre-incubation with DEL (c) or GEN (d) on the generation of ROS caused by a 1 h incubation with either 1 mM H₂O₂ or 50 μM AOH. Data are expressed as mean + SD of at least 4 independent experiments. Significant differences to the respective no-effect level were calculated by one-way ANOVA, followed by Fisher's LSD ($p < 0.05$), and are indicated with "a" or "b". Significant differences between the controls were calculated using Student's *t*-test, and are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

the formation of ROS induced by incubation for 1 h with 50 μ M AOH.

4 Discussion

Very recently, Fernandez-Blanco et al. investigated the influence of the polyphenol quercetin on the toxicity of AOH and AME, and found no protective effects [35]. However, this study focused on cytotoxicity, while we would expect interactions also to manifest at the genotoxic level, which—in the case of AOH and AME—seems to be the more concerning health issue.

DEL, which was previously described as an agent preventing TOPO to bind to the DNA [7, 15], slightly increased the DNA-bound levels of TOPO II β in the ICE assay (Fig. 6A). Based on this observation, it might be speculated that in addition the latter effect, in a higher concentration range the anthocyanidin also interferes to some extent with the stability of already formed DNA-topoisomerase-intermediates. However, in line with earlier publications of our group [7, 8], DEL interacted antagonistically with the TOPO-poisoning properties of AOH at concentrations of 25 μ M and higher (Fig. 6A), and also reduced oxidative stress induced by the mycotoxin at concentrations as low as 1 μ M (Fig. 7A). This is reflected at the level of genotoxicity, as DEL antagonized the DNA-breaking potential of AOH (Fig. 5A) at concentrations of 25 μ M or higher. Thus, our results indicate that a high intake of DEL might help to protect from genotoxic damages caused by AOH in the gut, and also suggest a mechanism that involves both interactions with the TOPO inhibition and the generation of oxidative stress.

It has to be considered that the systemic bioavailability of DEL is usually very low due to the lack of resorption and the limited stability of the compound in non-acidic environments. Thus, the antagonistic effects we observed *in vitro* might *in vivo* be limited to the gastrointestinal tract, where sufficient concentrations of anthocyanins seem entirely possible [36], especially if it is taken into account that DEL is not the only anthocyanidin taken up via nutrition. Usually also others, in particular cyanidin, is expected in DEL-containing fruits and berries [37]. Those compounds have been attributed similar effects regarding TOPO inhibition [38, 39] and antioxidative properties [13], so it seems conceivable that not the concentration of DEL alone, but rather the total anthocyanin level should be taken into account regarding possible interactions with mycotoxins. Furthermore, it cannot be excluded that degradation products of DEL, e.g. gallic acid or phloroglucinol aldehyde [18], play a role regarding the interaction with mycotoxins. Respective studies are currently in progress.

As demonstrated in the ICE assay, GEN, albeit stabilizing cleavable complexes itself, interacts antagonistically with the TOPO-poisoning properties of AOH at a concentration of 100 μ M (Fig. 6B). Markovits et al. linked the ability of a compound to inhibit tyrosine kinases, something GEN is

very capable of, with the ability to catalytically inhibit topoisomerase II, and explained their hypothesis with the similarity of the ATP-binding domains of those proteins [40]. Mizushima et al. recently suspected GEN to compete with ATP-binding and therefore to be a catalytic inhibitor of TOPO II as well [41]. Thus, it seems conceivable that GEN is both a catalytic inhibitor of TOPO II and a TOPO II poison. Catalytic inhibitors lead to a reduction of the formation of cleavable complexes, and therefore are expected to interact antagonistically with TOPO poisons in general. So if GEN is indeed a catalytic inhibitor of TOPO II as well as a TOPO II poison, this could explain the observed antagonism in our experiments.

GEN, at concentrations of 25 μ M and 100 μ M, also interfered with AOH regarding DNA damage in the comet assay (Fig. 5B), although not as strong as DEL, which is consistent with the observed effects on topoisomerase poisoning, where also DEL showed more prominent interactions. However, there is a significant antagonism at the level of genotoxicity, and in contrast to DEL it could also manifest systemically, as for GEN much higher systemic concentrations can be reached [42], especially when the possibility of an intake of isoflavones as food supplement is considered. Concerning ROS-generation, no interactions between GEN and AOH were found (Fig. 7B), so we conclude that the mechanism of the antagonistic effect on the genotoxicity of the mycotoxin can be primarily attributed to the interaction regarding TOPO II targeting.

In contrast to the combinatory effects with respect to genotoxicity, protective effects of the polyphenols were not reflected at the level of cytotoxicity, as both GEN and DEL interacted synergistically with AOH after 24 h of co-incubation. DEL showed this synergism only in the SRB assay, not in the WST-1 assay, and only at one concentration (50 μ M, Fig. 3B), so there might be reasonable doubts about the potency and relevance of this interaction. GEN, however, expressed synergistic interactions with AOH in both cytotoxicity assays over a broad concentration range (Fig. 3C and D), which was also confirmed with CI analysis (Fig. 4), underlining the effect. One might assume that genotoxicity is not the driving force behind the cytotoxic effects of AOH, and the responsible mechanism for the latter seems to be amplified by GEN. However, it has to be taken into consideration that in general genotoxicity but not cytotoxicity is the major concern with respect to the toxicological relevance of mycotoxins.

5 Conclusion

We conclude that the dietary polyphenols GEN and especially DEL might aid in protecting cells from AOH-induced genotoxicity, especially in the gut, with DEL being the more potent compound. Our results further suggest that observed antagonistic interactions arise from interference with the topoisomerase-poisoning properties of AOH and also—in

the case of DEL—from a reduction of AOH-induced oxidative stress. These findings provide an initial basis for subsequent studies on the *in vivo* situation.

GA was involved in the planning of the studies, conducting ICE assay and DCF assay experiments, statistical analysis and writing. JB carried out SRB/WST-1 and comet assays. DM was involved in study planning, supervision of the project and refining the manuscript.

We thank Bededikt Warth for proof-reading the manuscript, Heidelinde Schweighofer for designing Fig. 2 and Giorgia Del Favero for her help with finalizing the figures for submission.

The authors state no conflict of interest.

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Publication 3: Delphinidin protects colon carcinoma cells against the genotoxic effects of the mycotoxin altertoxin II.

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Abstract

Alternaria spp. are ubiquitous molds that are able to produce toxic secondary metabolites which may contaminate food globally. One of those is the mycotoxin altertoxin II (ATX-II), a genotoxic and mutagenic compound. In recent years, different flavonoids that can co-occur with mycotoxins in food were demonstrated to temper toxic effects of molds, mostly through their anti-oxidant properties. Thus, in this study, we assessed the influence of the berry anthocyanidin delphinidin on the toxicity of ATX-II in HT-29 colon carcinoma cells. We performed coupled SRB/WST-1 cytotoxicity assays which revealed only weak antagonistic interactions, and single-cell gel electrophoresis (“comet”) assays, where we observed a potent protective effect of delphinidin on the DNA-damaging properties of ATX-II. Furthermore, we investigated the mechanism for this interaction. In the DCF assay delphinidin was found to reduce intracellular oxidative stress levels, which might contribute partly to the latter protection. However, LC-MS experiments showed that a co-incubation of the mycotoxin with either delphinidin or its potential degradation product phloroglucinol aldehyde significantly decreased ATX-II concentrations in aqueous solutions, indicating that a direct chemical reaction of ATX-II with these components is likely responsible for the observed loss of toxicity. Our results provide evidence that delphinidin – and possibly other anthocyanins and polyphenols as well – might play a role in the protection of the gut from *Alternaria*-induced genotoxicity.

Keywords

food contaminant, antagonism, anti-oxidant, degradation, combinatory effects, food safety

Abbreviations

AOH, alternariol; ATX-II, altertoxin II; DCF, dichlorofluorescein; DEL, delphinidin; EV, expected value; FPG, formamidopyrimidin-DNA-glycosylase; GA, gallic acid; PGA, phloroglucinol aldehyde; ROS, reactive oxygen species; SRB, sulforhodamine B; WST, water soluble tetrazolium

1. Introduction

Fungi of the genus *Alternaria* are ubiquitous food contaminants which produce a large variety of different mycotoxins. While other molds have been studied extensively with respect to their toxic effects, less data are available regarding health concerns towards *Alternaria* spp. Therefore, in 2011 the European Food and Safety Authority already declared the need for more studies on the toxicity of *Alternaria* Toxins (EFSA, 2011). Some of the most abundant *Alternaria* toxins, i.e. alternariol (AOH) and its monomethyl ether, have been shown to be cytotoxic or genotoxic (Ostry, 2008). However, an extract of *Alternaria*-infested rice was found to be much more genotoxic than these substances, due to the presence of altertoxin II (ATX-II, figure 1a) as the major DNA-damaging compound (Schwarz et al., 2012b). The mechanism of this effect could not be fully elucidated yet. At the comparatively low concentrations where genotoxicity was observed, there were no indications of reactive oxygen species (ROS) production, GSH depletion or topoisomerase inhibition (Jarolim et al., 2016; Jarolim et al., 2017), which are already identified modes of action of other *Alternaria* toxins. Due to the high induction of formamidopyrimidin-DNA-glycosylase (FPG) sensitive sites observed in the comet assay, there is some speculation that ATX-II might be able to form DNA adducts in a similar way than other compounds possessing functional epoxy-groups, e.g. the infamous epoxy-metabolite of aflatoxin B1 (Schwarz et al., 2012b). However, this hypothesis still awaits experimental confirmation.

In general, mycotoxin contaminations of foods are often linked to the onset of oxidative stress or even genotoxicity. However, in food the possible co-occurrence of biologically active components might be taken into consideration. Polyphenols, defined as compounds possessing an aromatic ring system with at least two adjacent hydroxyl groups, have in general been linked with anti-oxidative properties and chemopreventive effects. One particular interesting subclass of polyphenols is constituted by the anthocyanins, deeply red or blue pigments that grant their intense color to fruits and berries. They consist of a flavonoid – the anthocyanidin – and a glycosidically bound sugar residue that is cleaved after ingestion by the intestinal microbiota. Biological effects described for these polyphenols include anti-oxidative and radical scavenging properties (Rice-Evans et al., 1996), reduction of cancer growth (Cooke et al., 2005; Zafra-Stone et al., 2007) and cardiovascular disease risk (Wallace et al., 2016). Anthocyanidins are not very stable under physiological conditions, as they spontaneously dissociate to a phenolic acid and the phloroglucinol aldehyde (PGA) (Keppler and Humpf, 2005). Thus, their systemic bioavailability is very low, which limits their presumable site of action mainly to the gut (Bitsch et al., 2004).

Consequently, the question arises whether co-occurring polyphenols and mycotoxins are interacting with each other, and the screening of polyphenols regarding possible protective effects against mycotoxins is

an emerging field. The existing studies mainly focus on anti-oxidative mechanisms, and a number of flavonoids were shown to protect against the toxicity of mycotoxins like ochratoxin A (Corcuera et al., 2012; Costa et al., 2007), patulin (Song et al., 2014) and deoxynivalenol (Kalaiselvi et al., 2013) in different experimental models. Concerning *Alternaria* toxins, data on interactions with polyphenols is yet limited to AOH. The polyphenolic fraction of virgin olive oil was found to protect against pro-oxidative and cytotoxic effects of AOH (Chiesi et al., 2015). However, the main toxicological concern of AOH is not cytotoxicity, but genotoxicity. Very recently, we could show that two polyphenols, namely the soy isoflavones genistein and especially the anthocyanidin delphinidin (DEL, figure 1b), antagonize genotoxic effects of this mycotoxin in a colon carcinoma cell line via both interaction with the generation of oxidative stress and with its ability to poison topoisomerases (Aichinger et al., 2017).

The relevance of the polyphenol subclass of anthocyanidins regarding interactions with *Alternaria* toxins is exceptionally high. The intense color of the compounds results in the comparably dark appearance of fruits and berries with outstanding anthocyanin content, e.g. blueberries or aubergines. *Alternaria* spp. are characteristically black molds, and are thus not easily visible on those dark colored foods, which might result in a higher consumption of *Alternaria* toxins with anthocyanin-containing edibles. For example, in an extensive study of mold and yeast growth on fruits from American supermarkets, Tournas and Katsoudas found 46 % of blueberry samples and around 20 % of black grape samples contaminated with *Alternaria* molds (Tournas and Katsoudas, 2005). Thus, it seems crucial to include eventual protective effects of anthocyanidins against *Alternaria* toxins in risk assessment, but also to further address the question, whether a polyphenol-rich diet might be beneficial regarding the protection from mycotoxins. Hence, in this study, we assessed interactions of DEL with ATX-II at the levels of cytotoxicity, genotoxicity, generation of oxidative stress and chemical degradation. We used HT-29 colon carcinoma cells for our experiments to account for the limited systemic bioavailability of the anthocyanidin.

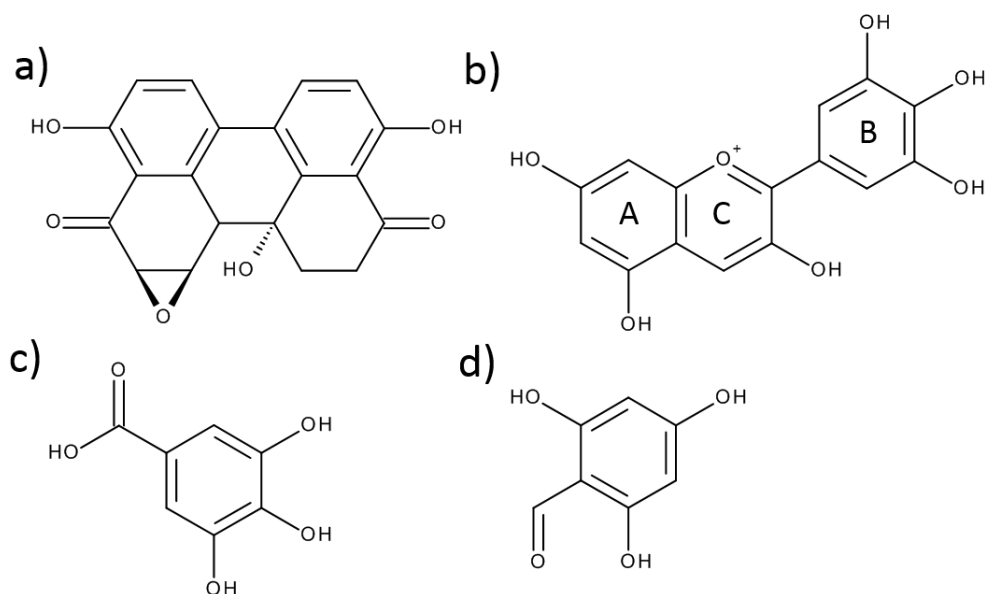


Figure 5: Chemical structures of a) alteroxin II (ATX-II), b) delphinidin (DEL), c) gallic acid (GA) and d) phloroglucinol aldehyde (PGA)

2. Materials and Methods

2.1. Chemicals and enzymes

DEL chloride was obtained from Extrasynthese (Genay, France). Gallic acid, phloroglucinol aldehyde (2,4,6-trihydroxybenzaldehyde), catalase (from bovine liver), sulforhodamine B and dichlorofluorescein-diacetate (DCF-DA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). WST-1 reagent was obtained from Roche (Basel, Switzerland) and formamidopyrimidin-DNA-glycosylase (FPG) from New England Biolabs (Frankfurt, Germany). Methanol, water and ammonium acetate (all LC-MS grade) were purchased from Fluka (Buchs, Switzerland). ATX-II was extracted from *Alternaria*-infested rice as previously described (Schwarz et al., 2012a). Media and supplements for cell culture were obtained from Invitrogen™ Life Technologies (Karlsruhe, Germany).

2.2. Cell Culture

The cell line HT-29, initially established from a human colorectal carcinoma, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) heat inactivated fetal calf serum and 1% (v/v) penicillin/streptomycin, under humidified conditions, with 5% CO₂, at 37°C. Monitoring of potential mycoplasma contaminations was routinely performed. For our experiments, test compounds were dissolved in dimethylsulfoxide (DMSO) and added to the incubation media, resulting in a total concentration of 1% (v/v) DMSO. Previously, anthocyanidins were found to generate H₂O₂ in cell culture

medium, accelerating the chemical degradation of the compounds and possibly causing oxidative cell damage (Kern et al., 2007). To prevent these effects, 100 u/ml catalase was added to all incubation solutions.

2.3. Coupled WST-1 and SRB cytotoxicity assay

The assay was performed as previously described (Aichinger et al., 2017). Briefly, 7500 HT-29 cells per well were seeded in 96-well plates and after 48 h incubated with the test compounds for 24 h. Subsequently, they were incubated with WST-1 reagent and absorption was detected at 450 nm. Upon washing, the cells were fixed with trichloroacetic acid, stained with sulforhodamine B and analysed by measuring the extinction at 570 nm. Effects on cytotoxicity were referred to the solvent control (1% (v/v) DMSO).

2.4. Single cell gel electrophoreses ("comet assay")

The assay was performed according to the guidelines of Tice et al. (Tice et al., 2000) as recently described (Aichinger et al., 2017). Briefly, 150,000 HT-29 cells were seeded in petri dishes, allowed to grow for 48 h. Then they were pre-incubated with DEL for 30 min and subsequently incubated with combinations of DEL and AOH for 60 min. UV-B radiation was used as positive control. Afterwards, cells were washed, singularized and embedded on object slides in agarose. After lysis, half of the slides were treated with FPG and then electrophoresis was performed under alkaline conditions (pH 10). DNA was stained with ethidium bromide and for microscopic image analysis, 100 cells ("comets") per object slide were scored.

2.5. Dichlorofluorescein (DCF) assay

The assay was performed according to the method of Keston and Brandt (Keston and Brandt, 1965) as recently described (Aichinger et al., 2017). Briefly, 20,000 HT-29 cells per well were seeded in black 96-well plates and cultured for 48 h. After washing with PBS, cells were incubated with 100 µl/well of a 50 µM DCF-DA solution for 30 min. Cells were washed again and then incubated with DEL and ATX-II alone and in combinations in non-colored DMEM (1% (v/v) DMSO, 100 u/ml catalase. After 1 h, fluorescence was measured at 485/535 nm (excitation/emission) with a victor 3^v plate reader. H₂O₂ without catalase was used as a positive control.

2.6. LC-MS

Stock solutions of the investigated compounds were prepared in DMSO. Individual test solutions and mixtures were diluted to a final concentration of 1 μ M ATX-II and 100 μ M DEL, GA and PGA in DMSO, PBS and DMEM and incubated for 1 h at 37°C to evaluate their interactions. Following incubation, ATX-II concentrations were determined by LC-ESI-MS/MS analysis, using external calibration.

Briefly, an UltiMate 3000 UHPLC system connected to a TSQ Vantage triple quadrupole mass spectrometer equipped with a heated electrospray ionisation interface (HESI, Thermo Fisher Scientific, Germany) and Xcalibur software 3.0 was used to operate the system. Chromatographic separation was realized on a Supelco Ascentis Express column (C18, 2.7 μ m, 10 cm x 2.1 mm) with a binary gradient elution using an aqueous 5 mM NH_4Ac solution (pH 8.7) as eluent A and pure methanol as eluent B (Tölgyesi et al., 2015). At a flow rate of 0.3 mL/min the following elution gradient was performed: from 0-1 min eluent B was held at 10%, from 1-11 min eluent B was continuously increased to 100% B and maintained at 100% for 4 minutes before reconstituting the column at 10% eluent B for additional 3 minutes. The column oven temperature was set to 30°C and the injection volume was 3 μ L. The mass spectrometer was run in negative ionization mode performing multiple reaction monitoring (Q1 peak width [FWHM], 0.7; Q3 peak width [FWHM], 0.7). The mass transitions m/z 349-331 and m/z 349-313 were used for identification and quantification of ATX-II, applying collision energies of 15 V and 23 V, respectively. Optimized HESI parameters were the following: spray voltage 3 kV, sheath gas flow 35 Arb, auxiliary gas flow 20 Arb, ion sweep gas flow 5 Arb, vaporizer temperature 400°C, capillary temperature 325°C.

Subsequently to the HPLC-ESI-MS/MS analysis, the samples prepared in PBS were additionally analyzed utilizing a high resolution MS full scan method for an advanced screening of m/z ratios obtained from potential reaction products.

Therefore, 10 μ L of the samples were injected to an UltiMate 3000 UHPLC system connected to a Bruker quadrupole time-of-flight (LC-QTOF) mass spectrometer equipped with an electrospray ionization interface (maXis; Bruker Daltonics, Bremen, Germany). The conditions of the chromatographic separation were the same as for the LC-ESI-MS/MS analysis described above.

MS detection was performed in the full scan mode in the mass range of m/z 50 to 1600 and at a scan rate of 1.0 Hz for both positive and negative ionization mode. Compass 1.3 micrOTOF-SR1 software (Version 3.0, Bruker) was used for the data acquisition and Compass Data Analysis software (version 4.0, Bruker) was used for data processing and analysis.

2.7. Interaction analysis

Per definition, an “Interaction” is observed if the effect of a mixture of two or more compounds differs from the additive effect of the single compounds. The calculation of additive effects of compounds that do not follow a linear dose-response relationship requires mathematical models. One of the most popular mathematical models, that we also used in our group’s recent combination studies (Aichinger et al., 2017; Aichinger et al., 2016; Vejdovsky et al., 2016) is the so-called “independent joint action” that was established by Webb in 1963 (Webb, 1963).

An expected additive value (EV) is calculated from the effects of the single compounds by using the formula:

$$f_{ab} = f_a + f_b - f_a f_b$$

f_{ab} is the combinatory effect and f_a, f_b are the effects of the single compounds. The EV ($=1-f_{ab}$) can then be compared to the measured combinatory value (MV) with Student’s *t*-test to determine synergistic ($EV > MV$) or antagonistic ($EV < MV$) interactions.

3. Results

3.1. Cytotoxicity

After 24 h of incubation, ATX-II and DEL/ATX-II combinations showed significant cytotoxic effects on the cells at concentrations of 5 μ M or higher (figure 2). No interactions between the two compounds manifested in the SRB assay, albeit at 10 μ M ATX-II the cell viability value was almost ($p=0.08$) significantly different to the expected additive value. In the WST-1 assay, a significant antagonistic interaction was detected at 10 μ M ATX-II with the measured value being clearly higher than expected.

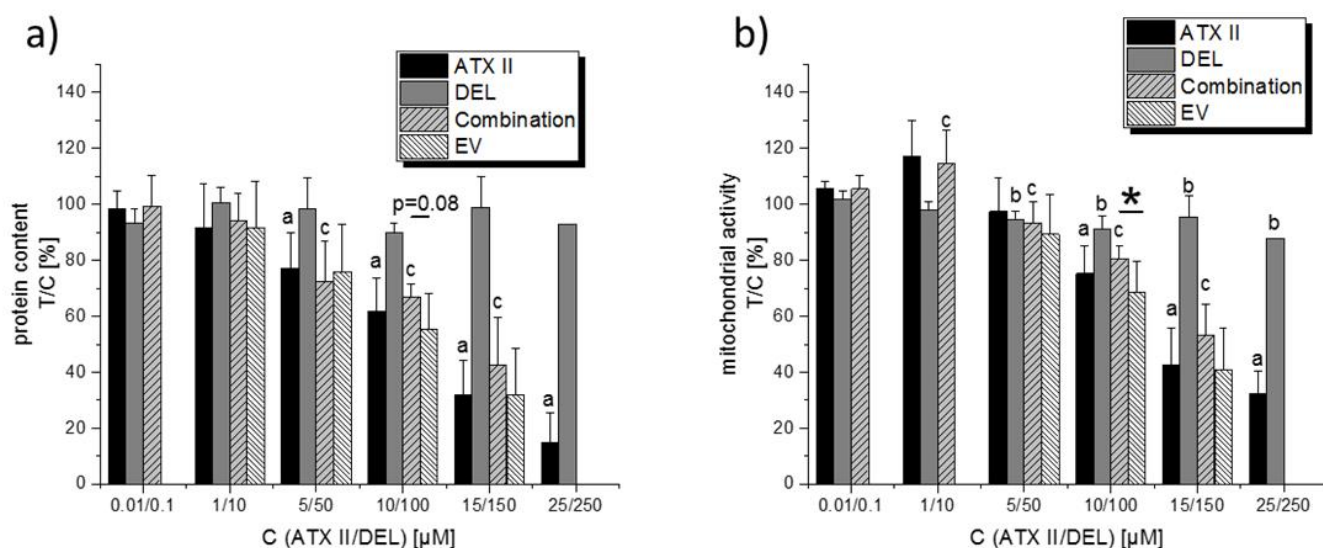


Figure 6: Cytotoxicity of a 24 h incubation with ATX-II, DEL and a combination of the compounds on HT-29 cells, measured with the a) SRB and the b) WST-1. Data is presented as mean + SD of at least 5 independent experiments. Significant differences ($p < 0.05$) to the respective no-effect level (0.01 μ M ATX-II and/or 0.1 μ M DEL) were calculated by one-way ANOVA, followed by Fisher's LSD post-hoc test, and are indicated with a (ATX-II), b (DEL) and c (combination). Expected combinatory values (EV) were calculated according to the model of "independent joint action" as described in the material and methods section. Significant differences between measured and expected combinatory cytotoxicity of the same concentrations were calculated by Student's t-test and are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

3.2. Genotoxicity

In line with previous publications, a 1 h incubation with 1 μ M ATX-II caused DNA damage in the comet assay, an effect that could be further increased by treating the DNA with FPG, thus indicating a damage of DNA bases (figure 3). Of note, the presence of DEL lead to a significant reduction of ATX-II – induced genotoxicity at concentrations of 50 μ M and 100 μ M, both with and without FPG treatment. Furthermore, measured combinatory values at 50 μ M and 100 μ M DEL were significantly lower than the expected values, indicating an antagonistic interaction according to the model of independent joint action. However, when cells were pre-incubated with DEL for 24 h, followed by ATX-II treatment, the genotoxic effects of 1 μ M of ATX-II were not affected (figure 4).

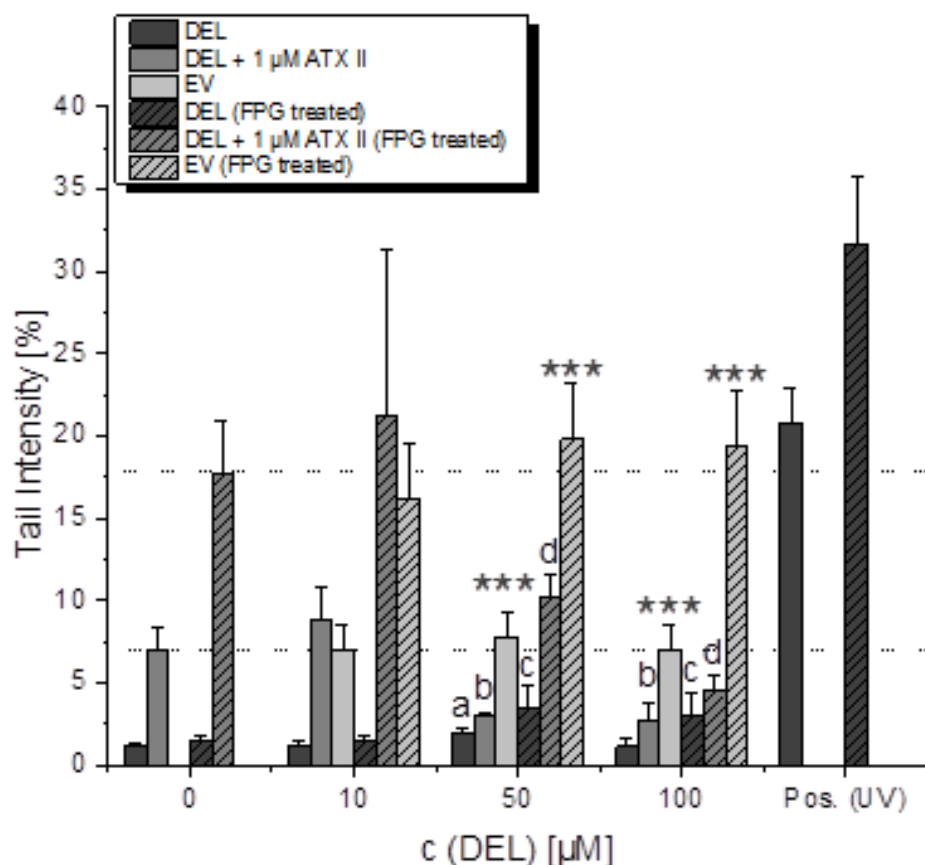


Figure 7: Generation of DNA strand breaks by a 1h co-incubation of ATX-II, DEL and combinations of the compounds in HT-29 cells, as measured with the comet assay. Data is presented as mean + SD of at least 5 independent experiments. Significant differences ($p < 0.05$) to the respective controls were calculated by one-way ANOVA, followed by Fisher's LSD post-hoc test, and are indicated with a (different to solvent control (0 μM DEL, 1% v/v DMSO) without FPG treatment), b (1 μM ATX-II without FPG), c (0 μM DEL with FPG) and d (1 μM ATX-II with FPG). Expected combinatory values (EV) were calculated according to the model of "independent joint action" as described in the material and methods section. Significant differences between measured and expected combinatory values of the same concentrations were calculated by Student's t-test and are indicated by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

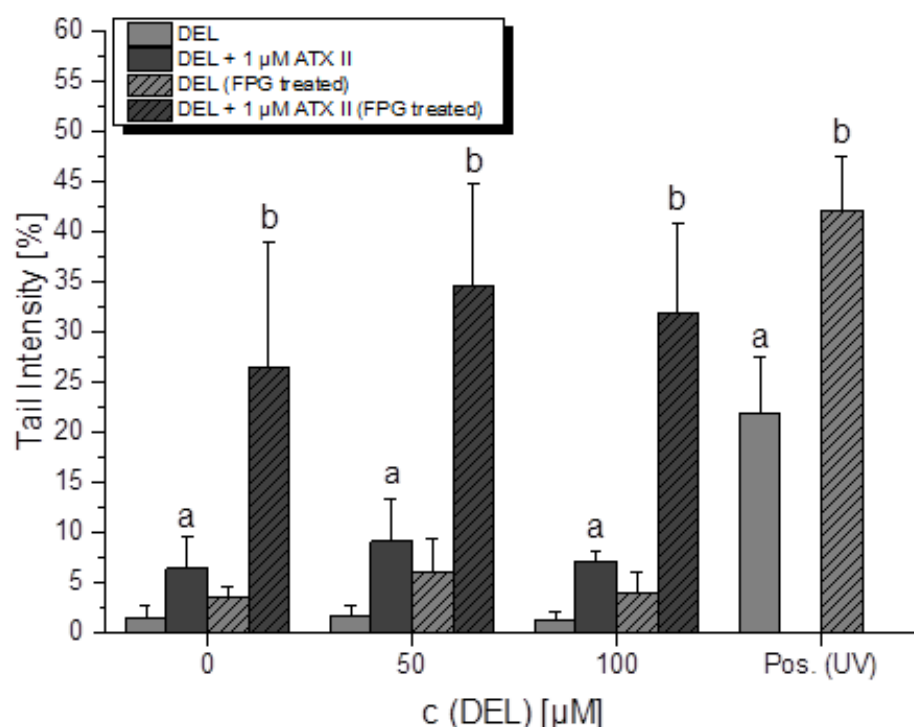


Figure 8: DNA strand breaks after a 24h pre-incubation with 50 µM or 100 µM DEL, followed by 1 h incubation with 1 µM ATX-II, measured with the comet assay in HT-29 cells. Data is presented as mean + SD of at least 4 independent experiments. Significant differences ($p < 0.05$) to the respective solvent control (0 µM DEL) were calculated by one-way ANOVA, followed by Fisher's LSD and are indicated with the letters "a" (untreated) and "b" (FPG treated). No significant differences between tail intensities of the ATX-II samples and the combination samples were observed.

3.3. Oxidative Stress

DEL, as described recently (Aichinger et al., 2017), reduced oxidative stress levels at concentrations of 10 µM or higher in the DCF assay (figure 5). In line with earlier publications, incubation with high concentrations (10 µM) of ATX-II generated reactive oxygen species (ROS) in HT-29 cells (Jarolim et al., 2017). In combination, DEL significantly counteracted this effect starting at a concentration of 1 µM.

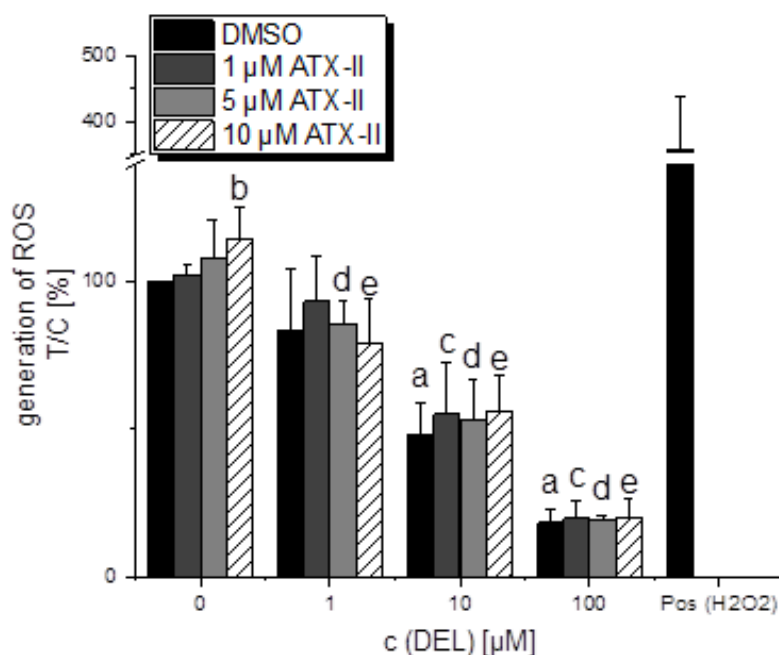


Figure 9: Intracellular levels of ROS after 1 h of incubation with DEL, ATX-II or respective combinations, as measured with the DCF assay. Data is presented as mean + SD of at least 4 independent experiments. Significant differences ($p < 0.05$) were calculated by one-way ANOVA, followed by Fisher's LSD and are indicated with the letters "a" (DEL samples vs. solvent control), "b" (ATX-II samples vs. solvent control), "c" (vs 1 μ M ATX-II), "d" (vs 5 μ M ATX-II) or "e" (vs 10 μ M ATX-II).

3.4. Potential chemical interactions evaluated by LC-MS

The measurement of ATX-II concentrations after 1 h at 37°C in phosphate buffered saline (PBS) showed a significant decrease of ATX-II levels in solutions containing 100 μ M of DEL, PGA or GA in addition to 1 μ M of ATX-II (figure 6). Of notice, ATX-II does not seem to be very stable in cell culture medium (DMEM), as after 1 h at 37 °C close to 50 % of the compound was not recovered. However, in the sample containing 100 μ M of DEL, it further decreased significantly to 44 % as compared to the 1 μ M ATX-II solution in PBS. In DMSO, the organic solvent used to create stock solutions, no loss of ATX-II was observed.

In order to determine potential reaction products of ATX-II and DEL, HR-MS full scan spectra of the incubation solutions of ATX-II with or without DEL and PGA were overlaid and directly compared. Total ion current (TIC), base line peak and extracted ion chromatogram (XIC) segments of 5 min were subsequently collated and scanned manually for differing mass signals.

Applying negative ionization mode, the signals for ATX-II (m/z 349.0715 $[M-H]^-$, retention time 9.1 min) were found in all samples at stable retention times and relative intensities in accordance with the results

of the quantitative LC-ESI-MS/MS analysis. In positive ionization mode, ATX-II signals (m/z 351.0870 $[M+H]^+$) could be detected only at very low intensities due to the limited ionization efficiency of ATX-II (Schwarz et al., 2012b). DEL could be detected neither in positive nor in negative ionization mode, presumably due to the basic chromatographic conditions, which are unsuitable for a positively charged polyphenol. Low signals for PGA (m/z 153.0195 $[M-H]^-$, retention time 1.5 min) and GA (m/z 169.0137 $[M-H]^-$, retention time 1.5 min) were detected in the DEL sample, indicating the partial degradation of DEL in solution. Higher PGA and GA signals were found in samples incubated with PGA and GA, accordingly. Both PGA and GA signals could not be detected in positive ionization mode as expected.

In the sample incubated with PGA one prominent additional signal could be detected at m/z 319.0455 (retention time 5.9 min), which corresponds to the chemical formula of a single isotope exact mass of $C_{15}H_{11}O_8$, $[M-H]^-$. However, the low number of carbon atoms compared to ATX-II ($C_{20}H_{14}O_6$) makes it rather unlikely to include fragments of ATX-II, but suggests for instance some dimerization of PGA forming a 1,3-diketone including a methylene-bridge. Apart from that, no significant differences in mass signals could be detected between HR-MS full scan spectra of the incubation solutions of ATX-II with or without DEL and PGA.

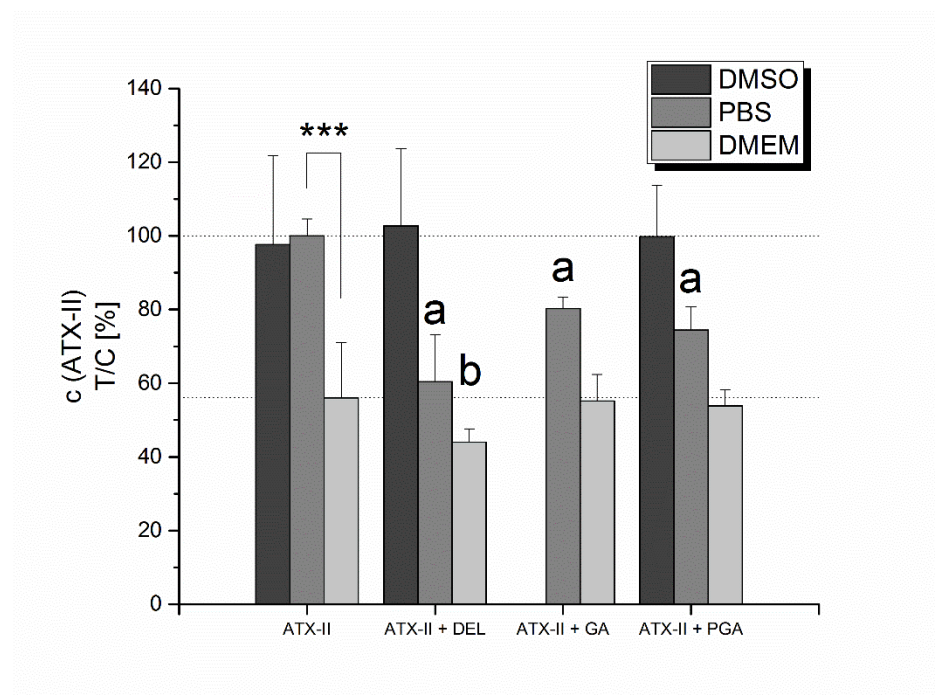


Figure 10: Concentrations of ATX-II assessed by LC-MS after incubation of 1 μ M ATX-II with and without 100 μ M of DEL, GA or PGA for 1 h at 37 °C in three different solvents. Data is presented as mean + SD of test/control (T/C) values of at least 4 replicates, with 1 μ M of ATX-II dissolved in PBS serving as control for the relative quantification. Significant differences ($p < 0.05$) to the measured concentration after 1 h incubation of 1 μ M ATX in the respective solvent were calculated by one-way ANOVA, followed by Fisher's LSD and are indicated with the letters "a" (PBS) and "b" (DMEM). Significant differences between samples solved in DMSO did not occur. The significant difference between the ATX-II samples solved in

either PBS or DMEM was calculated with Student's t-test. Dotted lines indicate ATX-II concentrations measured after 1h incubation of the single compound in cell culture medium (DMEM) and PBS.

4. Discussion

ATX-II, was previously described not to act as a topoisomerase poison (Tiessen et al., 2013) and not to cause oxidative stress (Jarolim et al., 2017) at the concentration where genotoxicity was detected in this study (1 μ M). Recently, we described DEL to antagonize toxic effects of a different *Alternaria* toxin, alternariol, by interaction with these two pathways (Aichinger et al., 2017). Hence, prior to this study we did not expect to see a comparable effect on the toxicity of ATX-II. However, the antagonistic interaction of DEL with ATX-II was found to be even more potent than observed for AOH. Albeit showing only moderate antagonistic interactions on cytotoxicity (figure 2), we found the anthocyanidin to strongly antagonize the genotoxic properties of ATX-II by significantly reducing the amount of induced DNA strand breaks by up to 75 % (for 100 μ M DEL, with FPG treatment, figure 3).

As reasonable ways of action, the influence of DEL on the cellular anti-oxidant defense system or on transporter proteins seemed plausible. If one of the latter modes would be responsible for the pronounced antagonistic interaction that we observed, a decrease of ATX-II induced genotoxicity should have been detectable after a pre-incubation with DEL. Therefore, we pre-incubated the cells with DEL for 24h, washed the cells and further incubated with ATX-II for 1 h before performing the comet assay. However, in this experimental setup no impact of DEL on the DNA-breaking properties of the mycotoxin was observed (figure 4). Therefore, the mechanisms described above seem to be highly unlikely as the simultaneous presence of both compounds appears to be essential for a protective effect.

Thus, we re-assessed the effects of the compounds on the oxidative stress level. In line with previous publications, ATX-II did not affect the cellular redox status at the concentration of 1 μ M that we used for our genotoxicity assays, but increased the concentration of ROS at high concentrations (figure 5). ROS generation in these experiments was slightly lower than in previous reports (Jarolim et al., 2017). However, this can be explained by the presence of catalase in our experimental system, which was added for the reasons described in the materials section. DEL, as expected, significantly decreased oxidative stress levels in a concentration-dependent matter. No difference was observed between cells either treated or not treated with ATX-II. It cannot be fully excluded that the suppression of oxidative stress by DEL contributes to the antagonistic interaction on genotoxicity. However, if there is an influence at all, it is only minor, as there was no induction of ROS generation detectable at a concentration of 1 μ M ATX-II.

Taken together, we could not find a conceivable biological mechanism for the extraordinary strength of the observed interaction. Thus we took a look at the chemistry of ATX-II, which has a functional epoxy

group adjacent to the ring system (figure 1a). Epoxides are known to be highly reactive compounds (Parker and Isaacs, 1959). Thus, it seemed conceivable that a spontaneous reaction with DEL, which would lead to a decrease of ATX-II concentrations and in turn to lower biological effects, might take place. In watery solutions with neutral pH, like cell culture medium, the structure of DEL (figure 1b) undergoes an opening of the “C”-ring followed by degradation (Keppler and Humpf, 2005) to gallic acid (GA, figure 1c) and PGA (figure 1d). The required nucleophile for a reaction with the epoxy raisin of ATX-II could be delivered by either of the latter compounds. Therefore, in a cell-free approach, we incubated ATX-II with and without DEL, GA and PGA under the conditions applied in the comet assay (1 h at 37 °C) in three different solvents (DMSO, PBS, DMEM) and then quantified the ATX-II levels by LC-MS/MS (figure 6). Indeed, we found that the presence of either DEL, PGA or GA significantly decreases the concentration of the toxin in aqueous solutions (PBS, DMEM), but not in DMSO, the solvent used for preparing stock solutions. Even as the hypothesis of a potential adduct formation of the polyphenolic compounds with ATX-II could not be confirmed in our full scan MS experiments, the significant reduction of ATX-II concentrations in the presence of those compounds suggests some sort of chemical interaction. It seems possible that potentially produced adducts could not be detected due to technical issues (e.g. inadequate chromatographic separation, limited ionization efficiency or limited sensitivity under the chosen conditions) or due to the fact that the chemical reaction lead to very small degradation products or undetectable oligomerization products. Thus, our results support the hypothesis that DEL, possibly after degradation to PGA and GA, reacts with ATX-II under physiological conditions. The significance of this finding seems even more relevant considering that PGA is also formed during the degradation of other anthocyanins and anthocyanidins in the intestine (Keppler and Humpf, 2005), where pH conditions are also mildly acidic to mildly basic. Hence, not only the concentration of DEL, but rather the total anthocyanin level might be contributing to a decrease of adverse mycotoxin action *in vivo*.

Furthermore, as ATX-II seems to be active enough to react with other compounds in the cell culture medium, it should not be excluded that other food constituents could also temper *Alternaria*-induced genotoxicity by enhancing ATX-II degradation. This pronounced reactivity could enlighten the question why until now ATX-II was found mostly in *Alternaria* cultures grown on cereal-based medium, and not in fruit samples, while other *Alternaria* toxins occur much less host-specific (Ostry, 2008; Scott, 2001). It seems possible that during the mash-up of fruit samples for analytical sample preparation, ATX-II gets into contact with polyphenols and other potential reaction partners, leading to enhanced “quenching” or even complete loss of the toxin.

5. Conclusions

We hereby demonstrated the anthocyanidin DEL to protect colon cells against genotoxic effects of ATX-II, one of the main genotoxic mycotoxins produced by *Alternaria spp.* Our data suggests that the underlying mechanism probably involves a direct chemical reaction of the two compounds, including reactions with the two degradation products of DEL, the GA and the PGA. This study provides an initial base on interactions of anti-oxidant food constituents with ATX-II. Further research, especially concerning the interactions between polyphenols and *Alternaria* toxins *in vivo*, is warranted. Also the implications of these findings on future methods for the quantification of ATX-II in food samples have to be considered carefully.

Funding Sources

This work was solely funded by the Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Austria.

Acknowledgements

We thank Christine Tiessen and Victoria Dostal for the extraction of ATX-II, and Lena Binder for her help with executing some comet assays. LC-MS analyses were performed at the Mass Spectrometry Center of the Faculty of Chemistry, University of Vienna. In particular, we would like to acknowledge the support of Anna Fabisikova and Martin Zehl.

Conflict of interest statement

The Authors state no conflict of interest.

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Appendix - Abstract

In recent years much research was dedicated to the impact of bioactive food constituents on health and disease prevention. In particular, the class of polyphenols has been in the spotlight as many compounds were found to possess anti-oxidative and chemopreventive properties through a spectrum of different mechanisms. Yet until now, considerably less attention was given to the possibility that this compounds might interact with other bioactive agents like drugs or food contaminants.

One field where such interactions could have a tremendous impact is chemotherapy, where the aim is to suppress the proliferation of cancer cells. If those were protected by food constituents this might negatively influence the outcome of the therapy. But also beneficial effects seem conceivable, as some polyphenols – for example anthocyanins - have a very low systemic bioavailability, limiting their site of action mostly to the gut. Here, they might hypothetically protect non-cancerous cells from severe side effects of drugs without interfering with the therapy targeting a different organ. One interesting new family of chemotherapeutic drugs is the class of receptor tyrosine kinase (RTK) inhibitors, which are preventing those receptors to engage in growth signalling. But also some polyphenols, for example the soy isoflavone genistein (GEN) or the berry anthocyanidin delphinidin (DEL), were previously described to inhibit RTKs, including the epidermal growth factor receptor (EGFR). Thus, in the frame of this thesis, *in vitro* experiments were conducted combining erlotinib (ERL), an EGFR inhibitor approved for the treatment of late stage lung and pancreatic cancer, with the latter polyphenols. The focus hereby lay on interactions at the level of cell growth inhibition by conducting SRB assays and of the inhibition of EGFR phosphorylation by SDS-PAGE/Western blot. GEN, a polyphenol-rich bilberry extract, its major anthocyanin delphinidin-3O-glucoside and the corresponding anthocyanidin DEL were observed to antagonistically influence cell growth inhibition by erlotinib. For GEN this effect could be related to an interference with EGFR inhibition, while for the other compounds a different mechanism has to be considered.

But polyphenols might also alter the toxicity of food contaminants. For example, molds of the genus *Alternaria* are frequently found to contaminate different foods and produce a wide range of different mycotoxins, of which some have been found to be genotoxic. Alternariol (AOH) was recently described to act both as an inducer of oxidative stress and as a poison of topoisomerase II (Topo II), an enzyme crucial for maintaining DNA integrity, and thus to induce DNA damage. Additionally, both GEN and DEL were previously described to inhibit Topo II. DEL was also found to decrease the effects of Topo-poisoning chemotherapeutic agents like irinotecan or doxorubicin. Thus, a similar protective effect of the latter polyphenols against AOH-induced genotoxicity seemed conceivable, even more so as polyphenols in general and especially anthocyanidins are also known to counteract oxidative stress. *In vitro* experiments were conducted with HT-29 colon carcinoma cells to assess such interactions at the levels of cytotoxicity with sulforhodamine B (SRB) and water soluble tetrazolium (WST-1) assays, of genotoxicity with the comet assay, of topoisomerase poisoning with the *in vivo* complex of

enzyme assay and of oxidative stress with the dichlorofluorescein assay. While only weak interactions were observed regarding cytotoxicity, both polyphenols were found to interact antagonistically with the DNA-breaking properties of AOH. Both polyphenols reduced the amount of topoisomerase/DNA complexes stabilized by AOH in the ICE assay, thus interacting with the mycotoxin's prime mode of action, the topoisomerase poisoning. Furthermore, DEL protected from the induction of oxidative stress by AOH in the DCF assay. Taken together, this study gave a first insight in protective effects of polyphenols against an *Alternaria*-induced toxic effects.

Another mycotoxin produced by *Alternaria spp.* is altertoxin II (ATX-II), which was recently shown to be the main genotoxic compound of an extract from *Alternaria alternata* – infested rice. When DEL was co-incubated with this toxin, its protective impact against ATX-II - induced DNA damages by far exceeded the antagonistic effect with AOH in the comet assay experiments. In search of a mechanism, DEL and its degradation products were found to significantly decrease ATX-II levels in aqueous solutions, indicating a direct chemical reaction with the mycotoxin.

Conclusively, this thesis demonstrates the high potential of polyphenols, in particular anthocyanins, to interfere with other bioactive compounds. Interactions at a range of different biological levels could be observed, including cytotoxicity, genotoxicity, EGFR activation, topoisomerase poisoning, oxidative stress and chemical degradation. In general the collected data indicates that polyphenols exert mainly antagonistic, i.e. protective, effects. While this seems desirable when it comes to food contaminants it should be taken into account in case of drug interactions, in particular with chemotherapy.

Appendix - Zusammenfassung

Polyphenole sind eine bedeutende Klasse von sekundären Pflanzenstoffen und vielen Substanzen aus dieser Klasse werden antioxidative und chemopräventive Wirkungen zugeschrieben. Während sich die Wissenschaft in den letzten Jahrzehnten intensiv mit diesen Effekten und den zugrundeliegenden Mechanismen beschäftigt hat, wurde weit weniger in die Aufklärung der Frage investiert, inwieweit Polyphenole in der Lage sind, andere bioaktive Substanzen in ihrer Wirkung zu beeinflussen.

Ein Bereich, in dem solche Wechselwirkungen von besonderer Signifikanz wären, ist die Chemotherapie. Nachdem ihr Ziel darin besteht, das Wachstum von Krebszellen zu unterbinden, wäre es unerwünscht, wenn Polyphenole diese beschützen würden. Umgekehrt wäre eine Verstärkung der medikamentösen Effekte durch natürliche Komponenten mit sehr geringen Nebenwirkungen von großem pharmazeutischen Wert. Außerdem wäre es denkbar, dass Polyphenole mit sehr geringer Bioverfügbarkeit, wie z.B. Anthocyane, den Gastrointestinaltrakt vor unerwünschten Nebenwirkungen der Chemotherapie schützen könnten, aufgrund ihrer Abwesenheit in den Zielorganen aber den therapeutischen Erfolg nicht schmälern würden.

Eine interessante, junge Klasse von Chemotherapeutika wirkt über die Hemmung von Rezeptortyrosinkinasen (RTK), eine Familie von Rezeptoren, die vor allem für die Vermittlung von Wachstumssignalen eine bedeutende Rolle spielen. Eine der wichtigsten RTKs ist der epidermale Wachstumsfaktorrezeptor („epidermal growth factor receptor“, EGFR), der in Tumorgewebe oft überexprimiert wird und dadurch zum Ziel für therapeutische Ansätze wurde. Ein prominentes Beispiel für einen EGFR-inhibierenden Wirkstoff ist Erlotinib (ERL), der bereits zur Behandlung von fortgeschrittenen Pankreas- sowie bestimmten Lungentumoren zugelassen wurde. Auch für einige Polyphenole, wie z.B. für das Isoflavon Genistein (GEN), das Anthocyanidin Delphinidin (DEL) bzw. für einen DEL enthaltenden Beerenextrakt wurde kürzlich eine Hemmung des EGFRs nachgewiesen. Daher wurden im Rahmen dieser Arbeit eventuelle Auswirkungen dieser Komponenten auf den zytostatischen Effekt von ERL an einer humanen Krebszelllinie untersucht. Auf der Ebene der Zellwachstumshemmung konnten mittels Sulforhodamin B (SRB) Assay antagonistische Wechselwirkungen eines Heidelbeerextrakts, dessen Hauptanthocyane DEL-3-O-Glukosid, DELs sowie GENs auf die zytostatische Wirkung des Chemotherapeutikums nachgewiesen werden. Des Weiteren konnte für GEN mittels SDS-PAGE/Western Blot als Mechanismus eine Beeinträchtigung der Fähigkeit ERLs, die Phosphorylierung des EGFRs zu unterdrücken, belegt werden.

Eine weitere Gruppe von bioaktiven Substanzen, bei der Interaktionen mit Polyphenolen denkbar sind, sind Lebensmittelkontaminanten, wobei insbesondere Mykotoxine von großer Bedeutung sind. Daher wurde im Rahmen dieser Arbeit untersucht, inwieweit Polyphenole in der Lage sein könnten, Zellen vor toxischen Auswirkungen von durch *Alternaria spp.* verursachten Kontaminationen zu schützen. Letztere bilden eine Gattung von ubiquitär verbreiteten Schimmelpilzen, die abhängig von den Wachstumsbedingungen eine große Anzahl verschiedener Mykotoxine produzieren. Besonders bedeutend ist Alternariol (AOH),

ein Toxin, das sowohl oxidativen Stress verursacht als auch als Gift der Topoisomerase II (Topo II), einem Enzym zur Regulation des Verdrillungsgrades der DNA, wirkt und dadurch DNA-Schäden verursacht. Polyphenole werden generell mit antioxidativen Eigenschaften in Verbindung gebracht, und einige Vertreter, z.B. DEL und GEN, sind darüber hinaus auch für die Inhibierung der Topo II bekannt. Für DEL wurden außerdem bereits antagonistische Wechselwirkungen mit chemotherapeutischen Topo II Hemmstoffen beschrieben. Daher wurde der Einfluss von DEL und GEN auf die Zyto- bzw. Genotoxizität von AOH in humanen Kolonkarzinomzellen untersucht. Während nur leichte Interaktionen auf der Ebene der Zytotoxizität feststellbar waren, wurden auf Ebene der DNA-Schädigung mittels Einzelzell-Gelelektrophorese („comet assay“) signifikante antagonistische Wechselwirkungen beider Polyphenole mit AOH festgestellt. Die mechanistische Aufklärung ergab einen antagonistischen Einfluss auf die Mykotoxin-induzierte Giftung der Topo II durch beide Flavonoide, sowie eine deutliche Verringerung des induzierten oxidativen Stresses durch DEL.

Weiters wurde auch eine eventuell protektive Wirkung DELs gegenüber Altertoxin II (ATX-II), das kürzlich als hauptgenotoxische Komponente in einem Extrakt aus auf Reis kultivierten *Alternaria alternata* identifiziert wurde, untersucht. Hierbei war ein überraschend starker protektiver Effekt DELs auf Ebene der Genotoxizität zu beobachten. Beim Versuch einer mechanistischen Aufklärung dieses Effekts konnte mittels LC-MS gezeigt werden, dass eine simultane Inkubation mit DEL und/oder dessen Abbauprodukten signifikant verringerte ATX-II Konzentrationen in wässrigen Lösungen zur Folge hat. Dieses Ergebnis deutet darauf hin, dass eine direkte chemische Reaktion zwischen dem Polyphenol und dem Mykotoxin stattfindet, die zur Verringerung der Toxizität führt.

Zusammenfassend demonstriert diese Doktorarbeit anhand ausgewählter Beispiele das hohe Potential von Polyphenolen, die Wirkung anderer bioaktiver Verbindungen zu beeinflussen. Die beobachteten Wechselwirkungen finden auf unterschiedlichen Ebenen statt. So konnte ein Einfluss der untersuchten Polyphenole auf die Zytotoxizität, die Genotoxizität, die RTK- und Topo- Inhibierung, die Induktion von oxidativem Stress sowie auf die chemische Stabilität der anderen Testkomponenten beobachtet werden. Generell deuten die Daten darauf hin, dass Polyphenole meist einen antagonistischen, also abschwächenden Einfluss auf die Toxizität anderer Substanzen ausüben. Dieser Umstand ist im Falle von Lebensmittelkontaminanten sicherlich erfreulich, sollte jedoch mit Vorsicht bedacht werden, wenn die Kombination pharmazeutische Wirkstoffe und insbesondere Chemotherapeutika betrifft.